Modulation of Androgen Receptor Transcriptional Activity

Hao Yun Wong

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transcriptionele activiteit

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List of abbreviations

AD	activation domain
AF	activation function
AIS	androgen insensitivity syndrome
AR	androgen receptor
ARE	androgen response element
ATP	adenosine triphosphate
BAF	Brg1-associated factor
CAIS	complete AIS
CaM II	calmodulin-dependent protein kinase II
CBP	CREB-binding protein
CDK (Cdk)	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CK II	casein kinase II
CPA	cyproterone acetate
CRC	chromatin remodelling complex
CREB	cAMP-response element-binding protein
DBD	DNA binding domain
DHT	5α-dihydrotestosterone
E_2	estradiol
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERK	extracellular signal-regulated protein kinase
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
FSK	forskolin
GR	glucocorticoid receptor
GST	glutathione S-transferase
hBRM	human Brahma
hCG	human chorionic gonadotrophin
HSD	hydroxysteroid dehydrogenases
HME	histone modifying enzyme
hsp	heat shock protein
JNK	JUN N-terminal kinase
kb	kilo base
kDa	kilo Dalton
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
MEKK1	MAP kinase kinase 1
MR	mineralocorticoid receptor
NCoA	nuclear receptor co-activator
N-CoR	nuclear receptor co-repressor
NR	nuclear receptor
NTD	NH_2-terminal domain
OH-F	hydroxyflutamide
ORF	open reading frame
p300/CREB	p300/cAMP response element-binding protein
P/CAF	p300/CBP-associated factor
PAIS	partial AIS

PC	prostate cancer
PIAS	protein inhibitor of activated STAT
PK A	protein kinase A
Pol II	RNA polymerase II
PP2A	protein phosphatase 2A
PR	progesterone receptor
PSA	prostate specific antigen
SRC1	steroid receptor coactivator 1
SHBG	sex hormone binding globulin
SHR	steroid hormone receptor
SRE	steroid response elements
T	testosterone
TIF2	transcription intermediary factor 2
TIP60	HIV-Tat interacting protein 60
TSA	trichostatin A
UTR	untranslated region

Abbreviations of recruited proteins described in Chapter 4 are explained in the corresponding tables in the same chapter.

CHAPTER 1

INTRODUCTION
AND
SCOPE OF THIS THESIS

1.1 PHYSIOLOGY OF ANDROGEN ACTION

Androgens play important roles during fetal male sexual differentiation, but also in development and maintenance of secondary male characteristics, and during initiation and maintenance of spermatogenesis (George and Wilson, 1994). In addition, androgens have a role in female physiology. Androgen action is primarily exerted by testosterone (T), but also by its more active metabolite 5α-dihydrotestosterone (DHT). The conversion of T to DHT occurs in some androgen target tissues (e.g. prostate) by the enzyme 5α-reductase type 2. Although both hormones act via binding to the same androgen receptor (AR), T and DHT have quite separate roles during male sexual differentiation. T acts on development of muscles, the larynx (male voice), the Wolffian duct and its derived structures (epididymides, vasa deferentia, seminal vesicles and ejaculatory ducts). DHT plays a role in other androgen target tissues, like the skin, hair follicles, the urogenital sinus, the urogenital tubercle, the urogenital fold, the urogenital swelling and their derived structures (prostate gland, scrotum, urethra, glans, shaft, penis) (Fig. 1-1). Both hormones act on spermatogenesis and can influence sex drive and behaviour (McLachlan et al., 2002; Wilson, 2001; Gooren and Kruijver, 2002). The precise reasons for the different roles of DHT and T are not understood. Although T and DHT bind the AR with similar high affinity, DHT can induce a higher AR transcriptional activation compared to T (Wilson and French, 1976; Askew et al., 2007). The higher AR activity is likely caused by the twofold higher affinity for co-factors when the AR is bound to DHT instead of T (Askew et al., 2007). In addition, the lower affinity for co-factors of T liganded AR accounts for a three to four times faster dissociation of T compared to DHT (Askew et al., 2007).

T and DHT are often rapidly converted *in vitro* into inactive metabolites during androgen-responsive reporter gene assays performed in cell lines. Therefore, stable synthetic androgenic agonists such as methyltrienolone (RU1881; 17β-hydroxy-17α-methyl 4,9,11,-estratrien-3-one) and mibolerone (7α,17α-dimethyl-19 nortestosterone) are predominantly being used in such assays.

An important tool in studying androgen action are anti-androgens. These compounds act by binding to the AR and inducing a conformational change resulting in an inactive or in a partially inactive receptor. Two anti-androgens with partial agonistic and partial antagonistic properties are cyproterone acetate (CPA) and mifepristone (RU38486), whereas hydroxyflutamide (OH-F), nilutamide and bicalutamide are pure anti-androgens (Neumann and Topert, 1986; Raynaud and Ojasoo, 1986; Furr et al., 1987). Because of their inhibitory action, anti-androgens are used extensively in the treatment of several diseases and disorders, such as prostate cancer, and hypervirilisation syndromes.

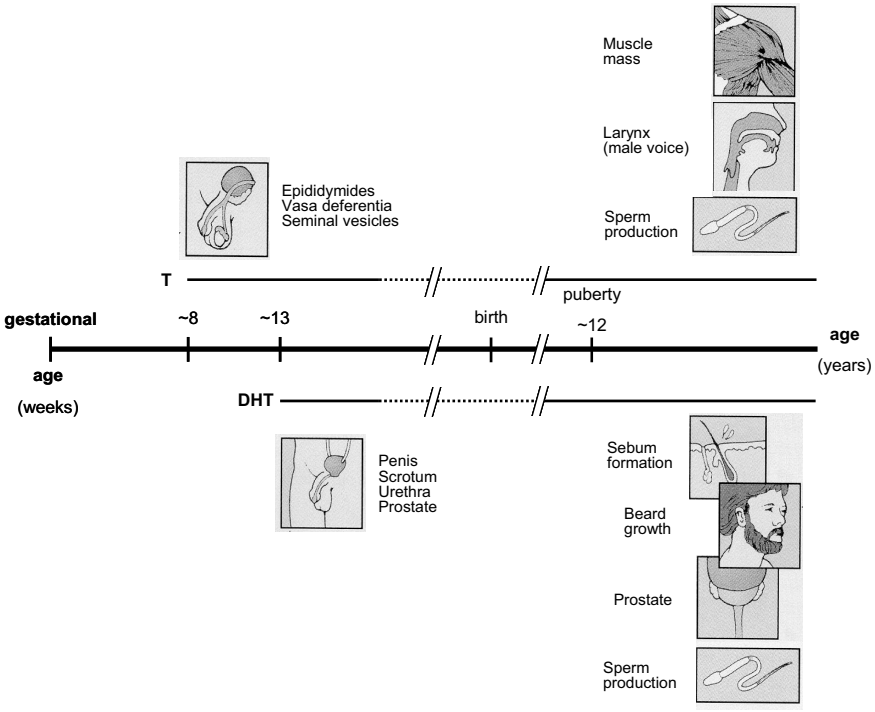

Figure 1-1
Schematic overview of androgen dependent development of different target tissues initiated by testosterone (T) and/or dihydrotestosterone (DHT). Adapted from Berne and Levy; Principles of human physiology, 3rd Ed., 2000, Mosby Inc., St. Louis, USA, Chapter 49, Figure 49-5.

1.2 THE ANDROGEN RECEPTOR (AR): CLONING, GENOMIC ORGANISATION, mRNA, PROTEIN AND FUNCTIONAL DOMAIN STRUCTURE

The AR belongs, together with progesterone (PR), glucocorticoid (GR), estrogen (ER) and mineralocorticoid (MR) receptors, to the steroid hormone receptor (SHR) family. This family in turn belongs to the superfamily of nuclear receptors (NRs). As the name nuclear receptor indicates, these receptors, play a role in the nucleus and once activated they act as transcription factors to regulate genes positively or negatively.

The human AR cDNA was cloned in 1988 by several groups just a few years after cloning of the GR, ERα, MR and PR cDNAs (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988). The *AR* gene is located on the human X chromosome at q11.1-q12 and consists of 8 exons (Fig. 1-2). The gene expands 186,587 kilo bases (kb) in total (Brown et al., 1989; Kuiper et al., 1989; www.genecards.org). Two mRNAs of 10.6 and 8.5 kb respectively, are transcribed from the *AR* gene and both contain a 1.1 kb 5'-untranslated region (UTR) and a 2.7 kb open reading frame

(ORF) (Faber et al., 1991; Tilley et al., 1990; Trapman et al., 1988). Furthermore, the 10.6 kb transcript contains a 3'-UTR of 6.8 kb, whereas the 8.5 kb transcript lacks part of the 3'-UTR (Faber et al., 1991). There are no indications for any preferential use of either one of the two transcripts and neither for a specific function. In the human prostate and in genital skin fibroblasts predominantly the 10.6 kb mRNA is being expressed.

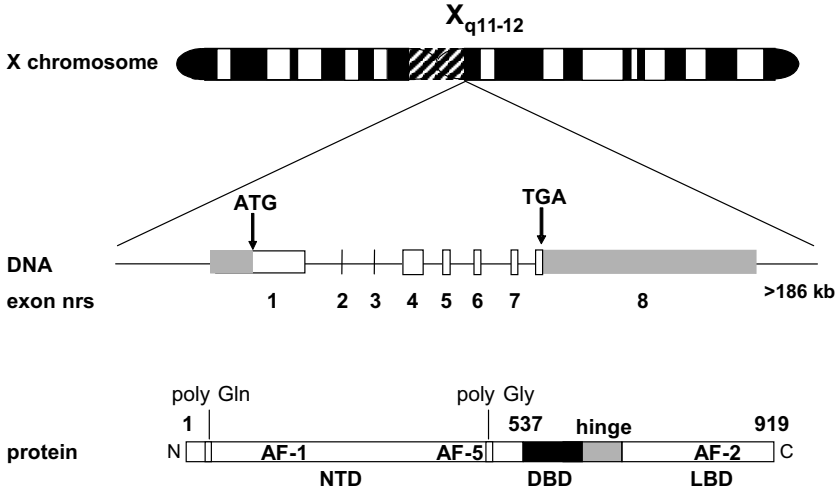

Figure 1-2
Genomic organization and protein domain structure of the human androgen receptor
The *AR* gene is located on the human X chromosome at X_{q11-12} and spans more than 186 kb. The gene encodes a protein of approximately 919 amino acids depending on the length of the variable polyglutamine and polyglycine stretches in the NH_2-terminal domain (NTD). The NTD, the DNA binding domain (DBD), the hinge region, the ligand-binding domain (LBD), activation functions (AF), and polyglutamine and polyglycine repeats are indicated in the figure.

The number of amino acid residues in the AR protein varies between individuals due to the polymorphic polyglutamine stretch and the less variable polyglycine stretch in the NH_2-terminal domain (NTD) (Faber et al., 1989; Sleddens et al., 1993). Throughout this thesis the numbering of the AR is based on 919 amino acid residues according to the AR-data base (www.mcgill.ca/androgendb; Gottlieb et al., 2004b). On SDS-PAGE the AR appears as a 110 - 112 kilo Dalton (kDa) doublet. However, in the presence of androgens a 114 kDa band also appears. Those three bands represent different phosphorylated isoforms (Kuiper and Brinkmann, 1995; Jenster et al., 1994; Kuiper et al., 1991; Wong et al., 2004). The AR protein is expressed in the male as well as in the female reproductive tract and in tissues not involved in reproductive functions (Quigley et al., 1995). In these tissues the expression of the AR protein is regulated by androgens at the transcriptional and post-transcriptional level, and by follicle-stimulating hormone (FSH) (Wolf et al., 1993; Blok et al., 1989; Dai and Burnstein, 1996).

1.2.1 Functional domain structure of the AR

Similar to the other SHRs, the AR consists of 4 different functional domains (Fig. 1-2): an NTD, a DNA binding domain (DBD), a hinge region and a ligand binding domain (LBD). Besides this similarity in the organisation of functional domains, the amino acid homology between the DBDs of SHRs is relatively high (approximately 80%) and for the LBD moderately high (approximately 50%). However, the homologies between the different NTDs and between the different hinge regions are relatively low (<15%) (Faber et al., 1989; Trapman et al., 1988; Evans, 1988).

1.2.1.1 NH_2-terminal domain

This domain is also named the transactivation domain, since it contains the major transcriptional activation functions. It determines to a large extent the androgen-specific regulation of gene expression, because of its unique amino acid composition, which differs extensively from that of the NTD of the other SHRs (Grad et al., 2001). It consists of 538 amino acid residues, which are encoded by the 3'-part of exon 1 (Faber et al., 1989; Kuiper et al., 1989). Two Transcription Activation Units (TAUs) exist within this domain: residues 51-217 are designated as TAU-1 and residues 376-510 are named TAU-5 (Jenster et al., 1991; Jenster et al., 1995). Remarkably, TAU-5 regulates transcription activation if the LBD is deleted, which suggests that TAU-5 in the full length AR is suppressed by the LBD (Jenster et al., 1995).

Another functional characteristic of the NTD is its interaction with the LBD once hormone has been bound, the so-called NH_2-/COOH-terminal domain interaction (Langley et al., 1995; Doesburg et al., 1997; Ikonen et al., 1997; Berrevoets et al., 1998). The NH_2-terminal region involved in the interaction with the LBD has been mapped to the first 36 amino acids, which contain the ^{23}FQNLF^{27} motif. A second motif in the NTD with the sequence ^{433}WHTLF^{437} might bind to a different region of the LBD (He et al., 2000). Interaction of these NH_2-terminal domain motifs with the LBD decreases the dissociation rate of bound androgen. Furthermore the NH_2-/COOH-terminal domain interaction plays a role in stabilisation of the AR dimer via intermolecular interactions (Zhou et al., 1995b). More details about the NH_2-/COOH-terminal domain interaction will be described in paragraph 1.2.1.4 in the section on the LBD.

1.2.1.2 DNA binding domain

The core DBD of the human AR consists of amino acids 554-636 and is encoded by exons 2 and 3 of the *AR* gene (Brinkmann et al., 1989; Kuiper et al., 1989). The DBD has two zinc clusters. Each cluster contains 4 cysteine residues involved in coordination binding of a zinc atom. Each cluster has its own specific function. The

first zinc cluster contains the P(roximal)-box sequence (^{577}GSCKV^{581}), which is involved in the recognition of specific androgen response elements (AREs) in the DNA (Claessens et al., 2001). The second zinc cluster harbours the D(istal)-box (^{596}ASRND^{600}) and other residues, and plays a role in the DNA-dependent dimerisation of the AR (Luisi et al., 1991; Freedman, 1992). Another functional characteristic of the DBD is its non-classical nuclear export signal, a 15 amino acid residues motif located between the two zinc clusters at position 580-595 (Black et al., 2001).

As a transcription factor, the AR can bind to AREs in promoter or enhancer regions of androgen target genes. High affinity steroid hormone response elements (HREs) consist of two half-sites organised as an inverted repeat with a variable three nucleotides spacer, 5'- AGAACAnnnTGTTCT-3'. AR, PR, GR and MR bind as homodimers to this element due to identical P-boxes (Beato, 1989). Therefore, hormone and tissue specific responses of different receptors are induced by additional determinants in receptor specificity. Important for specificity are DNA sequences flanking the hormone response element, receptor interactions with other proteins and receptor concentrations. It has been shown that the effect of co-factors on AR functioning might be different on either a general HRE or on a specific ARE (Geserick et al., 2003). It was previously thought that the inverted repeat of the HRE induces a tail-to-tail dimerisation of the AR and that a direct repeat induces a head-to-tail dimerisation (Schoenmakers et al., 1999). However, this has been disapproved by crystal structure studies of a direct ARE repeat complexed with an AR dimer, which binds in a tail-to-tail fashion (Shaffer et al., 2004). Although a number of specific AREs have been identified in AR target gene promoters and in enhancer sequences, there is not a common specific ARE consensus sequence. Therefore, AR binding response elements can be divided into 4 groups: (A) high affinity specific and (B) high affinity non-specific elements and (C) low affinity specific and (D) low affinity non-specific elements (Claessens et al., 2001; Table 1-1).

1.2.1.3 Hinge region

The hinge region is located between the DBD and the LBD, and stretches roughly between amino acid residues 622-670, which are encoded by parts of exons 3 and 4. It is considered to be a flexible spacer, and its size and composition is not conserved between the different SHRs. After deletion of the hinge region, the AR appears to be more stable and displays an increased transcriptional activity, suggesting that the hinge region has a negative influence on AR stability (Tanner et al., 2004). A similar inhibitory effect on the activation function 2 (AF2) sequence region in the LBD has also been found for a particular part of the hinge region located between amino acid residues 628-646 (Wang et al., 2001). The hinge region has been implicated in several other functions such as DNA binding, Nuclear

receptor co-repressor (N-CoR) binding, protein-protein interactions and nuclear localisation via its bipartite nuclear localisation signal (Haelens et al., 2003; Schoenmakers et al., 1999; Simental et al., 1991; Jenster et al., 1993; Zhou et al., 1994; Beitel et al., 2002; Fu et al., 2002; Fu et al., 2000).

Table 1-1 Classification of androgen response elements

(*A*) *High affinity, non-specific*	*sequence*	*reference*
GRE	GGTACA *aac* TGTTCT	(Beato, 1989)
C3(1) ARE	AGTACT *tga* TGTTCT	(Claessens et al., 1989)
PSA ARE1	AGAACA *gca* AGTGCT	(Riegman et al., 1991)
SLP-HRE-3	GAAACA *gcc* TGTTCT	(Loreni et al., 1988)
(*B*) *High affinity, AR-specific*		
PB-ARE2	GGTTCT *tgg* AGTACT	(Rennie et al., 1993)
SLP-HRE-2	TGGTCA *gcc* AGTTCT	(Loreni et al., 1988)
SC ARE1.2	GGCTCT *ttc* AGTTCT	(Verrijdt et al., 1999)
(*C*) *Low affinity, non-specific*		
PB-ARE1	ATAGCA *tct* TGTTCT	(Rennie et al., 1993)
MVDP pARE	TGAAGT *tcc* TGTTCT	(Darne et al., 1997)
GPX5	ATCCTA *tgt* TGTTCT	(Lareyre et al., 1997)
CRP2	AGAAGA *aaa* TGTACA	(Devos et al., 1997)
(*D*) *Low affinity, AR-specific*		
SC ARE	AGCAGG *ctg* TGTCCC	(Haelens et al., 1999)
SARG+4.6	TGTGCT *aac* TGTTCT	(Steketee et al., 2004; Haelens et al., 1999)

Based on gel shift assays as well as transfection studies (Claessens et al., 1989; Schoenmakers et al., 1999; Vanaken et al., 1996; Schoenmakers et al., 2000), AREs can be divided into four classes: high affinity, non-specific (A), high affinity, specific (B), low affinity, non-specific (C) and low affinity, specific (D). Typical examples with names, sequences and their references are given. Adapted from (Claessens et al., 2001).

1.2.1.4 Ligand binding domain

The LBD is encoded by part of exon 4, exons 5 – 7 and part of exon 8, and consists of amino acid residues 671-919 (Brinkmann et al., 1989; Kuiper et al., 1989). This region is the second most conserved part of SHRs (approximately 50%) and its 3D structure is highly conserved within the NR family (Wurtz et al., 1996). The 3-dimensional structure of the AR LBD has been determined in the presence of DHT and R1881, and predicts 11 alpha-helices (helix 1 – helix 12; there is no helix 2) and four short beta-strands arranged in two anti-parallel beta-sheets (Matias et al., 2000; Sack et al., 2001). Helix 12 of the AR, amino acid residues 892-908, is longer than in other NRs, and is split into two shorter helical segments (Matias et al., 2000; Poujol et al., 2000; Sack et al., 2001). In the agonist-bound conformation, the folding of helix 12 allows a closure of the ligand binding pocket (LBP), also named binding function 1 (BF1) (Matias et al., 2000; Poujol et al., 2000; Sack et al., 2001; Estebanez-Perpina et al., 2007). Depending on the kind of hormone, this conformational change allows the AR to recruit selectively co-activators or co-

repressors and to communicate with proteins of the transcription initiation complex (Berrevoets et al., 2004; Wang et al., 2005c). Before the 3D crystallographic structure of the AR LBD was determined, its dynamic folding in the presence of different ligands was predicted by limited proteolytic digestion experiments (Kuil et al., 1995). Upon agonist binding a conformational change occurs, which is different from that found in the presence of anti-androgens (Kuil and Mulder, 1995).

The AR LBD also contains an AF2 region like the other NRs. However, this AF2 is much weaker compared to those in the other SHRs (He et al., 2004; Jenster et al., 1995). Nevertheless, the AF2 region is very important for the maximal transcriptional activity of the AR and functions as an important interaction domain for the NH_2-terminal domain and for co-factors (Doesburg et al., 1997; Berrevoets et al., 1998; Langley et al., 1998). The AF2 consists of 6-8 crucial amino acid residues, which form a hydrophobic cleft that can interact with short α-helices present in co-activators and the AR NTD. This interaction site is also named BF-2 (Estebanez-Perpina et al., 2007). The ^{23}FQNLF^{27} motif of the NTD, which is part of an amphipatic α-helix, interacts specifically with the BF-2 site (Berrevoets et al., 1998; He et al., 2000; Steketee et al., 2002; Dubbink et al., 2006). To mediate NH_2-/COOH-terminal domain interaction and co-activator binding, the charged residues surrounding the hydrophobic cleft interact with oppositely charged residues flanking the ^{23}FQNLF^{27} motif (He et al., 2000; Steketee et al., 2002; Dubbink et al., 2004).

Co-regulatory proteins (see section 1.5) also bind to the BF-2 to enhance the transcriptional activity of the AR (Glass and Rosenfeld, 2000). Most of these co-regulatory proteins have an LXXLL motif (Heery et al., 1997; Voegel et al., 1998). Also co-factors with a FXXLF motif can bind and co-activate the AR (Hsu et al., 2003). In the same way as the FXXLF motif, charged residues flanking the LXXLL motif interact with the charged residues surrounding the hydrophobic cleft in the LBD (He et al., 2000).

The dynamic behavior of the AR LBD is not restricted to a repositioning of helix 12 upon hormone binding. The crystal structures of either a FXXLF or a LXXLL motif embedded in peptides and bound to the AR LBD predict a difference in the positioning of just a few amino acid residues of the hydrophobic interaction surface (He et al., 2004). Binding of the FXXLF motif is more preferred than binding of the LXXLF motif. In the same study it was demonstrated that substitution of the AR AF2 surface amino acid residues by those of other NRs results in the transition of a FXXLF motif favourable binding into an LXXLL motif favourable binding (He et al., 2004). The preferred binding for the FXXLF motif by the AR LBD is also predicted by a computer model, in which the phenylalanine residue at position 27 fits in the deep groove of the AR hydrophobic cleft and not in the shallow groove of the ERα LBD (Dubbink et al., 2004).

Interestingly, a previously unknown regulatory surface cleft, named BF-3, has been recently identified in the AR LBD (Estebanez-Perpina et al., 2007). BF-3 comprises of Ile-672, Phe-673, Pro-723, Gly-724, Asn-727, Phe-826, Glu-829, Asn-

833, Glu-837 and Arg-840 (Estebanez-Perpina et al., 2007). The AR transcriptional activity and co-activator binding can be decreased by binding to the BF-3 of thyroid hormones triiodothyronine (T3) and TRIAC and three nonsteroidal anti-inflammatory drugs (Estebanez-Perpina et al., 2007). In addition, several mutations of the amino acid residues of BF-3 have been found in subjects with either androgen insensitivity syndrome (AIS, loss of function mutation) or in prostate cancer (gain of function mutation) (Gottlieb et al., 2004b). Mutational analyses have shown the requirement of several of these amino acid residues for AR transactivity. However, these analyses have been performed only in the presence of DHT (Estebanez-Perpina et al., 2007). The influence of each of these residues in the presence of T3, TRIAC or other nonsteroidal anti-inflammatory drugs is therefore unknown.

1.3 AR FUNCTION IN GENERAL

All SHRs, including the AR, have comparable 3D structures of LBD and DBD and have also a comparable modular domain structure. In addition the general mechanism of steroid hormone action display many features, which are also valid for the mechanism of androgen action. Testosterone freely diffuses through the cell membrane into the cytoplasm of an androgen target cell, the so-called free hormone hypothesis, and binds subsequently to the AR either directly or after conversion to DHT. Before hormone binding, the AR is mainly cytoplasmic and is kept inactive by a heat shock protein complex of Hsp90, Hsp70 and FKBP52 (Jenster et al., 1993; Veldscholte et al., 1992b; Cheung-Flynn et al., 2005). In the absence of hormone, the cytoplasmic AR is constitutively phosphorylated on Ser-650 (Wong et al., 2004). Another study has shown a constitutive phosphorylation of Ser-94 (Gioeli et al., 2002). Phosphorylation of the AR will be discussed extensively in section 1.4. Upon hormone binding the AR is further phosphorylated and the Hsp complexes are released (Veldscholte et al., 1992a). At the same time the AR translocates to the nucleus (Simental et al., 1991; Jenster et al., 1993; Jenster et al., 1991) and binds as homodimer to AREs in promoter and/or enhancer regions of androgen target genes to modulate gene transcription (Glass and Rosenfeld, 2000; Gobinet et al., 2002).

There are some indications that the above described free diffusion model might not be complete. Recently it has been shown that testosterone bound to sex hormone binding globulin (SHBG) can be taken up by target cells via megalin, an endocytic receptor in reproductive tissues (Hammes et al., 2005). The inhibition of megalin-mediated uptake resulted in less cellular testosterone ex vivo and in a failure of testicular descent in megalin knockout male mice (Hammes et al., 2005). This suggests that testosterone can reach the AR by another mechanism than diffusion. Hsp90 seems to be essential for AR nuclear import. The blockade of Hsp90 by geldanamycin resulted in an arrest of the AR in the cytoplasm (Georget et al., 2002).

1.4 POST-TRANSLATIONAL MODIFICATION OF THE AR

Hormone binding is not the only mechanism by which conformational changes and/or transcriptional activity of SHRs, including the AR, can be influenced. Modulation of the AR conformation and/or transcriptional activity can also occur via post-translational modifications, like acetylation, sumoylation, ubiquitylation and phosphorylation (Fig 1-3). In the following sections, these 4 different types of AR post-translational modifications are being described and discussed.

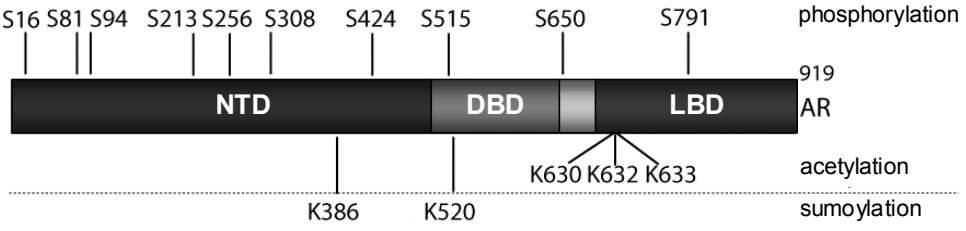

Figure 1-3
Post-translational modifications of the human androgen receptor
The known amino acid residues which can be acetylated, sumoylated, or phosphorylated are indicated in the schematic view of the AR.

1.4.1 Acetylation

Acetylation is the reversible attachment of an acetyl group to lysine residues catalysed by an acetyltransferase. The histone acetylases p300, p300/cAMP-response element-binding (p300/CREB) protein (Fu et al., 2000) and co-factor Tat-interactive protein 60 (Tip60) (Gaughan et al., 2002) can directly acetylate the AR *in vitro* and *in vivo* at a highly conserved ^{630}KLKK^{633} motif in the hinge region. Substitutions of the residues Lys-630, Lys-632 or Lys-633 by an alanine reduce the DHT-induced transactivation of androgen-responsive reporter genes (Fu et al., 2000) and co-activation by SRC1 (steroid hormone receptor co-activator 1), Ubc9, TIP60 and p300 (Fu et al., 2002). However, the acetylation mutants are still able to transrepress other androgen-responsive reporter genes and display a tenfold enhancement of the interaction with N-CoR (Fu et al., 2002). In another study, it was shown that compared to the wild-type AR, the AR lysine mutants display a marked delay in ligand dependent nuclear translocation (Thomas et al., 2004). A study, published already in 1993 showed, that the acetylation motif ^{630}KLKK^{633} is part of the bipartite nuclear localisation signal (Jenster et al., 1993). This suggests that

acetylation might play a role in AR nuclear localisation. Furthermore, the acetylation mutants undergo misfolding and form aggregates. They also co-localise within the cell with an ubiquitin-protein isopeptide E3 ligase carboxyl terminus of Hsc70 interacting protein (CHIP), and inhibit proteasome activity (Thomas et al., 2004).

Interestingly, AR lysine mutants mimicking neutral polar substitution acetylation (K630Q and K630T) display an enhanced p300 binding and their transactivation activity in the presence of DHT can not be influenced by the antagonist Flutamide, in contrast to that of the wild-type AR (Fu et al., 2003). This indicates that AR acetylation sites govern ligand sensitivity and specificity. Unfortunately, ligand binding characteristics of these mutants were not determined.

To summarise: acetylation of AR is determining its association with its co-regulators (co-activators and co-repressors), its proper folding and nuclear translocation. Furthermore, there is an indication that acetylation modulates AR ligand sensitivity and specificity.

1.4.2 Ubiquitylation

Ubiquitylation is the reversible coupling of ubiquitin, a small polypeptide, to lysines via an E3 ubiquitin ligase. The ubiquitylation sites of the AR have not been determined yet. E3 ubiquitin ligase Mdm2 (mouse double minute 2), Akt kinase and the AR are reported to form complexes in which the AR becomes initially phosphorylated (Gaughan et al., 2005; Lin et al., 2002), and subsequently ubiquitylated and finally degradated via the 26S proteasome. These processes are impaired in Mdm2 negative MEFs (mouse embryo fibroblasts) (Lin et al., 2002). Degradation results in an overall lower AR transcriptional activation (Lin et al., 2002). Another protein with E3 ubiquitin ligase activity, androgen receptor N-terminal-interacting protein (ARNIP) can interact with the AR in a ligand-independent manner (Beitel et al., 2002). However, no direct evidence for the ubiquitylation of the AR by ARNIP exists. ARNIP reduces the interaction between the NTD and the carboxy-terminal domain of the AR, but has no effect on the transactivation of the full-length receptor (Beitel et al., 2002). The finding that mice deficient in the E3 ubiquitin ligase E6-associated protein (E6-AP) show strongly reduced androgen responses also indicates a possible role of ubiquitylation for regulating AR function (Smith et al., 2002). These findings are in line with the suggested role of ubiquitylation in general, actually polyubiquitylation, which results in degradation of proteins (Thrower et al., 2000). Interestingly, another AR interacting protein, tumour susceptibility gene (TSG) 101, can induce monoubiquitylation of the AR and thereby enhances AR transactivation (Burgdorf et al., 2004). This transcriptional enhancement by monoubiquitylation has also been shown for other proteins (Schnell and Hicke, 2003)

To summarise: ubiquitylation determines either AR degradation (polyubiquitylation) or AR transcriptional activation (monoubiquitylation).

1.4.3 Sumoylation

Sumoylation is the reversible coupling of a small protein, small ubiquitin-like modifier (SUMO, also called Sentrin), to lysine residues of proteins. This coupling is mediated by a SUMO-E3 ligase. The sumoylation sites are in most cases embedded in a consensus sequence (I/L/V)KXE, where X represents any amino acid residue (Sternsdorf et al., 1999; Johnson and Blobel, 1999). In the AR several sumoylation sites have been identified. Substitution of Lys-386 by Arg in the AR reduces the sumoylation by SUMO-1 and results in an enhanced AR transactivation (Poukka et al., 2000). AR transactivation is even more enhanced when both Lys-386 and Lys-520 are mutated, whereas a single mutation of Lys-520 does not have an influence (Poukka et al., 2000). This finding indicates that sumoylation of AR results in repression of its transcriptional activity. However, the trans-repressing activity of the AR in presence of co-repressors is unchanged after mutation of Lys-386 (Poukka et al., 2000). Another study showed that mutation of Lys-386 or mutation of both Lys-386 and Lys-520, resulted in inhibited SMRT interaction and repression (Dotzlaw et al., 2002). Also DNA binding by Lys-386 Arg mutant AR is unaffected as demonstrated in a promoter interference assay (Poukka et al., 2000) and in an *in vitro* EMSA (electrophoretic mobility shift assay) (Callewaert et al., 2004).

Lysine residue 386 in the AR is sumoylated by SUMO-E3 ligases, PIAS1 (protein inhibitor of activated STAT) and PIASxα/ARIP3 (Kotaja et al., 2002; Nishida and Yasuda, 2002). Remarkably, the effect of AR sumoylation by ectopically expressed PIASxα/ARIP3 and PIAS1 is cell-type dependent (Kotaja et al., 2002). It can result in either a reduced or an enhanced AR transactivation, depending on the response element used in the assay (Geserick et al., 2003; Callewaert et al., 2004). In another study it was shown that a PIAS like protein, without the RING finger-like ligase domain, hZimp10, can interact with the AR and can enhance AR transactivation and sumoylation (Sharma et al., 2003).

A possible mechanism for the decreased transactivation observed after sumoylation of the AR, has been presented in a study. It was shown that *in vitro* sumoylation of AR can result in interaction with Daxx, which in turn reduces AR transactivation (Lin et al., 2004). Daxx can inhibit the transcriptional potential of several transcription factors and can therefore function as a kind of co-repressor. This effect is due to a reduced DNA binding of the AR as shown with EMSA and ChIP (chromatin immunoprecipitation) analyses (Lin et al., 2004). Furthermore, studies in human and rodent Sertoli cells consistently show an inverse correlation between the expression levels of AR and of SUMO-1 and their compartmentalisation as determined with fluorescence microscopy (Vigodner et al., 2005).

To summarise: sumoylation of the AR at a particular site can result in either a decreased or an increased transactivation potential of the AR protein, depending on the cell type and promoter context. The exact mechanism(s) remain to be elucidated.

1.4.4 Phosphorylation

Of all post-translational modifications of SHRs, phosphorylation is the most studied one. Phosphorylation by protein kinases in general is an important biological regulatory signalling mechanism in eukaryotic cells. In 1992, Fischer and Krebs received the Nobel Prize for medicine for their discovery of the impact of protein phosphorylation in a large number of cellular processes. About 518 kinases may be expressed in a cell, and one-third of all intracellular proteins may be phosphorylated, representing as many as 20,000 phosphoproteins, and approximately one of every 400 diseases is associated with a defective protein kinase-mediated pathway (Hubbard and Cohen, 1993). Phosphorylation is often a trigger for switching from one cellular activity state to another and can therefore also regulate the function of transcription factors. Phosphorylation of transcription factors can regulate, for example, their DNA binding capacity, their interaction with other proteins/transcription factors, their degradation or their shuttling between cytoplasm and nucleus (Whitmarsh and Davis, 2000).

1.4.4.1 Phosphorylation of the androgen receptor

One of the most studied post-translational modifications of the AR protein is phosphorylation. However, it remains unclear what exactly the impact is of phosphorylation on the AR transactivation potential. Much more is known about which sites in the AR protein are phosphorylated.

1.4.4.1.1 AR phosphorylation sites

Van Laar et al. showed that the AR is a phosphoprotein and that extra phosphorylation of the AR is induced when cells are exposed to androgens, in addition to the so-called basal AR phosphorylation observed in the absence of androgens (van Laar et al., 1990; van Laar et al., 1991; Gioeli et al., 2002; Wong et al., 2004). Basal and hormone-induced phosphorylation occurs predominantly at serine residues which are mainly located in the NH_2-terminal domain (Kuiper et al., 1993; Gioeli et al., 2002; Kuiper and Brinkmann, 1995). Furthermore, the hormone-induced increase in AR phosphorylation was not observed in the presence of anti-androgens (Kemppainen et al., 1992).

The first identified phosphosites of the AR, serine residues 81, 94, and 650, were found indirectly after mutational analyses in combination with SDS-PAGE (Jenster et al., 1994; Zhou et al., 1995a). Several years later the first non-mutational *in vivo* phosphosite Ser-308 was detected by mass spectrometry (Zhu et al., 2001). Soon, more phosphosites, serine residues 16, 81, 94, 256, 308, 424, and 650, were all identified and confirmed as phosphosites by mutagenesis, peptide mapping and mass spectrometry (Gioeli et al., 2002; Wong et al., 2004). However, cell-free *in*

vitro protein kinase assay studies on AR mutants also demonstrated serine residues 213, 515, and 791 as phosphosites (Lin et al., 2001; Wen et al., 2000; Yeh et al., 1999). Recently, with the use of phosphoserine antibodies it was demonstrated that serine residues 81, 213, and 650 are also phosphosites in the AR (Black et al., 2004; Gioeli et al., 2006; Taneja et al., 2005).

1.4.4.1.2 Phosphorylation and AR isoforms

The first experiment showing a correlation between AR phosphorylation and the appearance of AR isoforms, was established via SDS-PAGE gel electrophoresis and by the use of alkaline phosphatase (Kuiper et al., 1991). Dephosphorylation by alkaline phosphatase treatment in the absence of hormone resulted in the loss of the largest isoform. Substitution of phosphosite Ser-81 by a glycine residue resulted in the loss of the largest isoform irrespective of the presence of ligand (Jenster et al., 1994). Similar results were obtained for the double-mutant S81/94A (Zhou et al., 1995a). However, substitution of Ser-81 with an alanine residue resulted in the loss of one isoform only in the presence of hormone (unpublished data).

Thus, the influence of phosphorylation on the isoform appearance is somewhat complex. With reversed-phase HPLC of ^{32}P labelled AR it was demonstrated that after de novo AR synthesis, the 110 kDa isoform becomes immediately and predominantly phosphorylated on Ser-650 (Chapter 2). The larger isoform of 112 kDa displayed an additional phosphorylation at Ser-94 (Chapter 2). The relationship between phosphorylation of Ser-94 and the appearance of the 112 kDa isoform is in agreement with immunoblot studies in which the phosphosite mutant S94A caused disappearance of the 112 kDa isoform in the absence of hormone and disappearance of the 114 kDa isoform in the presence of hormone (Jenster et al., 1994; Fu et al., 2004). Furthermore, the appearance of the 114 kDa isoform induced by R1881 is directly linked to an overall increase in phosphorylation of several sites as compared with the 110 and 112 kDa isoforms (Chapter 2). Van Laar et al. (1991) has shown previously that in the presence of hormone in addition to a stabilisation of the AR protein also an extra 1.8 fold increase of AR phosphorylation can be induced.

In contrast with the S94A AR mutant, substitution of Ser-650 with an alanine residue does not influence the isoform pattern on SDS-PAGE (Fu et al., 2004; Wong et al., 2004). This is probably due to the fact that the Ser-650 site represents a basal phosphorylation site of the AR. Only changes in phosphorylation of other sites could likely contribute to the appearance of the isoforms. To emphasise the complex relation between phosphorylation and the isoform appearance, another study showed that substitution of serine residues 81, 256, 308, and 515 by alanine has no influence on the isoform pattern. However, the mutant S424A has only one isoform, irrespective of the presence of hormone (Fu et al., 2004). In conclusion, several

phosphosites are clearly involved in the relationship between phosphorylation and isoform pattern of the AR.

1.4.4.1.3 AR phosphorylation and function

As mentioned in the previous paragraphs the role of AR phosphorylation is not exactly clear. In this paragraph more details will be presented on the impact of every phosphorylated site on AR function. These data are also summarised in Table 1-2.

Table 1-2 AR serine phosphorylation sites and their function

site	identification method	function of phosphorylation	mutated isoform pattern	ligand induced phospho-rylation	potential kinase	reference
S16	mass spectrometry	not found	n.d.[1]	slight ↑	CaM II [2] not PKA	(Gioeli et al., 2002)
	phospho-antibody	n.d.	n.d.	slight ↑	n.d.	(Yang et al., 2007)
S81	mutagenesis	not found	only 110 kDa	n.d.	n.d.	(Jenster et al., 1994)
	mutagenesis	not found	unchanged	n.d.	n.d.	(Fu et al., 2004)
	mass spectrometry	not found	n.d.	n.d.	not PKC	(Gioeli et al., 2002)
	phospho-antibody	not found	n.d.	↑	n.d.	(Black et al., 2004)
	phospho-antibody	not found	n.d.	↑	CDK1 [3]	(Chen et al., 2006)
S94	mass spectrometry	not found	n.d.	basal, constitutive	Ser-Pro Kinases [4] not MAPK	(Gioeli et al., 2002)
	mass spectrometry	n.d.	n.d.	112 kDa↑ 114 kDa↑	n.d.	(Wong et al., 2004)
	mutagenesis	not found	1 isoform less	n.d.	n.d.	(Jenster et al., 1994)
	mutagenesis	n.d.	n.d.	n.d.	n.d.	(Zhou et al., 1995a)
	mutagenesis	DHT: wt activity DHT+TSA [5]: inactive p300+DHT: 50% active	-DHT: only 1 isoform	n.d.	n.d.	(Fu et al., 2004)
	phospho-antibody	not found	n.d.	basal, constitutive	n.d.	(Yang et al., 2007)
S213	*in vitro* kinase assay	n.d.	n.d.	n.d.	Akt	(Wen et al., 2000)
	in vivo +cAkt[6]	decreased AR activity	n.d.	n.d.	Akt	(Lin et al., 2001)
	phospho--antibody	+cPI3Kinase [7]→60% activity	n.d.	↑	PI3K/Akt pathway	(Taneja et al., 2005)
	mass spectrometry	n.d.	n.d.	no phospho-rylation	not Akt	(Gioeli et al., 2002)
S213 + S791	*in vivo* +cAkt	docking site for Mdm2 ubiquitin ligase	n.d.	n.d.	Akt	(Lin et al., 2002)
	S231D+ S791D [8]	decreased stability, no nuclear transfer	n.d.	n.d.	n.d.	(Palazzolo et al., 2007)
S256	mass spectrometry	not found	n.d.	↑	CK II [2]	(Gioeli et al., 2002)

	mutagenesis	not found	unchanged	n.d.	n.d.	(Fu et al., 2004)
S308	mass spectrometry	not found	n.d.	↑	Ser-Pro Kinases [4] not MAPK	(Gioeli et al., 2002)
	mass spectrometry	not found	n.d.	n.d.	n.d.	(Zhu et al., 2001)
	mutagenesis	not found	unchanged	n.d.	n.d.	(Fu et al., 2004)
	cyclin D3 +CDK11^{p58}	overexpressed CDK11^{p58} → AR activity↓	n.d.	n.d.	CDK11^{p58}	(Zong et al., 2007)
S424	mass spectrometry	not found	n.d.	↑	Ser-Pro Kinases [4] not MAPK	(Gioeli et al., 2002)
	mutagenesis	not found	1 isoform less	n.d.	n.d.	(Fu et al., 2004)
S515	*in vitro* kinase assay	HER2/Neu+DHT→ AR activity increased	n.d.	n.d.	MAPK	(Yeh et al., 1999)
	mass spectrometry	not found	unchanged	n.d.	n.d.	(Wong et al., 2004)
	mass spectrometry	n.d.	n.d.	no phospho-rylation	not MAPK	(Gioeli et al., 2002)
	mutagenesis	50% decreased activity	n.d.	↑	n.d.	(Ponguta et al., 2008)
S578	phospho-antibody	with EGF inactive	n.d.	n.d.	n.d.	(Ponguta et al., 2008)
S650	mass spectrometry	not found	n.d.	↑	CK II [2] PKC	(Gioeli et al., 2002)
	mass spectrometry	not found	unchanged	basal	n.d.	(Wong et al., 2004)
	phospho-antibody	decreased nuclear AR export	n.d.	+PMA: ↑	p38/JNK	(Gioeli et al., 2006)
	mutagenesis	AR activity 10 to 30% decreased	n.d.	n.d.	n.d.	(Zhou et al., 1995a)
	mutagenesis	not found	unchanged	n.d.	n.d.	(Fu et al., 2004)
S791	*in vitro* kinase assay	n.d.	n.d.	n.d.	Akt	(Wen et al., 2000)
	in vivo +cAkt	not found	n.d.	n.d.	Akt	(Lin et al., 2001)

This table summarises literature data available about serine phosphorylation sites of the AR. The identification method, functional consequences, isoform pattern after mutation, ligand induced phosphorylation, potential kinase and their references are represented.

[1]n.d.= not determined
[2]predicted kinase based on a consensus sequence
[3]CDK= cyclin-dependent kinase
[4]Ser-Pro kinases are serine-proline-directed kinases (Ser/Thr-Pro), MAPK and cyclin-dependent kinases
[5]TSA = trichostatin A, a histone deacetylase inhibitor
[6]cAKT = constitutive active AKT kinase
[7]cPI3Kinase = constitutive active PI3Kinase
[8]D=aspartic acid, a phosphorylation mimicking amino acid

Two-dimensional thin-layer electrophoresis and ascending chromatography was used to detect one of the phosphorylation sites. The phosphorylation status of phosphosite Ser-16 increases slightly when hormone is added (Gioeli et al., 2002). This slight increase was also observed with antibodies directed against the phosphorylated Ser-16 (Yang et al., 2005; Yang et al., 2007). However, upon mutation of this phosphosite, AR transactivation activity was similar to that of wt AR (Gioeli et al., 2002; Fu et al., 2004).

Phosphorylation of Ser-81 was studied in more detail. Mutation of this site does not change the transactivation of the AR in the presence of hormone (Gioeli et al., 2002; Fu et al., 2004; Chen et al., 2006). In the presence of the histone deacetylase inhibitor Trichostatin A (TSA) or of the co-activator p300 the transactivation activity of the Ser-81 Ala mutant displayed the same level of activation as the wt AR (Fu et al., 2004). The AR mutant F582Y found in androgen insensitivity syndrome (AIS), the export double mutant (F582A, F583A) or the ΔF582 mutant showed androgen dependent arrest in sub-nuclear foci and accumulation of histone acetyl transferase CREB binding protein (CBP). Remarkably, these mutants have a hypophosphorylated Ser-81 (Black et al., 2004). This might indicate that recruitment of CBP by the AR occurs before Ser-81 becomes phosphorylated. Furthermore, the arrest in the foci can be a consequence of the mutations together with the hypophosphorylated status of Ser-81. Unfortunately, the arrest in foci of the AR ΔF582 mutant or the F582A, F583A double mutant together with the S81A mutation has not been studied. Furthermore, based upon the late induced increase of both the Ser-81 phosphorylation and the *prostate specific antigen (PSA)* gene transcription, which occurs after approximately 4 hours of DHT stimulation, Chen *et al.* hypothesised that Ser-81 might contribute to the delayed transcription of the *PSA* gene (Chen et al., 2006). However, this hypothesis was not supported by experimental evidence.

In the presence of androgens, phosphorylation of Ser-94 increases (Wong et al., 2004). Also phosphorylation of this site in isoforms 112 and 114 kDa increases (see Chapter 2). Other research groups reported that phosphorylation of Ser-94 occurs constitutively and did not change after addition of hormone (Gioeli et al., 2002; Yang et al., 2005; Yang et al., 2007). Mutation of Ser-94 to alanine does not influence AR transactivation activity (Fu et al., 2004; Gioeli et al., 2002; Jenster et al., 1994). However, in the presence of TSA the mutant AR became inactive, while co-activation by co-factor p300 is decreased by 50% (Fu et al., 2004).

There are some contradictory reports about Ser-213, which makes it questionable whether Ser-213 is a phosphorylation site and what the effect is on AR activity of phosphorylated Ser-213. First, a research group found that constitutive active Akt kinase (cAkt) increases the activity of the mutant AR T877A present in LNCaP (Lymph Node Carcinoma of the Prostate) cells and that Ser-213 and Ser-791 were the candidate sites in an *in vitro* kinase assay (Wen et al., 2000). However, another study showed that co-transfection of AR and cAkt in DU-145 prostate

cancer cells resulted in a decrease of AR activity, which merely depends on whether Ser-213 is mutated (Lin et al., 2001). This was confirmed in a study using a human embryonic kidney cell line (293) with a constitutive active PI3 kinase, which is an activator of Akt (Taneja et al., 2005). The decrease in AR activity was due to the fact that phosphorylation of both Ser-213 and Ser-791 together with cAkt and the Mdm2 ubiquitin ligase resulted in the enhanced degradation of the AR protein (Lin et al., 2002). A less stable AR protein and a lower AR transactivation were also observed for the phosphorylation mimicking mutation of Ser-213 to aspartic acid (S213D), whereas the protein level and the AR transactivation were normal when the serine residue was mutated to an alanine (Palazzolo et al., 2007). Furthermore, the transfer of the AR S213D/S791D double mutant to the nucleus was arrested. On the contrary, the AR S213A/S791A double mutant was able to transfer to the nucleus similar as the wild-type AR (Palazzolo et al., 2007). The phosphorylation status of Ser-213 has not been studied for androgen induced nuclear transfer. Remarkably, with the use of a phosphosite-specific antibody against Ser-213 it was demonstrated that addition of R1881 is necessary for the phosphorylation of this site (Taneja et al., 2005). Furthermore, with immunohistochemistry it was shown that phosphorylated AR Ser-213 is already present in human prostate epithelial cells of 14-week old human tissue, but not in those of 24 weeks-old human foetuses (Taneja et al., 2005). This corresponded positively with the initially more active and higher expressed Akt kinase in the 14 weeks old human foetuses. In contrast to another study, it was shown after mass spectrometry analysis that only the non-phosphorylated Ser-213 existed (Gioeli et al., 2002). In addition, inhibition of the constitutive active PI3 kinase did not change the phosphorylation status of this residue (Gioeli et al., 2002). It can be concluded that the role of Ser-213 phosphorylation is still unclear. It has to be kept in mind that the majority of the studies on the phosphorylation status of Ser-213 has either not been verified *in vivo* or are based on the use of constitutively active kinases.

Although phosphorylation of Ser-256, Ser-308 and Ser-424 increases in presence of hormone, no change in AR activation has been found once these sites were mutated to alanine (Gioeli et al., 2002; Fu et al., 2004). Recently, it has been shown that overexpression of cyclin D3 and the 58 kDa isoform of cyclin-dependent kinase 11 ($CDK11^{p58}$) can repress AR transcriptional activity and this repression is abolished when Ser-308 is mutated to an alanine (Zong et al., 2007). In addition, the mutation abolished the cyclin D3+$CDK11^{p58}$ induced overall phosphorylation of AR (Zong et al., 2007). Unfortunately, the phosphorylation status of Ser-308 alone has not been studied (Zong et al., 2007). Remarkably, mutation of Ser-424 resulted in phosphorylation of another not identified peptide (Gioeli et al., 2002). This conditional phosphorylation could be caused by a more accessible surface for kinases after a conformational change due to mutation of Ser-424.

It is doubtful whether Ser-515 is being phosphorylated. The first indication that Ser-515 is a phosphosite came from an *in vitro* kinase study, which showed that the

NTD of the AR was phosphorylated in the presence of MAPK kinase (Yeh et al., 1999). Ser-515 was suggested to be a phosphosite after observation of a decreased activity of the AR mutant S515A in the presence of DHT and HER2/Neu, a MAPK pathway activator (Yeh et al., 1999). However, in this study the phosphorylation status of the mutant AR was not shown. Mass spectrometric analyses showed that Ser-515 is not phosphorylated and that the use of MAPK inhibitor did not change the phosphorylation of any site (Gioeli et al., 2002). Interestingly, mutation of this site did not result in the non-phosphorylated form of the expected peptide, but of the peptide containing Ser-650 (Wong et al., 2004). Opposite to what happens with mutation of Ser-424, probably a conformational change may have caused the absence of a phosphate group at Ser-650. Furthermore, mutation of this site does not influence AR transactivation (Yeh et al., 1999; Wong et al., 2004). However, when mutant Ser-515 Ala is being tested in the context of an extremely expanded polyglutamine stretch (112 glutamine residues instead of the normal number of approximately 20), then the mutant AR becomes less toxic and less prone to degradation. Also the mutation blocked AR induced cell death (LaFevre-Bernt and Ellerby, 2003). It can be concluded therefore that phosphorylation at serine 515 enhances the ability of caspase-3 to cleave the AR and generate cytotoxic polyglutamine fragments in the context of an expanded 112 polyglutamine stretch.

In addition, contradictory results were found for the function of phosphorylated Ser-650. We found that phosphorylation of Ser-650 is constitutive and did not change in each isoform relative to total AR phosphorylation (Wong et al., 2004). The first indication for the function of phosphorylated Ser-650 came from a study in which the mutated Ser-650 showed a 10 to 30% decreased activity (Zhou et al., 1995a). In addition, in another study it was found that this mutant displays less export from the nucleus to the cytoplasm, suggesting a role of Ser-650 in nuclear export of the AR (Gioeli et al., 2006). However, this mutation has not resulted in a different AR activity (Fu et al., 2004; Gioeli et al., 2002; Wong et al., 2004).

In conclusion, studies up till now do not show a clear relationship between phosphorylation of the AR and function, not even in double mutants like S81A/S650A or S81A/S94A (Zhou et al., 1995a; Gioeli et al., 2002). In addition, transactivation was comparable to the wt AR for an AR mutant in which serine residues 81, 94, 256, 308, 424 were mutated to alanine or to aspartic acid (Yang et al., 2007). Unfortunately, newly phosphorylated sites induced specifically by hormone could not be identified. These kinds of sites could have a higher impact on AR transactivation.

1.4.4.1.4 AR and kinases

Ser-16 is a candidate phosphorylation site for Calmodulin-dependent protein kinase II (CaM-II) and for protein kinase A (PKA) as predicted by NetPhosK 1.0 (Kemp and Pearson, 1990; Kennelly and Krebs, 1991; Blom et al., 2004). However, forskolin

(FSK) a protein kinase A activator did not increase the phosphorylation of Ser-16 (Gioeli et al., 2002).

The kinase involved in phosphorylation of Ser-81 is still unknown, but Ser-81 is embedded in a consensus sequence for PKC (Kishimoto et al., 1985). PKC is both a positive and a negative regulator of the AR and of the AR-responsive *PSA* gene (Andrews et al., 1992; de Ruiter et al., 1995). A study with a PKC activator, 4β-phorbol 12-myristate 13-acetate (PMA), showed that no extra overall AR phosphorylation was found (de Ruiter et al., 1995). Furthermore, another study showed that phosphorylation of this site was not changed in the presence of inhibitors, which can block certain PKC isoforms (Gioeli et al., 2002). However, not all PKC isoforms were blocked in this study. Recently, it was found that overexpression of CDK1 could increase the phosphorylation of Ser-81 (Chen et al., 2006).

The identified phosphosites Ser-94, Ser-308 and Ser-424 are so-called Ser-Pro sites, which can be phosphorylated by serine-proline-directed kinases (Ser/Thr-Pro), MAPK and cyclin-dependent kinases such as Cdc2 and CDKs. However, it has been shown that MAPK has no influence on AR phosphorylation (Gioeli et al., 2002). Furthermore, the overall phosphorylation of the AR is increased by overexpression of the 58 kDa isoform of CDK11 ($CDK11^{p58}$), which does not occur when Ser-308 is mutated to an alanine (Zong et al., 2007). However, the phosphorylation status of Ser-308 alone has not been determined.

Although, several studies described the involvement of constitutive active Akt in Ser-213 and Ser-791 phosphorylation (see section on AR function and phosphorylation, 1.4.4.1.3), a study showed that inhibition of the constitutive active PI3k/Akt in LNCaP cells did not change the AR phosphorylation status (Gioeli et al., 2002).

Ser-256 is predicted to be a consensus site for casein kinase II (Pinna, 1990). However, phosphorylation of this site and all other potential casein kinase II sites has not been investigated *in vitro* with the particular kinase.

It is uncertain whether Ser-515 is phosphorylated by MAPK. The phosphorylation status of Ser-515 in the presence of MAPK was not shown and inhibition of MAPK did not change the Ser-515 phosphorylation status (Yeh et al., 1999; Gioeli et al., 2002).

Which candidate kinase phosphorylates Ser-650 remains also unclear. Ser-650 is a specific consensus site for casein kinases 1 and 2. One study suggested that also PKC could be a candidate. PMA, a PKC activator, increased the phosphorylation of Ser-650 (Gioeli et al., 2006; Gioeli et al., 2002). This is in contradiction with another study (de Ruiter et al., 1995). There is an indication that PKC is not involved in the phosphorylation of Ser-650 directly, because the PMA induced phosphorylation was completely inhibited by the use of p38 and JNK kinase inhibitors (Gioeli et al., 2006). The same study showed that an increased amount of JNK and p38 resulted in more binding of the AR protein by the Ser-650

phosphosite-specific antibody. However, whether this *in vivo* also occurs, remains to be proven.

It has long been questioned whether phosphatases play a role in the dephosphorylation of the AR. Initially only *in vitro* studies showed that added alkaline phosphatase or phosphatase 2A resulted in the disappearance of two of the three phosphorylated AR isoforms (Wang et al., 1999; Jenster et al., 1994). Recently, it has been shown that simian virus 40 small t antigen (SV40 ST) in combination with the synthetic androgen R1881 can mediate the interaction between the liganded AR and phosphatase 2A (PP2A), followed by a decreased AR transcriptional activity and a decreased binding of the AR to AREs (Yang et al., 2005; Yang et al., 2007). However, the AR transactivity has not been studied in a PP2A knockout situation or after a mutational disruption of the PP2A-AR interaction. In addition, SV40 ST mediated the dephosphorylation of the liganded AR complexed with PP2A of serine residues 81, 94, 256, 308 and 424 (Yang et al., 2005). However, mutation of these serines did not change the AR transcriptional activity, which suggests that the decreased AR activity is not caused by dephosphorylation of the investigated serine residues (Yang et al., 2007).

Interestingly, there are indications that kinases can phosphorylate the AR before androgen binding and that phosphatases play a role in keeping the AR less phosphorylated in the unliganded state (Yang et al., 2007). Therefore, it has been suggested that phosphorylation of the AR is regulated by a balance between phosphatases and kinases, in which the unliganded AR is favoured by phosphatases and the liganded AR by kinases.

1.4.4.2 Phosphorylation of other steroid hormone receptors

Phosphorylation has been studied more extensively for other SHRs than for the AR. A picture emerges from the many studies that the function of phosphorylation is different for each receptor. However, a clear conclusion for the function of each SHR cannot be easily drawn, because of the complexity of phosphorylation on multiple sites.

1.4.4.2.1 Phosphorylation of the glucocorticoid receptor

As most of the SHRs the GR is phosphorylated predominantly on residues in the NTD. The human GR is only phosphorylated on five serine sites (Ser-131, Ser-141, Ser-203, Ser-211, Ser-226) whereas one threonine residue is also phosphorylated at position 159 and 171 in the mouse and rat GR, respectively (Ismaili and Garabedian, 2004).

The human GR with all five serine sites mutated to alanine showed dramatic reduction in phosphorylation level, when expressed in yeast, however, without any change in the transactivation of the hGR (Almlof et al., 1995). Only mutation of a

single phosphosite at Ser-203 to an alanine resulted in 50% reduction in hGR activity (Almlof et al., 1995). With the use of an antibody specific for this phosphosite it was shown that Ser-203 of the human GR is phosphorylated in the absence of hormone, whereas phosphorylation of the corresponding Ser-224 in rat GR is hormone dependent (Krstic et al., 1997; Pocuca et al., 1998; Wang et al., 2002b).

Although, phosphorylation of Ser-211 in the hGR is indeed hormone dependent as shown with a phosphosite specific antibody, once mutated, the transactivation activity of the mutant hGR is unaffected (Wang et al., 2002b; Almlof et al., 1995). Consequently, the hormone-induced phosphorylation site is only involved in GR transactivation, in the hormone-induced transactivation status.

Interestingly, immunohistochemistry with two different phosphosite-specific antibodies each interacting with phosphosite Ser-203 and Ser-211 respectively, revealed that there are different GR phosphorylated isoforms and that these isoforms are located at different regions within the cell at the same time (Wang et al., 2002b).

Phosphorylation status is also important for protein-protein interactions. For instance if the GR is hyperphosphorylated, the receptor displays an increased interaction with the co-activator DRIP150, which results in a further enhancement of GR transactivation (Wang and Garabedian, 2003). Furthermore, the transcription inhibitory protein TSG101 binds preferentially to hypophosphorylated and non-phosphorylated forms of the GR (S203A/S211A) and prevents in this way degradation of the unliganded GR (Ismaili et al., 2005). A significant decrease in degradation has also been shown for the mouse GR, when 7 to 8 phosphosites are mutated (Webster et al., 1997).

Two protein kinase families are known to be involved in phosphorylation of the GR. The GR can be phosphorylated in a cell cycle dependent way and thereby influencing GR transcriptional activation, because cyclin-dependent kinases (CDKs) are involved in this event (Bodwell et al., 1998; Ismaili and Garabedian, 2004). Family members of the superfamily of mitogen-activated protein kinases (MAPKs) can phosphorylate directly the GR as well. c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated protein kinase (ERK), can phosphorylate directly rat GR at Ser-246 (homologous site in hGR Ser-226), which results in inhibition of the transactivation activity of the hGR and in an increase of the nuclear export of the GR (Rogatsky et al., 1998; Itoh et al., 2002).

Indirect evidence exists for a role of phosphatases in the dephosphorylation of the GR. Okadaic acid, a serine/threonine protein phosphatase inhibitor, blocked the release of hsp90 complexed with the GR and thereby nuclear translocation of the GR in the presence of hormone and GR nuclear accumulation in the absence of hormone (Galigniana et al., 1999; Dean et al., 2001). Indirect evidence exists that decreased expression of phosphatases results in an increase in DNA binding and a tenfold increase in hormone induced transcription and in an increase in basal

activity in the absence of hormone (Zuo et al., 1999). However, the (de)phosphorylation status of the GR in that study has not been investigated.

Phosphorylation of GR seems to be involved in the downregulation of the GR protein, although the role of each phosphosite still has to be elucidated.

1.4.4.2.2 Phosphorylation of estrogen receptors

Phosphorylation of human (h)ERβ has not been studied in detail, in contrast to the hERα. This protein can be phosphorylated on serine residues in the NTD (Ser-104, Ser-106, Ser-118, Ser-167) and in the DBD (Ser-236). In addition, a tyrosine residue in the LBD can also be phosphorylated. Tyrosine phosphorylation in the estrogen receptor was first extensively described by the group of Ferdinando Auricchio (Migliaccio et al., 1989; Auricchio et al., 1987; Migliaccio et al., 1986; Migliaccio et al., 1984). The phosphorylation at tyrosine 537 in the LBD predominantly stabilises this region, but is not required for hormone binding (Arnold et al., 1997; Weis et al., 1996). Phosphorylation of Ser-104 and Ser-106 can be induced by the cyclin A-CDK2 complex, resulting in an enhanced transactivation (Rogatsky et al., 1999). Ser-167 can be phosphorylated in a hormone-dependent way by Casein kinase II and by constitutive active Akt kinase, which changes the antagonistic characteristics of Tamoxifen to a more agonistic one (Campbell et al., 2001; Arnold et al., 1994). However, the study of *Le Goff et al.* showed the absence of phosphorylation at this particular site when estradiol was added (Le Goff et al., 1994).

Estradiol can also induce phosphorylation of the ER on Ser-118. However, antagonists 4-OHT and ICI 164,384 have the same effect, although to an lesser extend (Ali et al., 1993). This site can be phosphorylated by kinases, such as MAPK or CDK7 (Bunone et al., 1996; Joel et al., 1995; Kato et al., 1995; Chen et al., 2002). In addition, recruitment of splicing factor (SF)3a p120 can take place via this phosphorylated site (Masuhiro et al., 2005). Normally the estrogen receptor migrates as a doublet in the presence of estradiol. However, migration of the ER mutant S118A results in the absence of the largest isoform (Joel et al., 1995). Many studies have focused on Ser-118 phosphorylation, but have shown conflicting results about the effect of phosphorylated Ser-118 on the transactivation activity of the ER (Lannigan, 2003).

Ser-236 in the DBD can be phosphorylated by protein kinase A, which regulates dimerisation and DNA binding of ER (Chen et al., 1999b). Phosphorylation of Ser-305 blocks acetylation of Lys-303, which results in an enhanced transactivation (Cui et al., 2004).

Only indirect evidence is present for the involvement of phosphatases in the possible dephosphorylation of the ER. Phosphatase Cdc25B can interact with the ER and enhance ER transactivation, but its phosphatase activity is not required for this enhancement (Chavez et al., 2001). Another phosphatase, protein phosphatase 2A (PP2A) can interact with the ER via its catalytic subunit and its

dephosphorylation capacity could be shown in an *in vitro* kinase assay (Lu et al., 2003). However, the role of this phosphatase *in vivo* has not been shown.

There are conflicting results about the function of the ER phosphorylated sites. Therefore, no clear conclusion can be drawn about the effect of phosphorylation of the ER and its transactivation. An extensive overview of ER phosphorylation has been published by Lannigan (Lannigan, 2003).

1.4.4.2.3 Phosphorylation of progesterone receptors

The human PR-A of 97 kDa has seven phosphosites in the NTD (Ser-190, Ser-213, Ser-294, Ser-345, Ser-400, Thr-430, Ser-554) and one phosphosite in the hinge region (Ser-676). The longer PR-B protein of 120 kDa has six additional phosphosites in the unique NTD region (Ser-20, Ser-25, Ser-81, Ser-102, Ser-130, Ser 162). Most of these sites are being phosphorylated *in vivo* with the exception of Ser-25, Thr-430 and Ser-554, which can be phosphorylated by Cdk2 *in vitro* (Knotts et al., 2001; Zhang et al., 1997; Zhang et al., 1994; Zhang et al., 1995).

Ser-81, 162, 190, 213 and 400 are defined as “basal” phosphorylation sites and are constitutively phosphorylated in the absence of hormone (Zhang et al., 1997; Knotts et al., 2001). Ser-102, 294 and 345 are hormone-dependent phosphorylation sites and become maximally phosphorylated *in vivo* one to two hours after progestin stimulation (Zhang et al., 1995). The phosphorylation stoichiometry of residues Ser-20, Ser-130 and Ser-676 has not been studied.

The phosphosites Ser-294 and Ser-400 have been studied in somewhat more detail. Phosphorylation of Ser-294 together with an active p42/p44 MAPK is the signal for the hormone-dependent degradation by the 26S proteasome (Lange et al., 2000). The activation of p42/p44 MAPK is induced by MEKK1 (MAPK kinase kinase 1), which is also involved in the phosphorylation of Ser-294 and the enhanced transactivation of the PR (Shen et al., 2001). Mutation of this serine site to alanine decreases the MEKK1 and the ligand-induced transactivation of the PR dramatically (Shen et al., 2001). In addition, nuclear export of this mutant does not take place (Qiu and Lange, 2003).

Phosphorylation of Ser-400 is involved in the Cdk2-induced, but not in the ligand-induced, transactivation of the PR (Pierson-Mullany and Lange, 2004). This event depends on the Ser-400 necessity for nuclear translocation, because mutation of Ser-400 to Ala results in a dramatic decrease in Cdk2 –induced nuclear import of PR (Pierson-Mullany and Lange, 2004). However, in the presence of hormone, the Ser-400-Ala mutant has only a delayed nuclear accumulation, (Pierson-Mullany and Lange, 2004). Therefore, Ser-400 seems to be only necessary for hormone-independent activation.

Phosphorylation of Ser-162 and Ser-294 in the hPR is cell-cycle dependent as was shown with the use of site-specific phospho-antibodies. Phosphorylation is absent in the G2/M phase along with a decrease in transcriptional activation of the

PR (Narayanan et al., 2005). However, a functional analysis with mutant Ser-162 has not been performed. Another study revealed that mutation of either phosphorylation sites Ser-190 or a cluster of serines just upstream of the DBD or Ser-676 inhibit transcription by 20-50% depending on cell- and/or promoter context (Takimoto et al., 1996). In the same study was also shown that after mutation of all serines unique to the NTD of the PR-B to alanine, the transactivation activity of this mutant construct is equal to that of the wt PR-B.

Studies on the PR from other species indicated more functions associated with the phosphorylation status of the PR. The rabbit PR in the presence of hormone or the antagonist RU 486 was found to be hyperphosphorylated (Chauchereau et al., 1991). This hyperphosphorylated PR has a decreased electrophoretic mobility (Chauchereau et al., 1991). Furthermore, the constitutive active receptor (without the LBD) exhibited only a low basal level of phosphorylation (Chauchereau et al., 1991). When Ser-530 of the chicken PR was mutated to alanine, it reduced the transcriptional activity of the PR at low hormone concentrations but did not affect maximal activity (Bai et al., 1994). Mutation of Ser-211 to alanine resulted in a decrease in the transcriptional activity of the receptor and affects the phosphorylation-dependent decrease in mobility on a SDS-PAGE gel (Bai and Weigel, 1996).

1.4.4.2.4 Phosphorylation of the mineralocorticoid receptor

Phosphorylation of the MR has not been studied in very much detail, despite the fact that the MR contains numerous potential phosphorylation sites. One potential tyrosine phosphorylation site, at position 73 in NTD of the rat MR from the Fisher 344 strain is a cysteine at the same position in the MR of the Brown Norway rat, a strain which is insensitive to adrenalectomy. The Y73C substitution leads to a gain of function for the rat MR with a stronger transactivation activity in the presence of aldosterone, and interestingly also by progesterone (Marissal-Arvy and Mormede, 2004; Marissal-Arvy et al., 2004). However, it remains to be proven that Y73 is being phosphorylated in the Fischer 344 rat MR and therefore reduces its transactivation potential. Aldosterone can induce a rapid (already within 15 minutes) MR phosphorylation on serine and threonine (but not tyrosine) residues which is mediated at least in part through the PKCα-signalling pathway (Le Moellic et al., 2004).

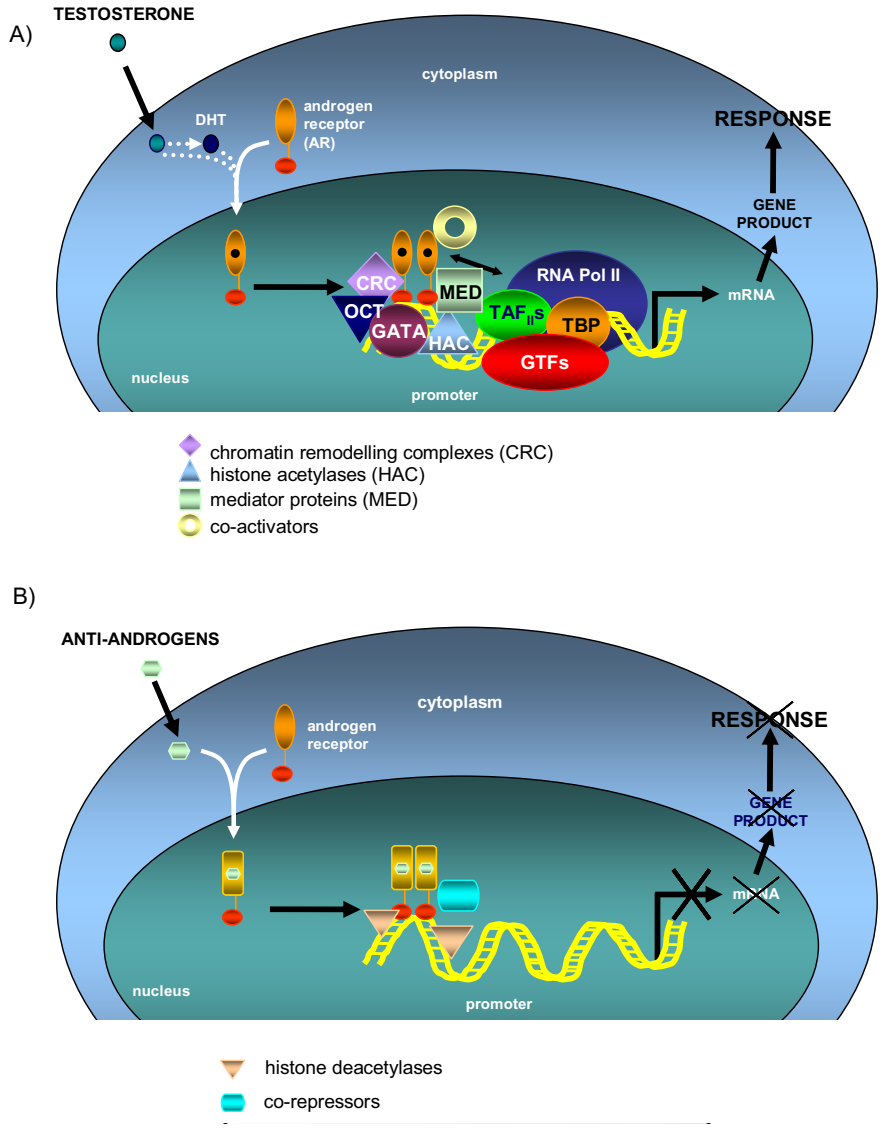

Figure 1-4

Mechanisms of androgen and anti-androgen action

A) Agonist bound AR translocates to the nucleus where it binds to the promoter regions and enhancers of androgen-responsive genes and recruits activating co-factors, such as chromatin remodelling complexes, histone acetylases and mediator complexes. By recruiting RNA polymerase (Pol) II, TATA binding protein (TBP), several TBP-associated factors ($TAF_{II}s$) and general transcription factors (GTFs), transcription initiation of a target gene can start. **B**) Antagonist bound AR has a conformation favourable for recruiting co-repressors and histone deacetylases. This complex inhibits transcription of genes.

1.5 TRANSCRIPTION REGULATION

The AR can bind to AREs in the promoter and/or enhancer regions after hormone binding and subsequent migration to the nucleus, as outlined in section 1.2.1.2, and can initiate transcription activation or repression of target genes. Bound to the response elements the AR is the DNA binding activator protein that serves as a nucleation site for the recruitment of co-factors, general transcription factors (GTF), other transcription factors and RNA polymerase II (Pol II) (Fig. 1-4). At the same time the activator together with co-factor complexes coordinates transcription by Pol II (Kraus and Wong, 2002; Lemon and Tjian, 2000; Roeder, 2005).

Eukaryotic DNA is approximately two meters in length and is compacted to chromatin together with nucleoprotein complexes (Widom, 1998). At the first level of organisation, 146 bp of DNA is wrapped in 1.75 superhelical turns around a histone octamer with two copies of four histones (H2A, H2B, H3 and H4) (Kornberg and Lorch, 1999; Chakravarthy et al., 2005). These octamers, nucleosomes, are in a dynamic equilibrium between a fully wrapped state and a set of partially unwrapped states (Mellor, 2005). The wrapped state prevents the initiation of transcription by blocking the access for Pol II and general transcription factors. The concept is that SHRs and other NRs act as pioneer factors in the initiation of the chromatin remodelling process to unwrap the DNA. Besides the wrapped state of DNA, it has recently been discovered that there are pre-existing regions of open chromatin to which a SHR, GR, can bind. This has been suggested to play a role in the determination of tissue-selective receptor function (John et al., 2008).

The access of SHRs to nucleosomes is gained by recruitment of 2 groups of chromatin modifying enzymes. Group 1 contains histone modifying enzymes (HME), which relieve the DNA-histone interaction, and group 2 consists of ATP-dependent chromatin remodelling complexes (CRC) that displace or remove the nucleosomes. Beside chromatin modifying enzymes three other groups of co-factors can be recruited. Group A: Mediator complexes, which are essential for recruitment of GTFs and Pol II. Group B: Co-activators, which can enhance transcriptional activation. Group C: Co-repressors, which repress transcription. These co-factors will be discussed in more detail for the AR in the following sections.

1.5.1 Chromatin remodelling complexes

Chromatin remodelling ATPase complexes are the first group of chromatin modifying enzymes to prepare transcription initiation by remodelling nucleosomes. Based on the identity of the ATPase subunit, the ATP-chromatin remodelling complexes can be subdivided into three distinct families, namely, the SWI/SNF, ISWI and the Mi-2/NuRD complexes (Narlikar et al., 2002; Becker and Horz, 2002). These chromatin remodelling complexes use the energy stored in adenosine

triphosphate (ATP) to mobilise or structurally alter nucleosomes (Kingston and Narlikar, 1999).

1.5.1.1 SWI/SNF

The SWI/SNF family was originally discovered in yeast (Yoshinaga et al., 1992). In humans there are two ATPase catalytic subunits, brahma related gene 1 (BRG1; also named BAF or hSwi/Snf-A) and human brahma (hBRM; also named PBAF or hSwi/Snf-B) (Kwon et al., 1994; Wang et al., 1996a; Wang et al., 1996b). Each of these ATPase subunits belong to a complex of approximately 2 MDa molecular mass with 10–12 additional protein subunits termed as Brg-1-associated factors or BAFs (Kwon et al., 1994; Wang et al., 1996a; Wang et al., 1996b). Purification and characterisation studies of the separate complexes demonstrate differences in activity and subunit composition suggesting distinct regulatory roles (Bultman et al., 2000; Lemon et al., 2001).

The mouse mammary tumour virus (MMTV) promoter is widely used as a model system for studying the molecular mechanisms of SHR-dependent transcriptional regulation in a chromatin context (Hager, 2001). The MMTV-long terminal repeat (LTR) of retroviral DNA is organised in six nucleosomes if integrated in mammalian chromosomes (Richard-Foy and Hager, 1987). *In vitro* studies with the MMTV-LTR assembled into chromatin in Xenopus oocytes and in cell-based transfection assays suggest a requirement for BRG1 during AR-dependent transcription and chromatin remodelling of the MMTV promoter (Huang et al., 2003). In this study, ChIP analysis showed that BRG1 is recruited to the MMTV promoter in the presence of the AR ligand R1881. In addition, AR ligand-dependent activation of the MMTV promoter was impaired in SW13 cells, which lack BRG1, supporting a role for BRG1 complex in AR dependent gene regulation. Transient transfection studies have suggested that the AR differentially requires hBRM to activate the probasin promoter *in vivo* (Marshall et al., 2003). However, addition of PSA-enhancer to the core PSA promoter bypassed SWI/SNF requirement (Marshall et al., 2003). Recently, it has been shown that BAF57 directly binds to hormone stimulated AR and is recruited on AR targets, which increases AR transactivation via hBRM (Link et al., 2005). Another member of the SWI/SNF family, AR interacting protein (ARIP4) can also moderately enhance AR transactivation on minimal promoters (Rouleau et al., 2002). Interestingly, it was demonstrated that the co-repressor Krab-associated protein 1 (KAP-1) together with HDAC-1/2 and SWI/SNF were components of the nuclear co-repressor complex (N-CoR), suggesting a possible dual function for SWI/SNF (Underhill et al., 2000).

1.5.1.2 ISWI

Many studies provide substantial evidence for a role of the BRG1 complex in SHR-dependent chromatin remodelling and transcriptional regulation. However, less evidence exists for the ISWI/hSNF2h ATP-dependent complexes. ACF (ATP-dependent chromatin assembly and remodelling factor) a chromatin remodelling complex that contains the ISWI subunit, did not support NR-dependent activated transcription in *in vitro* transcription assays (Lemon et al., 2001). Another study showed that synergistic transactivation by PR and NF1 of an MMTV promoter was enhanced by recombinant ISWI, but in absence of SWI/SNF (Di Croce et al., 1999). ISWI, unlike BRG1 always exists as subunit in other complexes (NURF, CHRAC and ACF) and a role is suggested in transcription termination by stimulation of phosphorylation of the CTD in RNA Pol II (Ito et al., 1999b; Morillon et al., 2003).

1.5.1.3 Mi-2/NURD

The third class of ATP-dependent remodelling complexes is the Mi-2/NURD complex. Evidence suggests that complexes such as the Mi-2/NURD complex that contain the Mi-2 ATPase subunit are involved in transcriptional repression when recruited by DNA binding proteins to specific DNA sequences (Knoepfler and Eisenman, 1999; Solari and Ahringer, 2000). The Mi-2 was shown to be a subunit of the nucleosome remodelling and deacetylation (NURD, NuRD or NRD) complex. NURD complexes include members of histone deacetylases (HDAC1/2), histone H4-interacting proteins RbAp46/48, methyl-binding proteins MBD2/MBD3 and Mi-2 as the ATPase subunit (Zhang et al., 1998; Wade et al., 1999). A common feature of the Mi-2 complex is the presence of the members of the metastatic-associated protein gene family (Zhang et al., 1998; Wade et al., 1999). A role for the Mi-2/NuRD complex in SHR-dependent transcription regulation has not been studied extensively. Mi-2/NURD mediated repression of NR-dependent transcription was suggested in a study showing that the co-repressor KAP-1 together with HDAC-1/2 and SWI/SNF were components of the nuclear co-repressor complex (N-CoR) (Underhill et al., 2000). KAP-1 was shown to associate with an isoform of Mi-2 protein, an integral component of the NURD complex (Schultz et al., 2001). Two independent laboratories have found MTA1 and MTA3 components of the Mi-2/NURD complex, to be involved in ER-dependent transcription repression (Mazumdar et al., 2001; Fujita et al., 2003).

1.5.2 Histone modifying enzymes

Although ATP-dependent chromatin remodelling is required for NR-dependent transcription regulation, it is not sufficient (Dilworth et al., 2000; Li et al., 1999). Chromatin remodelling complexes may allow the access for the second group of chromatin modifying enzymes, the histone modifying enzymes (Dilworth et al., 2000;

Li et al., 1999). Extensive post-translational modifications of histones, either by acetylation, methylation, phosphorylation, ubiquitylation/ubiquitination and/or ADP-ribosylation determine whether particular nucleosomes remain wrapped and hence repressed or become unwrapped, resulting in gene activation (Loury and Sassone-Corsi, 2003; de Murcia et al., 1988; Sun and Allis, 2002). It has been suggested that a certain pattern of histone modifications, the “histone code”, functions as a read out for certain proteins, including transcription factors and co-activators (Strahl and Allis, 2000). Recently, evidence has been provided that the 5’-specific and 3’-specific regulation of nucleosome composition and the histone code are important for the location and kinetics of transcriptional initiation in *Saccharomyces cerevisiae* (Lieb and Clarke, 2005).

1.5.2.1 Acetylation

Numerous studies in yeast and higher eukaryotic organisms have demonstrated the link between acetylation of the N-terminal tails of histones and the activation of transcription (Wade et al., 1997). Acetylation of lysines neutralises the positive charge of the histone tails and thereby decreases their interaction with the negative charged DNA (Hong et al., 1993). This is directly related to an increased transcription activity. Several subgroups of histone acetyl transferases (HATs) have been identified.

p300/CBP was found to acetylate histones and to be tightly associated with RNA-Pol II (Ogryzko et al., 1996). p300/CPB is also able to acetylate the AR as already mentioned in section 1.4.1. It can enhance the transactivation of the AR and other SHRs, (Chakravarti et al., 1996; Aarnisalo et al., 1998; Heinlein and Chang, 2002).

A second subgroup is the p300/CBP-associated factor (P/CAF), which was initially identified as a p300/CBP interacting factor (Yang et al., 1996). It can acetylate the AR as well, which also enhances AR transactivation (Fu et al., 2000).

The third group of HATs is the group of p160-co-activators, which consists of steroid hormone receptor co-activator (SRC)-1, (SRC)-2 and (SRC)-3 (or nuclear receptor co-activator NCoA-1, -2 and -3) (Anzick et al., 1997; Chen et al., 1997; Tan et al., 2000; Yeh et al., 1998; Onate et al., 1995; Spencer et al., 1997; Yao et al., 1996). SRC-2, also known as TIF2, has in contrast to SRC1 and SRC-3 no HAT activity (Hong et al., 1997; Voegel et al., 1996; Chen et al., 1997; Spencer et al., 1997). The HAT activity of p160-co-activators is much weaker than that of p300/CBP, which probably functions differently (Clements et al., 1999). Besides they can interact with the AR, they can also associate with other HATs, p300/CBP and P/CAF and other factors, for example methyltransferases (Chen et al., 1999c). The SRCs appear to function as bridging factors.

1.5.2.2 Deacetylation

Deacetylation of histones is performed by histone deacetylases HDACs. Histone deacetylation is directly correlated with a decreased transcription activation (Pazin and Kadonaga, 1997; Hu and Lazar, 2000). There are 8 different HDACs (HDAC 1-8). HDAC 1 and 2 are both subunits in two repressor complexes, the mSin3 and NuRD (nucleosome remodelling and deacetylation complex) complex. HDAC3 was found in an 1.5-2 MDa complex complexed with two co-repressors, nuclear co-repressor (N-CoR) and silencing mediator of retinoid and thyroid receptors (SMRT) and it requires the interaction with N-CoR or SMRT for its deacetylase activity (Guenther et al., 2000; Li et al., 2000). These co-repressors can interact with the AR (Shang et al., 2000; Berrevoets et al., 2004). In fact it has been shown by ChIP technology that HDAC-1 and HDCA-2 are being recruited by the AR via SMRT or N-CoR at the promoter region of the *PSA* gene (Shang et al., 2002).

The AR has been found together with HDAC1 and Mdm2 as a trimeric complex, which results in a decreased AR transactivation (Gaughan et al., 2005). HDAC1-DJBP (DJ-1-binding protein) complex can interact with the AR and represses AR transactivation (Niki et al., 2003). Pod-1 can repress AR transactivation by recruiting HDAC1, but not HDAC4 (Hong et al., 2005). The AR can recruit HDAC4 via ARR19 (AR corepressor-19 kDa), but not HDAC1 nor HDAC5 and ARR19 represses AR transactivation only in the presence of HDAC4 (Jeong et al., 2004).

1.5.2.3 Methylation

Methylation of arginine and lysine residues of histones can either result in gene activation or gene repression (Lee et al., 2005). Gene activation or repression depends not only on the methylation status of histones, but also on the number of methyl groups (mono-, di- and trimethyl). Two arginine methyltransferases, co-activator-associated arginine (R) methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) are known to enhance transactivation of AR and other NRs. This enhancement is only obvious in the presence of p160-co-activators and ligand (Koh et al., 2001; Chen et al., 1999a). It has been shown that CARM1 also binds to beta-catenin and can function in synergy with beta-catenin and p300 as co-activators for AR (Koh et al., 2002). In addition to histone methylation, PRMT1 can also methylate other proteins, such as the RNA-interacting proteins heterogeneous ribonuclein particles (hnRNP) A and B, RNA, fibrillarin and nucleonin (Najbauer et al., 1993; Lee et al., 2005).

Transactivation by the AR can result in a different methylation status of histone 3. Activation of *PSA* gene transcription by androgens can lead to reduced H3 Lys-4 methylation in the promoter region, but in an increase of this modification in the coding region (Kim et al., 2003). Recently, a study showed that the lysine methyltransferase G9a is involved in AR transactivation. G9a enhanced AR

transactivation synergistically with NR co-activators TIF2, CARM1 and p300 (Lee et al., 2006).

1.5.2.4 Demethylation

Only recently, demethylases, the counterpart of methyltransferases, were identified (Kubicek and Jenuwein, 2004). Arginine demethylases are not involved in AR transactivation, but a lysine-specific histone demethylase, LSD1, is. Originally it was found as KIAA0601/NPAO in several co-repressor complexes, the CtBP, NRD, Co-REST, and subsets of the HDAC complexes (Hakimi et al., 2002; Hakimi et al., 2003; Humphrey et al., 2001; Shi et al., 2003; Tong et al., 1998; You et al., 2001). LSD1 specificity and activity is regulated by associated protein co-factors (Lee et al., 2005; Metzger et al., 2005; Shi et al., 2005). The AR or its associated factor belongs to proteins that are able to regulate LSD1 specificity. LSD1 can demethylate histone H3 at Lysine-9 in the presence of AR, but not histone H3 at Lysine-4. This loss of repressive methylation revealed the co-activator function of LSD1 since it enhances AR transactivation. Interestingly, this binding and co-factor function does not occur with the estrogen receptor or the retinoic acid receptor (Metzger et al., 2005).

1.5.3 Mediator complexes

A mediator is an evolutionary-conserved complex that contains approximately 25-30 subunits and is required for activator-dependent transcriptional activation (Conaway et al., 2005). The first Mediator complex was identified as thyroid hormone receptor associated proteins (TRAPs), which enhanced the transactivation by liganded-TR (Fondell et al., 1996). The TRAP complex turned out to be highly similar to other mediator complexes. The vitamin D receptor interacting proteins (DRIP), activator-recruited co-factor (ARC) and SRB/MED containing co-factor complex (SMCC) are identical to TRAP (Ito et al., 1999a; Gu et al., 1999; Rachez et al., 1998; Naar et al., 1999). These complexes were later renamed as Mediator (MED)-complex according to a unified nomenclature (Bourbon et al., 2004).

Studies with transcription activators, like NRs, in systems with DNA templates and purified RNA polymerase II and corresponding general initiation factors, revealed that mediators were required for transcription activation but not for basal (activator-independent) transcription (Flanagan et al., 1991). Mediator proteins can interact with general transcription factors and RNA-Pol II. This interaction facilitates RNA-Pol II recruitment and promotes formation of the preinitiation complex (PIC). Furthermore, MED-complexes can also modulate the function of Pol II (Malik and Roeder, 2005).

A recent study elegantly showed that both recruitment and postrecruitment steps in transcription initiation required Mediator proteins. This was demonstrated in

Med23 negative cells, in which histone acetylation, methylation, and chromatin remodelling complex association at the *Egr1* promoter were equivalent to that of wild-type cells, but *Egr1* induction was largely reduced (Wang et al., 2005a). Knockouts of Mediator subunit confirmed the suggested general role for these complexes, since these knockouts result in embryonic lethal phenotypes (Ito et al., 2000).

Several subunits of the TRAP complex, TRAP220, TRAP170 and TRAP100, can interact with the AR LBD *in vitro* and their overexpression results in an enhanced AR transactivation. Furthermore, Chromatin immunoprecipitation (ChIP) assays show that TRAP220 is recruited to the *prostate-specific antigen (PSA)* gene promoter in AR stimulated LNCaP cells (Wang et al., 2002a).

1.5.4 RNA polymerase II and general transcription factors

Prior to the initiation of transcription the preinitiation complex (PIC) is assembled at the core promoter. The giant PIC, consisting of RNA-Pol II, general transcription factors (GTFs), and Mediator complexes, is an assembly of minimal 48 proteins, with a total molecular mass of approximately 2.5 MDa. The general transcription factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH are assembled in a particular order (Roeder, 1996; Buratowski et al., 1989). TFIID consists of TATA binding protein (TBP) and several TBP-associated factors (TAF_{II}s) and binds to the TATA-box to position the other proteins of the PIC at the core promoter. The other GTFs either stabilise intermolecular interactions and the interaction of TFIID with DNA or destabilise non-specific RNA-Pol II DNA interactions. TFIIH has helicase activity to unwind the DNA. The assembly and stability of the PIC is usually controlled by a second class of transcription factors, such as the AR, which can be found on the promoter region and/or on distant enhancer regions. It is not clear how the AR regulates the PIC assembly and its stability. But two studies nicely showed the tracking of the RNA-Pol II from the PSA enhancer over approximately 4 kilobases to the PSA promoter upon androgen induction (Louie et al., 2003; Wang et al., 2005c).

1.5.5 Other transcription factors

Recently, studies on the PSA promoter and enhancer have shown the necessity of recruitment of transcription factors other than the GTFs (Wang et al., 2007). GATA2 and Oct1 are cooperating in androgen mediated *PSA* gene expression (Wang et al., 2007). After binding of DHT to the AR, the receptor interacts with GATA2 in order to be recruited to the PSA enhancer, whereas Oct1 acts at a step subsequent to GATA2 action (Wang et al., 2007).

1.5.6 Co-activators

The Group B of non-chromatin modifying enzymes (see paragraph 1.5), the so-called co-activators, do not bind to DNA, but interact indirectly through association with DNA binding proteins, such as SHRs. A list of co-activators has been published at www.nursa.org. Lists of AR interacting co-activators are also published (Heinlein and Chang, 2002; Jasavala et al., 2007; Heemers and Tindall, 2007). Many co-activators interact with domains of the AR via their LXXLL motifs (Savkur and Burris, 2004). The highly similar FXXLF motif, which is present in the AR NH_2-terminal domain, is also present in the co-activators ARA70, ARA55, ARA54 and FHL2 (Kang et al., 1999; Fujimoto et al., 1999; Muller et al., 2000; Yeh and Chang, 1996). The AR favours the interaction with the FXXLF motif more than with the LXXLF motif, in contrast to other SHRs (He et al., 2004; Dubbink et al., 2006).

The primary function of co-activators is to facilitate AR directed target gene expression and to enhance AR transactivation. For example, a component of the Hsp90 chaperone heterocomplex, BAG-1L, is presumed to enhance the AR transactivation by promoting the appropriate folding of the AR (Froesch et al., 1998). AR protein stability can also be enhanced by co-activators (Moilanen et al., 1998). Several co-activators, like ARIP (PIASαx), SRC1 and TIF2 are capable to enhance NH_2-/COOH-terminal domains interaction (Moilanen et al., 1999; Spencer et al., 1997; Berrevoets et al., 1998). Furthermore, DNA binding of the AR can also be enhanced by co-activators, as shown for the co-activator RAF (Kupfer et al., 1993) Some of the mentioned co-activators and many other co-activators act through multiple mechanisms to influence AR transactivation and harbour more than one function. p300/CPB is such a co-activator. It can enhance AR transactivation by acetylation of the AR and of the nucleosome. Furthermore, co-activators together can enhance AR transactivation in a synergistic way, probably by facilitating each other's recruitment. An example of the synergistic action of co-activators on AR mediated transcription activation is the cooperative action of FHL2, CBP/p300 and β-catenin (Labalette et al., 2004).

In general, co-activators are proteins. The only exception so far is SRA, which is a RNA transcript. SRA is able to co-activate AR transactivation. It is present in ribonucleoprotein complexes together with SRC1 (Lanz et al., 1999).

1.5.7 Co-repressors

Co-activators are recruited to activate gene transcription. For the fine tuning of cellular processes, repression of gene transcription by co-repressors is also essential. Two of the most well known nuclear co-repressors N-CoR, and SMRT, can inhibit AR transactivation by recruiting HDACs, which in turn results in deacetylation of histones to make the DNA less accessible for factors necessary for gene transcription (Privalsky, 2004). However, the AR can also recruit HDAC1 by

itself (Gaughan et al., 2002). When either N-CoR or SMRT is overexpressed, interaction of the co-repressor with agonist bound AR can occur, which consequently results in transactivation inhibition (Cheng et al., 2002; Liao et al., 2003). However, partial agonist and antagonist bound AR can recruit more efficiently N-CoR and SMRT. Interestingly, N-CoR can compete with TIF2 for AR binding sites, which has led to an equilibrium model, in which the protein level of co-activator and co-repressor determines whether AR activity is enhanced or repressed (Berrevoets et al., 2004). Similarly, this competition between TIF2 and SMRT has also been shown. Furthermore the NH_2-/COOH-terminal domain interaction appears to be inhibited by SMRT (Liao et al., 2003). Cyclin D1 is another co-repressor which is able to inhibit the association between the co-activator P/CAF and the AR (Reutens et al., 2001). hRad9, a member of the checkpoint Rad protein family, can also interfere with the NH_2-/COOH-terminal domain interaction of the AR (Wang et al., 2004). Other mechanisms to repress AR transactivation activity is by interference of AR DNA binding as illustrated by studies with calreticulin (Dedhar et al., 1994). An overview on AR co-repressors has been published recently (Wang et al., 2005b).

Thus an agonist bound AR recruits merely co-activators and less co-repressors, which is the opposite of an antagonist bound AR which merely recruits co-repressors. All these results are based on experiments with synthetic antagonists. It remains therefore questionable whether a repressive mechanism is operating, since the existence of natural AR antagonists is not evident.

1.5.8 The transcriptional clock

Results from studies with ChIP assays illustrated the dynamics of the recruitment and dissociation of the estradiol (E2) liganded ERα together with several co-factors involved in transcription activation at a target promoter. These studies revealed that estradiol (E2) liganded ERα in synchronised cells can associate with the cathepsin D-promoter or the *pS2* gene promoter in a cyclic way with cycles of approximately 45 minutes. In the first cycle, approximately 15 minutes after addition of E2, in addition to ERα, p300, SCR-3 (AIB1), MED1, CPB, PRMT1 and RNA Pol II were detected on the promoter (Shang et al., 2000; Metivier et al., 2003; Reid et al., 2003). The sequential and combinatorial assembly of a transcriptionally productive complex on a promoter has been defined as a “transcriptional clock”.

For ERα three different cycles can be distinguished (Metivier et al., 2003). In the initial unproductive cycle without RNA Pol II, SWI/SNF chromatin remodellers were recruited, followed by PRMT1 and HATs (Tip60 and p300). Thereafter, several components of the basal transcription machinery are recruited. In the next step ERα was targeted to the proteasome and degraded. After the initial cycle the transcriptionally productive cycle was started with the recruitment of p68 RNA

helicase, which is assumed to serve as an adapter protein to attract AF2 co-activators (Endoh et al., 1999). Next, the histone modifications were started with a large complex containing HMTs (PRMT1 and/or CARM), HATs and p160-co-activators that serve as scaffold proteins. Subsequently, $TAF_{II}250$, $TAF_{II}130$ and mediator-complexes were attracted to the promoter to start transcription elongation. This is followed by the targeting of ERα to proteasomes, and recruitment of HDACs and SWI-SNF complexes, which are probably needed for reorganising the nucleosomes in the subsequent cycles. At the end of the second productive cycle, approximately 180 min after the start of the unproductive cycle, NuRD components were recruited, presumably to remove TFIIA/TBP. A reinitiation cycle is then occurring in which remodelling of the nucleosomal structure is needed prior to the start of new cycles.

1.5.9 The concept of the transcriptional clock in the mechanism of androgen action

A similar cyclic pattern as described for ERα was also found for the AR and several co-activators, although it has not been studied extensively. However, the transcriptional clock ticks slower during the AR cycle. The first two cycles of the AR and RNA Pol II binding on the PSA and KLK2 promoters take approximately 85 min each. Remarkably, after the second cycle, maximum binding of AR, RNA Pol II binding and some modified histones was reached and did not change for at least 300 min, whereas only two cycles were found on the enhancers of both genes (Kang et al., 2004; Kang et al., 2002). However, time points for measuring protein recruitment were taken every 15 min or 25 min. instead of 1 min. as in the ERα study. More timepoints would reveal a far more detailed protein recruitment. Another study showed that the amount of AR, SRC1, GRIP1, AIB1, p300, TRAP230, CARM1 and TBP recruited on the PSA promoter increases for at least 16 hours (Wang et al., 2005c). Interestingly, AR, p160, CBP, p300 and RNA Pol II are preferentially recruited to the PSA enhancer on androgen stimulation (Louie et al., 2003). Furthermore, in the presence of the pure anti-androgen bicalutamide, AR associated with the promoter regions and with components belonging to co-repressor complexes, but RNA Pol II and HATs were not detected on the promoters (Kang et al., 2004; Kang et al., 2002; Masiello et al., 2002; Shang et al., 2002). The sequential and cyclic recruitment of proteins shows that a highly regulated process at the molecular level is involved in transcription regulation mediated by the AR.

1.6 ANDROGEN RECEPTOR DISORDERS

1.6.1 Androgen insensitivity syndrome

Disorders of androgen action can be caused either by defective androgen synthesis or metabolism, or by defective AR functioning. A defective functioning AR can result in a partial or complete androgen resistance in androgen target tissues. This disorder is called androgen insensitivity syndrome (AIS). AIS is characterised by a mild or severe undervirilisation in 46,XY individuals due to a mutation in the *AR* gene (McPhaul et al., 1993; Quigley et al., 1995; Boehmer et al., 2001; Gottlieb et al., 2004a; Brinkmann et al., 1996). The clinical phenotypic spectrum ranges from a female phenotype to an undervirilised male phenotype or an infertile but otherwise normal male phenotype, and strongly depends on the type of mutation. The complete form of AIS (CAIS) is characterised by a female phenotype with breast development, but absence of axillary and pubic hair, no uterus, no ovary, no fallopian tubes, a blind ending vagina and the presence of testes in the abdomen. The 46,XY individuals with partial AIS (PAIS) have phenotypes ranging from a predominantly female external appearance, with mild cliteromegaly and some fusion of the labia, to phenotypes with ambiguous genitalia or to a predominantly male phenotype with micropenis, perineal hypospadias, and cryptorchidism. Individuals with the mildest form of AIS have mild undervirilisation and are infertile (Hiort et al., 2000).

The different mutations in the *AR* gene causing AIS are often single base substitutions. However, deletions (1-6 base pairs), partial or complete gene deletions (>10 base pairs), insertions or duplications are also found. Mutations are compiled in the AR database (Gottlieb et al., 2004b; www.mcgill.ca/androgendb). Most of the mutations are found in the LBD (approximately 62%). Many mutations in this domain are affecting ligand binding resulting in a non-active or partially active AR. Approximately 15% of all mutations are found in the DBD, causing defective DNA binding of the AR, and consequently a defective activation of androgen-responsive genes. Only 17% of all mutations are found in the NTD (Gottlieb et al., 2004b). Most mutations in this region are associated with CAIS, mainly due to premature stop codons, although PAIS phenotypes have also been described.

1.6.2 Spinal and bulbar muscular atrophy (Kennedy's disease)

Kennedy's disease, also known as spinal and bulbar muscular atrophy (SBMA), is a slowly progressing degeneration of lower motor neurons, resulting in muscle weakness in adult males (Kennedy et al., 1968; Greenland and Zajac, 2004; Arbizu et al., 1983). These patients may also develop gynaecomastia and sometimes reduced fertility, indicating a defect in AR functioning. La Spada and colleagues were the first to demonstrate a direct correlation of SBMA with an extension of the

$(CAG)_nCAA$- repeat in exon 1, which encodes the polymorphic polyglutamine stretch in the AR NTD (La Spada et al., 1991). In the normal human population the repeat is between 11 and 38, also depending on the ethnic background (Caskey et al., 1992; Sleddens et al., 1992; Edwards et al., 1992). In SBMA, the number of glutamine residues varies between 40 and 63.

1.6.3 Prostate cancer

Prostate cancer is the second leading cause of death by cancer in men in Western countries (Gronberg, 2003). Most prostate cancers express relatively high levels of AR protein (van der Kwast et al., 1991). Initially prostate cancer is androgen dependent, because removal of androgens or blocking the AR by anti-androgens results in growth arrest of the tumour. However, tumour growth arrest is only temporarily, because most tumours undergo a transition to an androgen unresponsive state.

Despite many suggestions for a possible mechanism for the development of the androgen unresponsive state of prostate tumours, the exact mechanism underlying the transition to androgen independency is still unclear (Feldman and Feldman, 2001; Grossmann et al., 2001). Since the AR is expressed in androgen independent prostate tumours, it is assumed that the AR is still involved in some way in tumour growth. One mechanism may be a higher AR protein expression, caused by amplification of the *AR* gene (Edwards et al., 2003a; Edwards et al., 2003b; Visakorpi et al., 1995). Under extremely low androgen levels (by hormone deprivation) the AR can still be activated. Also somatic mutations in the *AR* gene can result in a more active receptor protein (www.mcgill.ca/androgendb) or may broaden the ligand specificity towards anti-androgens or other steroid hormones, such as found for the AR mutant T877A (Veldscholte et al., 1990; Zhao et al., 2000). This mutation is found frequently in androgen independent prostate tumours. Another mechanism that has been proposed is the increased expression of AR specific co-factors, resulting in an enhanced AR activity and consequently in enhanced tumour growth. Finally, a mechanism has been suggested involving ligand-independent activation of the AR. This might be achieved by cross-talk with other activated signal transduction pathways.

1.7 SCOPE OF THIS THESIS

To better understand the various actions of androgens in different cellular systems, and the dysregulation of such actions, it is important to obtain more information on processes and factors which modulate AR transcriptional activity. Modulating processes and factors have been described in a number of studies, but the picture

is far from complete. The aim of the experiments described in this thesis is to gain more insight in the effect of several AR processes and modulating factors.

Phosphorylation is involved in many processes via modulation of enzymes or proteins in signal transduction pathways. This posttranslational modification also has been shown to influence the function of several SHRs. The AR is known to be phosphorylated, but the effect of phosphorylation on AR function is largely unknown. Chapter 2 describes the studies in which an attempt has been made to answer the following question:

Question 1: *What is the influence of AR phosphorylation on AR isoforms and AR transactivation?*

Androgen insensitivity is predominantly caused by mutations in the *AR* gene. However, the correlation between a specific AR mutation and the AIS phenotype is not always consistent. After a novel mutation was found in an individual with PAIS, we addressed the following question in Chapter 3:

Question 2: *What is the effect of the novel mutation F826L on AR transactivation?*

Most, if not all, of the AR binding co-factors have been isolated by yeast-two-hybrid assays, GST pull-down, or co-immunoprecipitation methodology. DNA binding does not play a role in these methods, whereas there are indications that DNA can influence the AR conformation and co-factor recruitment. In Chapter 4, we describe the set up and the use of an assay with DNA-bound AR as target in which both DNA and AR are expected to play a role in co-factor recruitment. This research was done to answer the following question:

Question 3: *Can existing and novel AR co-factors be isolated when complexed with the AR in an oligonucleotide-based in vitro assay?*

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CHAPTER 2

Phosphorylation and androgen receptor isoforms

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Phosphorylation of androgen receptor isoforms

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Phosphorylation of the human AR (androgen receptor) is directly correlated with the appearance of at least three AR isoforms on an SDS/polyacrylamide gel. However, it is still not clear to what extent phosphorylation is involved in the occurrence of isoforms, which sites are phosphorylated and what are the functions of these phosphosites. The human AR was expressed in COS-1 cells and AR phosphorylation was studied further by mutational analyses and by using reversed-phase HPLC and MS. The reversed-phase HPLC elution pattern of the three isoforms revealed that Ser-650 was phosphorylated constitutively. After *de novo* synthesis, only Ser-650 was phosphorylated in the smallest isoform of 110 kDa and both Ser-650 and Ser-94 were phosphorylated in the second isoform of 112 kDa. The hormone-induced 114 kDa isoform shows an overall increase in phosphorylation of all the isolated peptides. The activities of the Ser–Ala substitution mutant S650A (Ser-650 → Ala) was found to be identical with wild-type AR activation in four different cell lines and three different functional analyses, e.g. transactivation, N- and C-terminal-domain interaction and co-activation by transcriptional intermediary factor 2. This was also found for mutants S94A and S515A with respect to transactivation. However, the S515A mutation, which should eliminate phosphorylation of the potential mitogen-activated protein kinase site, Ser-515, resulted in an unphosphorylated form of the peptide containing Ser-650. This suggests that Ser-515 can modulate phosphorylation at another site. The present study shows that the AR isoform pattern from AR *de novo* synthesis is directly linked to differential phosphorylation of a distinct set of sites. After mutagenesis of these sites, no major change in functional activity of the AR was observed.

Key words: androgen receptor, isoform, MS, phosphorylation, reversed-phase HPLC, transactivation.

INTRODUCTION

The AR (androgen receptor) is a ligand-dependent transcription factor belonging to the family of steroid hormone receptors. Similar to other members of the steroid hormone receptor family including the oestrogen receptor, progesterone receptor, glucocorticoid receptor and mineralocorticoid receptor, the AR becomes activated after ligand binding. This results in stabilization, a conformational change and tight nuclear binding of the receptor and, eventually, in a positive and/or negative transcription regulation of target genes.

Ligand binding is not the only regulatory event in the functions of steroid hormone receptors. Post-translational modification of steroid hormone receptors by proteins participating in other signal-transduction pathways plays a role in the regulation as well. Phosphorylation of oestrogen receptor α influences transactivation and association with the co-activators p160 and p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein] [1–3] or with the co-repressors N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) [4]. Transactivation of the glucocorticoid receptor is also regulated by phosphorylation [5–7]. A hormone-dependent phosphosite in the progesterone receptors A and B plays a role in receptor degradation [8], transactivation [9] and nuclear export [10,11]. Thus phosphorylation of specific sites in steroid hormone receptors has been shown to play a role in various processes.

Post-translational modifications such as acetylation and sumoylation have been shown to influence the transactivation potential of the AR [12,13]. However, it is not clear whether phosphorylation has an effect on the properties and activity of the AR. It has been shown that the AR is a phosphoprotein [14,15] and extra phosphorylation of the AR is induced when cells are exposed to androgens, in addition to the so-called basal AR phosphorylation observed in the absence of androgens [15,16]. Phosphorylation occurs predominantly at serine residues [16,17], which are mainly located in the N-terminal domain [18]. Furthermore, phosphorylation is correlated with the three AR isoforms that appear on an SDS/polyacrylamide gel [19]. Within minutes after the start of *de novo* synthesis, the AR appears as a 110 kDa isoform, whereas generation of the second (112 kDa) isoform follows within 15 min as shown by radioactive methionine-labelling studies [20,21]. Only after hormone binding does the third (114 kDa) isoform appear [19]. The AR isoform pattern is correlated with AR phosphorylation as was shown in previous studies by using phosphatases. The dephosphorylation of AR by phosphatases resulted in the loss of one isoform either in the presence or absence of hormone [19]. This effect was also observed when AR phosphosites were mutated [19]. Furthermore, several phosphorylation sites have been identified. The first identified phosphosites, Ser-81, Ser-94 and Ser-650, were found by mutagenesis analyses in combination with SDS/PAGE [19,22]. Ser-308 was the first phosphosite identified by mutagenesis and MS [23]. Ser-16, Ser-81, Ser-94, Ser-256, Ser-308, Ser-424

Abbreviations used: AR, androgen receptor; CHO cell line, Chinese-hamster ovary cell line; dcc, dextran-coated charcoal; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MMTV, murine-mammary-tumour virus; RP, reversed phase; TIF2, transcriptional intermediary factor 2; WT, wild-type.

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and Ser-650 were all identified and confirmed as phosphosites by mutagenesis, peptide mapping and MS [16]. However, cell-free *in vitro* phosphorylation reaction studies on AR mutants also demonstrated Ser-213, Ser-515 and Ser-791 to be phosphosites [24–26].

It is still not clear to what extent phosphorylation is involved in the appearance of isoforms and which sites in the different isoforms are phosphorylated. In the present study, phosphorylation of the isoforms was further explored by studying the differential phosphorylation of the three AR isoforms. An attempt was made to identify all the phosphosites. Furthermore, the consequences of transcriptional activation of the identified and potential phosphosites were evaluated using functional assays, which tested the transactivation, N- and C-terminal-domain interaction and co-activation by TIF2 (transcriptional intermediary factor 2), and the functional assays were performed in COS-1 and CHO (Chinese-hamster ovary) cell lines and two prostate cancer cell lines (PC-3 and DU-145). In contrast with a previous study, which showed that Ser-94 was constitutively phosphorylated [16], our study showed that the Ser-650 is constitutively phosphorylated.

MATERIALS AND METHODS

Materials

Phosphate-free DMEM (Dulbecco's modified Eagle's medium) and goat anti-mouse agarose beads were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Media (GlutaMAX I-supplemented DMEM/F12 and RPMI 1640) and filtertop culture flasks were purchased from Invitrogen (Paisley, U.K.). Multiwell tissue culture plates were obtained from Nunc (Roskilde, Denmark). FCS (fetal calf serum) was obtained from Greiner (Frickenhausen, Germany) and the mixture of penicillin and streptomycin was from BioWhittaker (Walkersville, MD, U.S.A.). The oligonucleotides used in plasmid construction and sequencing were synthesized by Eurogentec (Liege, Belgium). FuGENE 6, Complete inhibitor EDTA-free, shrimp alkaline phosphatase and Rapid DNA Ligation kits were obtained from Roche (Basel, Switzerland) and Quik Change Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA, U.S.A.). Steady-Glo Luciferase assay system and sequencing-grade modified trypsin (specific activity, 16 000 units/mg) were obtained from Promega (Madison, WI, U.S.A.). [^{32}P]P_i was from Amersham Biosciences (Uppsala, Sweden). F39.4.1 is a mouse monoclonal antibody raised against amino acids 301–320 of the human AR [27] and SP197 is a rabbit polyclonal antibody [18]. The 10× Tris/glycine/SDS electrophoresis buffer and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). HPLC reagents were of sequencing grade and obtained from Merck (Darmstadt, Germany). Deltapack C18 column was purchased from Waters and C-18 Z-tips were from Millipore (Milford, MA, U.S.A.). NEN Life Science Products (Boston, MA, U.S.A.) supplied R1881 (methyltrienolone). MMTV-Luc reporter plasmid (where MMTV stands for murine-mammary-tumour virus) was kindly provided by Dr R. Dijkema (Organon, Oss, The Netherlands) and has been described previously [28]. SpeedVac concentrator was obtained from Thermo Savant (Division of Thermo Electron Corporation, Waltham, MA, U.S.A.).

Plasmid construction

Plasmid construction was performed according to standard methods [29] and, where indicated, the plasmids were rendered blunt-ended with Klenow. Constructs including a PCR-amplification step for preparation were sequenced to verify the correct reading frame and the absence of random mutations. All the AR amino acid numbers used in the present study are based on the National Center for Biotechnology Information accession number AAA51729, which refers to the AR consisting of 919 amino acids [30]. The mutants AR S650A (Ser-650 → Ala) and AR S515A were constructed by site-directed mutagenesis using PCR DNA amplification techniques. The following sense oligos were used for introducing the substitution with a Quik Change: AR S650A, 5′-CCAGCACCACC*GC*CCCCACTGAG-3′; and AR S515A, 5′-CCTATCCC*GC*TCCCACTTGT-3′. The mutations are indicated in italics and underlined. AR104 was described previously as pSVAR-104, which is the AR construct containing sequences encoding the C-terminal amino acids 537–919 [31]. The N-terminal construct, pSVAR(TAD_{1-494}), consists of the AR amino acids 1–503 [32]. AR104/S650A was prepared by the digestion of BHEXARS650A with *Sac*I and ligating the fragment into AR104 by using the Rapid DNA Ligation kit. TIF2 is described as a co-activator in [33,34].

COS-1 cell culture, transfection, metabolic labelling with [^{32}P]P_i and immunoprecipitation

COS-1 cells were cultured in DMEM/F12 supplemented with GlutaMAX I and 5 % (v/v) FCS treated with dextran-coated charcoal (dcc-FCS). For steady-state labelling, 1.5×10^6 COS-1 cells were grown overnight in an 80 cm^2 flask with 8 ml of DMEM/F12, followed by transfection with the following mix: 4 μg of the indicated plasmid with 12 μl of FuGENE 6 transfection reagent in 200 μl of serum- and antibiotics-free DMEM/F12. Transfections were performed according to the manufacturer's instructions for FuGENE 6. After 30 h, cells were washed twice with 0.9 % (w/v) NaCl and incubated in phosphate-free DMEM, supplemented with 50 mM Hepes buffer and 5 % dcc-FCS, which had been dialysed for 24 h against 0.9 % NaCl. Subsequently, cells were incubated with R1881 as indicated and [^{32}P]P_i (0.333 mCi/ml) for 16 h. Cells were harvested and lysed at 4 °C in immunoprecipitation buffer A [40 mM Tris/HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 % (v/v) glycerol, 10 mM sodium phosphate, 10 mM sodium molybdate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 0.6 mM PMSF, 0.5 mM Bacitracin, Complete inhibitor EDTA-free and 10 mM dithiothreitol], supplemented with 1 % (v/v) Triton X-100, 0.5 % (w/v) deoxycholic acid and 0.08 % (w/v) SDS. Subsequently, the lysate was centrifuged at 100 000 ***g*** for 30 min at 4 °C. The supernatant was then incubated at 4 °C with the antibody F39.4.1, which was linked to goat anti-mouse agarose. After 2 h, the agarose beads were washed as follows: three times with buffer A supplemented with 1 % Triton X-100, 0.5 % deoxycholic acid and 0.08 % SDS, three times with buffer A supplemented with 0.2 % Triton X-100 and 0.4 M NaCl and three times with buffer A without any additions. The immunoprecipitated AR was separated by SDS/PAGE (7 % gel). After fixing the gel in 10 % (v/v) acetic acid and 50 % (v/v) methanol, the gel was subjected to Coomassie Blue staining and destaining. Subsequently, the AR band was excised from the gel and digested with sequencing-grade modified trypsin.

In-gel digestion and RP (reversed-phase) HPLC analysis

The excised AR spot was in-gel-digested as described by Shevchenko et al. [35] with 20 units of sequencing-grade modified trypsin for 16 h at 37 °C. The amount of trypsin necessary to secure full digestion of the higher amount of AR protein in the presence of hormone was verified by varying the amount. The

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peptides were extracted as described by Shevchenko et al. [35] and dried in a SpeedVac for 1.5 h. The peptides were dissolved in 0.1 % (v/v) trifluoroacetic acid. Then, 25 μl of this solution was applied to a 2 mm × 150 mm Waters Deltapack C18 column. The flow was set to 0.18 ml/min and fractions were collected every 1.5 min until a gradient of 18 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid was reached.

Peptide gel

A 40 % (w/v) acrylamide alkaline peptide gel was cast and run as described by West et al. [36].

Characterization of the HPLC fractions with MS

AR tryptic peptides were separated by RP-HPLC. The fractions with retention times corresponding to ^{32}P-labelled tryptic phosphopeptides were collected and, after drying in a SpeedVac, they were dissolved in 20 μl of 50 % acetonitrile. With electrospray ionization MS and MS/MS, data were collected from the individual fractions using a Q-TOF (Micromass, Wythenshaw, Manchester, U.K.). The peptides were directly infused in the Q-TOF with a nanospray needle (Micromass) containing a 3 μl sample plus 0.3 μl of 10 % (v/v) formic acid. Low-energy collision-induced dissociation of selected precursor ions was used to obtain fragmentation spectra. These were deconvoluted (Masslynx software; Micromass) and used to identify the corresponding tryptic peptides, including modifications, of the AR.

CHO, PC-3, DU-145 and COS-1 cell culture, transfection and luciferase assay

CHO and COS-1 cells were maintained in DMEM/F12 culture medium, supplemented with 5 % dcc-FCS. PC-3 and DU-145 were cultured in RPMI 1640 medium, supplemented with 5 % FCS. For transcription activation experiments, the cells were plated in 24-well plates at a density of 2×10^4 cells/well (1.9 cm^2) in 500 μl of either DMEM/F12 or RPMI 1640 and grown overnight. Cells were transfected using 100 μl of either serum- and antibiotics-free DMEM/F12 or RPMI 1640 containing FuGENE 6 (FuGENE/DNA ratio of 3:1) with AR expression plasmids and, where indicated, with TIF2 reporter plasmids (50 ng/well) and pTZ19 carrier plasmid to a total DNA concentration of 250 ng/well. After 5 h, R1881 was added, followed by an overnight incubation; at the end of this incubation, the cells were harvested for a luciferase assay. Then, 50 μl of lysis buffer [25 mM Tris/phosphate (pH 7.8), 15 % glycerol, 1 % Triton X-100, 8 mM MgCl and 1 mM dithiothreitol] was added to the cells. After incubation for 10 min, 25 μl of the supernatants were transferred to white non-transparent 96-well assay plates, and 25 μl of 16 mg Steady-Glo Luciferase assay substrate per ml of Steady-Glo luciferase assay buffer was added. Luciferase activity was measured with a TopCount luminometer (Packard Bioscience, PerkinElmer Life Sciences, Zaventem, Belgium).

Western blotting

COS-1 cells were plated at a density of 1×10^6 cells/80 cm^2 flask and transfected with 4 μg of AR expression plasmid and 12 μl of FuGENE. After an overnight incubation with hormones, the cells were washed once with PBS, and immunoprecipitation buffer (see the COS-1 cell culture subsection), supplemented with protease inhibitors, was added. Lysates were centrifuged for 10 min at 400 000 ***g*** and AR was immunoprecipitated with the monoclonal antibody F39.4.1. Next, samples were subjected to SDS/PAGE and blotted on to a nitrocellulose membrane. AR was immunoblotted with the AR polyclonal antibody SP197 and visualized by chemiluminescence detection or by using an alkaline phosphatase-conjugated secondary antibody.

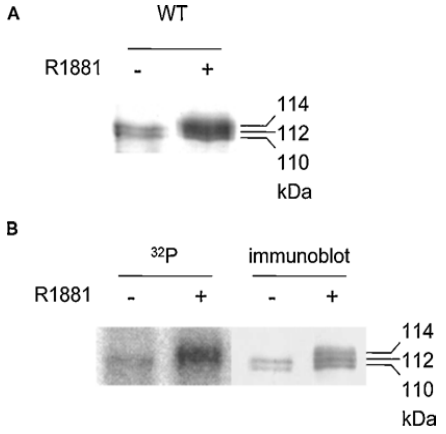

Figure 1 AR isoforms on a Coomassie Blue-stained SDS/polyacrylamide gel

WT AR was expressed in COS-1 cells and labelled with [^{32}P]P_i for 16 h in the absence or presence of 10 nM R1881 for 16 h. The same amount of total lysate of the two conditions was used for AR immunoprecipitation with the monoclonal antibody F39.4.1. The immunoprecipitated AR was separated by SDS/PAGE (7 % gel) and Coomassie Blue-stained (**A**) or blotted and immunostained with the polyclonal antibody SP197 (**B**). The corresponding autoradiogram is also shown in (**B**).

RESULTS

AR isoform expression

To verify the AR isoform pattern on an SDS/polyacrylamide gel, WT (wild-type) human AR was expressed by transient transfection into COS-1 cells and metabolically labelled with [^{32}P]P_i either in the absence or presence of the synthetic androgen R1881. The same amount of total lysate of the two conditions was used for AR immunoprecipitation. After immunopurification, the gel was stained with Coomassie Blue. In the absence of R1881, the two isoforms (110 and 112 kDa) were clearly visible and the presence of R1881 resulted in the appearance of the third isoform of 114 kDa (Figure 1A).

Lysates obtained by the same procedure as the previous experiment were also immunoprecipitated and blotted. The immunoblot showed the same isoform pattern (Figure 1B) as the Coomassie Blue-stained gel. An autoradiogram of the corresponding immunoblot showed an upshift of a phosphorylated band and an increase in phosphorylation (Figure 1B). This increase is partly due to stabilization of the AR. These results confirm that the AR is phosphorylated in the absence of hormone and that phosphorylation of the AR is increased in the presence of hormone [16]. Furthermore, this suggests that the upshift of a phosphorylated band corresponds to the 114 kDa isoform.

Changes in AR phosphorylation induced by R1881

To investigate whether androgenic activation increases the phosphorylation of existing phosphosites and/or induces phosphorylation of new sites, the AR was expressed by transient transfection into COS-1 cells and metabolically labelled with [^{32}P]P_i either in

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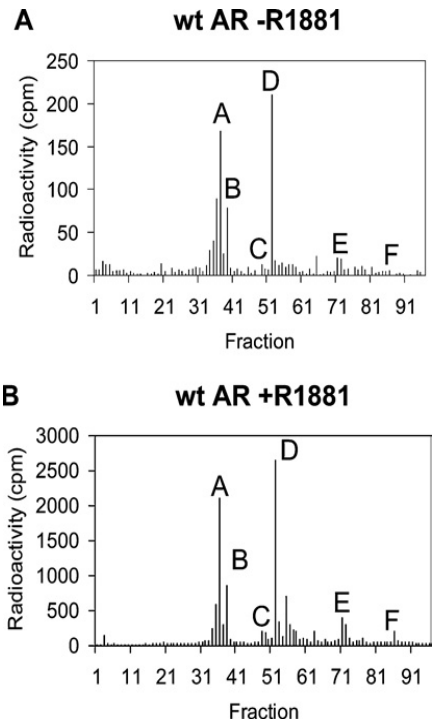

Figure 2 R1881-induced changes in AR phosphorylation pattern

AR was expressed in COS-1 cells and labelled with [^{32}P]P_i for 16 h in the absence (**A**) or presence (**B**) of 10 nM R1881. The same amount of total lysate of the two conditions was used for AR immunoprecipitation (with anti-AR monoclonal antibody F39.4.1). The precipitated AR was digested with trypsin and the peptides obtained were separated on an RP HPLC C18 column. HPLC fractions were collected and the amount of [^{32}P]P_i incorporated was determined. The different radioactive fractions are indicated with A–F. Note that the ordinates have a different scale.

the absence or presence of R1881. After immunopurification and subsequent digestion with trypsin, the resulting peptides were separated on an RP HPLC column. The RP HPLC elution pattern of AR in absence of R1881 showed that several fractions contained ^{32}P-labelled peptides (Figure 2A, fractions A, B, D and E). After stimulating the cells with 10 nM R1881, an increase in phosphorylation of the peptides in fractions A, B, D and E was observed (Figure 2B). In addition, the relative phosphorylation level of two peptides in fractions C and F was slightly increased (Figure 2B). It is important to note that the overall phosphorylation pattern was highly reproducible in ten independent experiments. These results are in agreement with a previous report that hormone binding results in increased phosphorylation of existing phosphorylated sites [16].

Differential phosphorylation of AR isoforms

To study differential phosphorylation of the three isoforms of 110, 112 and 114 kDa in more detail, COS-1 cells were transfected with AR and stimulated for 16 h with R1881 and labelled with [^{32}P]P_i. Each ^{32}P-labelled AR isoform was isolated separately from an SDS/polyacrylamide gel. The phosphorylation pattern of tryptic fragments of the individual isoforms revealed that the 110 kDa isoform is predominantly phosphorylated on the peptide in fraction D (Figure 3A). The phosphorylation level of fraction

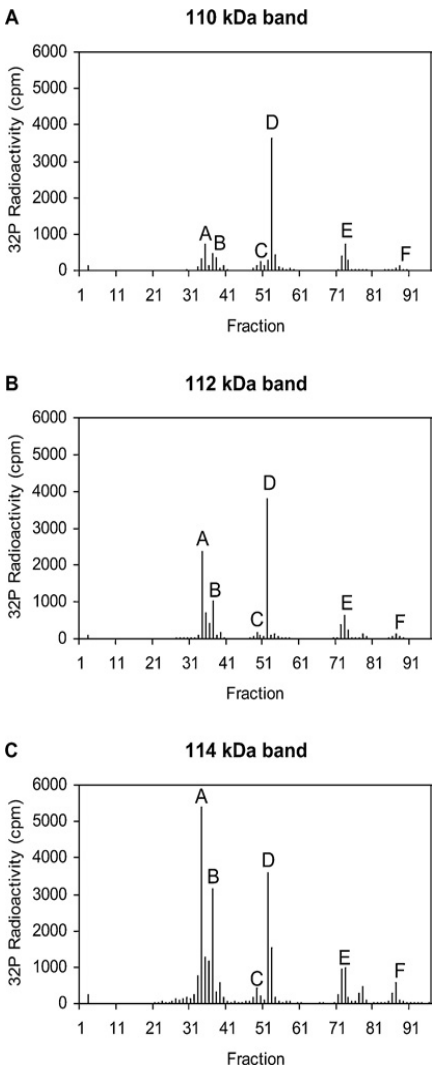

Figure 3 Differential phosphorylation of AR isoforms

AR was expressed in COS-1 cells and labelled with [^{32}P]P_i for 16 h in the presence of 10 nM R1881. AR was immunoprecipitated (with anti-AR monoclonal antibody F39.4.1) and the isoforms of 110 kDa (**A**), 112 kDa (**B**) and 114 kDa (**C**) were separately cut out from the same lane on an SDS/7 % polyacrylamide gel followed by trypsin digestion. The peptides obtained were separated on an RP HPLC column. HPLC fractions were collected and the amount of [^{32}P]P_i incorporated was determined. The different radioactive fractions are indicated with A–F.

D in the 112 kDa isoform was similar to that in the 110 kDa isoform (Figures 3A and 3B) and slightly increased further in the 114 kDa isoform (Figure 3C). Since fraction D contains the peptide with the phosphorylated Ser-650 (see below), these results indicate that Ser-650 is constitutively phosphorylated.

The phosphorylation level of the different RP HPLC fractions for each isoform was found to be different and to get an impression of quantitative changes, the fold increase has been calculated. First, the phosphorylation level of each peptide is based on the sum of the radioactivities in three consecutive fractions. Since phosphorylation of fraction D was shown to be more or less

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Table 1 Phosphorylation ratios of AR peptides

The phosphorylation ratio of different fractions is calculated relative to the intensity of phosphorylation of fraction D (1.0, see also text).

	Phosphorylation				
HPLC fractions	Fraction X/D (110 kDa isoform)	Fraction X/D (112 kDa isoform)	Fold increase (relative to 110 kDa)	Fraction X/D (114 kDa isoform)	Fold increase (relative to 110 kDa)
A	0.3	0.8	2.9	1.4	5.1
B	0.2	0.4	1.9	0.9	4.3
C	0.1	0.1	0.7	0.2	1.3
D	1.0	1.0	1.0	1.0	1.0
E	0.3	0.3	1.0	0.4	1.2
F	0.1	0.1	1.1	0.2	2.9

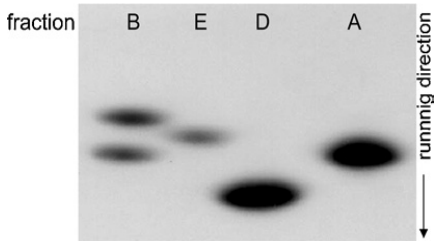

Figure 4 Separation of individual HPLC fractions on a 40% acrylamide alkaline peptide gel

Peptides from HPLC fractions were separated on a 40% acrylamide alkaline peptide gel. Note that the separation is according to size and charge. Phosphopeptides were visualized by using a PhosphorImager (Molecular Dynamics). The different radioactive fractions are indicated with A, B, D and E.

constant in the different isoforms, the level of phosphorylation in the other fractions was calculated relative to the level of phosphorylation in fraction D. Furthermore, the change in ratios for the 112 and 114 kDa isoforms were calculated relative to the ratios for the 110 kDa isoform, resulting in fold increase (Table 1). The fold increase of the fractions A, B, C, E and F obtained from the 110 kDa isoform were low. For the 112 kDa isoform, the fold increase of fractions A and B was 2–3-fold higher (Table 1). The fold increase of fractions C, E and F was the same in the 112 kDa isoform compared with the 110 kDa isoform.

Moreover, analysis of the 114 kDa isoform showed that, in the presence of R1881, the fold increase of fractions C and E was rather small, but the fold increase of phosphorylation in fractions A, B and F were higher (Table 1). These results confirm that a correlation exists between phosphorylation status and the SDS/PAGE migration pattern for the three AR isoforms [19]. In conclusion, increase in phosphorylation is correlated with a decreased migration rate.

Phosphopeptide analysis

To characterize the HPLC fractions in more detail, the most intensely phosphorylated fractions (A, B, D and E) were subjected to further analysis on a 40% acrylamide alkaline peptide gel. Peptide analysis revealed that fractions A, D and E each contained a single phosphorylated peptide and that each peptide was different from the others (Figure 4). However, fraction B contained two phosphorylated peptides. The peptide in fraction B, which migrated into the gel as far as the peptide in fraction A, is most probably identical with the phosphorylated peptide from fraction A and is present in fraction B due to incomplete resolution during HPLC separation. These data show that at least four different peptides derived from the AR are phosphorylated.

Peptide analysis by MS

To identify the phosphorylated amino acid residues, MS analysis was used to characterize first the phosphorylated peptides in the HPLC fractions. Out of the six HPLC fractions collected (fractions A–F), five contained tryptic peptides from the AR (Table 2). The identified peptides in fractions B, D and E were

Table 2 MS data of phosphorylated peptides in RP HPLC fractions

Fraction	Measured mass	Tryptic peptide fragment*	AR peptide	Putative phosphosite	Prediction†
A	962.4	T70–T71 Cys-852 with acrylamide adduct	847–854	Ser-851	0.962
B	2056.8	T7–T8 plus phosphate probably on Ser-94	84–100	Ser-94	0.572
C	n.d.‡				
D	2232.0	T50 plus phosphate probably on Ser-647 or Ser-650	639–658	Ser-647 Ser-650	0.760 0.997
E	1226.6	T28 plus phosphate probably on Ser-515; Cys-518 with carbamidomethyl	511–520	Ser-515	0.967
F	1554.7	T15	221–235	§	

* Peptide identification is based on comparison of the detected mass and the corresponding MS/MS information of the measured peptides with all possible tryptic fragments (T1–T79).
† NetPhos 2.0 [40] was used to search for possible phosphorylation sites. A prediction of 0.5 and higher was considered as representing a potential phosphorylation site.
‡ n.d., not determined.
§ No potential phospho-serine residue was present in the peptide.

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phosphorylated. The theoretical mass of these peptides was increased by the mass of one phosphate group (79.9799 Da) and, moreover, MS/MS data confirmed the presence of this modification, probably on a serine residue. The MS/MS data from fraction D could not discriminate between two possible serine phosphosites (Ser-647 and Ser-650). Both of the identified tryptic AR peptides in fractions A and F contained a serine residue, but were not phosphorylated. The results are summarized in Table 2 together with information on possible putative phosphosites. A small number of the fractions displayed a variable low level of phosphorylation, most probably due to contaminations, and were excluded from further analyses.

Phosphosite identification by site-directed mutagenesis

The peptide in fraction D (amino acid residues 639–658) contains two potential phosphorylation sites, Ser-647 and Ser-650 (Table 2). Substitution of Ser-650 with an alanine residue resulted in the disappearance of peptide phosphorylation in fraction D (Figure 5A). In contrast, substitution of Ser-647 to Ala-647 did not result in any change in phosphorylation (results not shown). This substantiates the observation that fraction D contains the peptide consisting of amino acids 639–658 and shows that Ser-650, but not Ser-647, is a phosphorylation site. Another peptide identified by MS and consisting of amino acid residues 84–100 (fraction B) contains the potential phosphosite Ser-94 (Table 2). Substitution of Ser-94 to Ala-94 resulted in a 40% loss of radioactive phosphate incorporation in both fractions A and B (Figure 5B and Table 3). A third phosphorylated peptide identified in these experiments is the peptide from fraction E consisting of amino acid residues 511–520 and containing the potential phosphosite Ser-515. Remarkably, substitution of Ser-515 to Ala-515 did not result in disappearance of radioactivity from fraction E. In contrast, fraction D, which contained the peptide with phosphosite Ser-650, was no longer radioactive after the Ser-515 to Ala-515 substitution (Figure 5C). This result indicates that changes in Ser-515 might exert an influence on the phosphorylation status of Ser-650. In fraction A, the peptide consisting of amino acids 847–854 has the predicted phosphosite Ser-851. Substitution of Ser-851 with an alanine residue did not result in a change in the phosphorylation pattern (results not shown). This indicates that the identified peptide consisting of amino acids 847–854 present in fraction A does not contain the predicted Ser-851 phosphosite. No phosphosites were predicted for the identified peptide present in fraction F and no peptides could be detected in fraction C.

Phosphorylation and AR transcriptional activity

Since the phosphorylation level of the AR was changed in the presence of R1881, AR phosphorylation might regulate the functional activity of the AR. The transactivation activity of AR mutants S515A, S650A and S94A was tested using an MMTV-Luciferase reporter assay in CHO cells. Substitution of Ser-650 to Ala-650 did not result in a change in transactivation of the MMTV-Luc reporter gene when compared with the WT AR (Figure 6A). The same neutral effect was found for the S515A and S94A mutants (results not shown). To exclude the possibility that CHO cells exert AR phosphorylation in a peculiar cell-type-specific manner, the same experiment was performed in COS-1 as well as PC-3 and DU-145 cells (both prostate cancer cell lines). No differences in AR transactivation activity for the three AR mutants could be detected in any of these three cell lines (results not shown). These results indicate that loss of AR phosphorylation on Ser-650, Ser-515 and Ser-94 does not have an effect on AR transactivation activity.

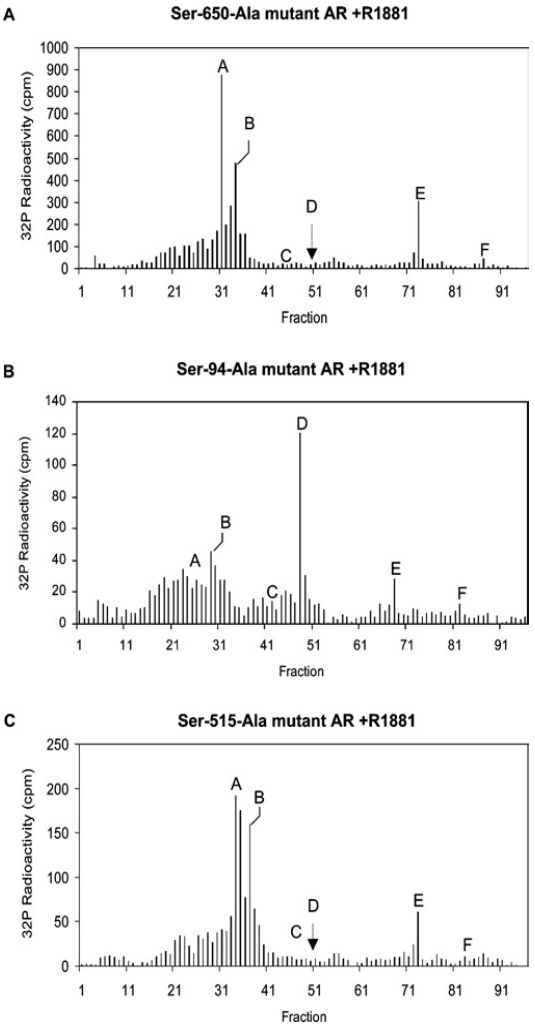

Figure 5 AR phosphorylation on Ser-650 and Ser-94 and the phosphorylation influenced by Ser-515

The AR mutant S650A (**A**), S94A (**B**) or S515A (**C**) was expressed in COS-1 cells and labelled with [^{32}P]P_i for 16 h in the presence of 10 nM R1881. The AR mutant was immunoprecipitated (with anti-AR monoclonal antibody F39.4.1) and digested with trypsin. The peptides obtained were separated on an RP HPLC C18 column. HPLC fractions were collected and the amount of [^{32}P]P_i incorporated was determined.

Table 3 Phosphorylation of S94A mutant

The phosphorylation of peptides in fractions A and B from the WT AR (Figure 2B) and the S94A mutant (Figure 5B) were normalized to their corresponding fractions D. Percentage change in phosphorylation between the mutant and WT for fractions A and B was calculated from the ratios.

	Phosphorylation		
Ratio	WT AR	Mutant S94A	Change in phosphorylation (%)
A/D	0.65	0.38	−42
B/D	0.42	0.23	−44

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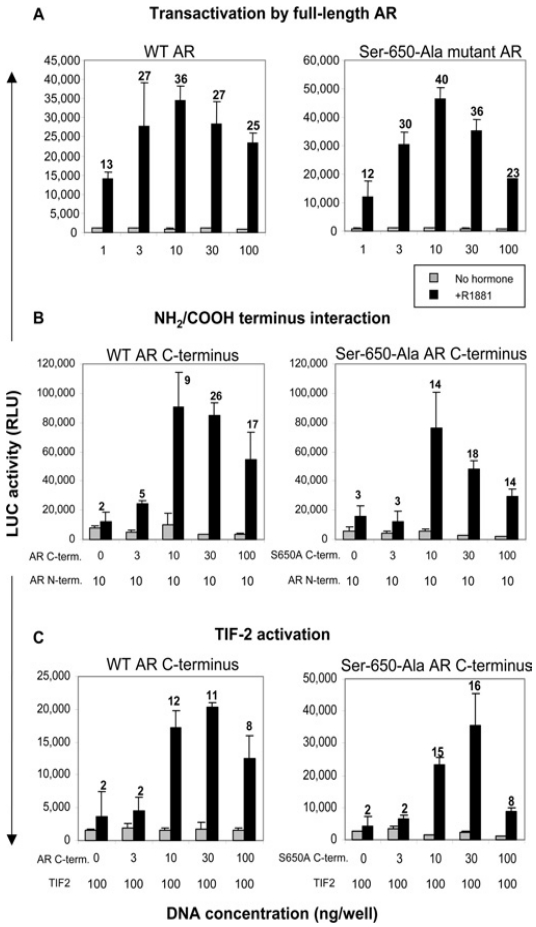

Figure 6 Functional studies on WT AR and the AR mutant S650A

CHO cells were transfected with 50 ng of reporter construct MMTV-Luc, different amounts of DNA plasmids from either the AR or AR mutant S650A (**A**), C-terminal construct AR104 or C-terminal mutant construct AR104/S650A together with 10 ng of N-terminal construct pSVAR(TAD_{1-494}) (**B**) or 100 ng of TIF2 cDNA (**C**). The cells were harvested 16 h after treatment with ethanol or 1 nM R1881. Fold induction is shown at the top of each bar and represents the ratio of activity determined after incubation in the presence or absence of R1881.

The AR protein can undergo conformational changes resulting in intra-molecular interaction between the N- and C-terminal domains [32,37] and this N- and C-terminal interaction can be influenced by several mutations in the AR, resulting in altered transcriptional activation activity of the AR [38,39]. To examine whether phosphorylation exerts an influence on this interaction, the C-terminal-domain construct containing the S650A mutant and the N-terminal-domain construct were co-transfected in CHO cells. As shown in Figure 6(B), the S650A mutant displayed a similar functional N- and C-terminal interaction as the WT AR C-terminal construct.

To investigate whether phosphorylation has an influence on TIF2 co-activation, CHO cells were co-transfected with constructs of AR C-terminal domain with or without the S650A substitution and TIF2. The mutant showed a similar functional interaction with TIF2 as the WT AR C-terminal domain (Figure 6C).

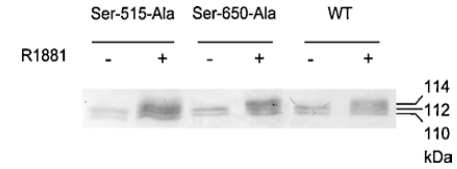

Figure 7 Immunoblot of S650A and S515A AR mutant isoforms

AR S650A and S515A mutants were expressed in COS-1 cells in the absence or presence of 10 nM R1881 for 16 h. The AR mutants were immunoprecipitated with the monoclonal antibody F39.4.1, separated by SDS/PAGE (7% gel), blotted and immunostained with the polyclonal antibody SP197.

AR isoforms on SDS/PAGE

It has been shown that substitution of the phosphosite Ser-94 by an alanine residue results in a loss of isoforms both in the absence and presence of hormone [19]. To determine whether there is a change in isoform pattern of the AR mutants S650A and S515A, these mutants were expressed in COS-1 cells and immunoprecipitated. The precipitated AR mutants were separated by SDS/PAGE. The isoform patterns of both mutants were similar to the WT AR (Figure 7). Both mutants expressed two isoforms in the absence of hormone and three isoforms in the presence of R1881. This indicates that Ser-515 and Ser-650 are not essential for the migration of 112 and 114 kDa isoforms in the absence or presence of R1881. Furthermore, these results demonstrate that loss of phosphorylation on certain sites does not always result in a change in isoform pattern.

DISCUSSION

The present study shows that the AR isoform pattern after AR *de novo* synthesis is directly linked to differential phosphorylation. It appeared that, after synthesis, the AR 110 kDa isoform is predominantly phosphorylated at Ser-650, and there is a higher phosphorylation level for other existing sites in the 112 and 114 kDa isoforms. These results reveal that Ser-650 is constitutively phosphorylated. Moreover, loss of phosphorylation on certain sites does not always result in a change in isoform pattern. Functional analysis of the identified phosphosites Ser-94, Ser-650 and Ser-515 revealed that substitution of these sites with alanine does not influence AR function.

Recently, several AR phosphosites were identified after transient expression in COS-1 cells, by sequencing AR peptides using tandem MS and Edmann degradation [16]. Our approach was different, i.e. RP HPLC was used instead of two-dimensional thin-layer electrophoresis and ascending chromatography. With the purified fractions of the tryptic peptides containing possible phosphosites, tandem MS can be a good tool for identification. However, it is not always possible to identify the exact position of the phosphate group. Fragmentation data may lack the specific fragment ions containing the phosphate group owing to the individual fragmentation behaviour of the peptide or by loss of their positive charges during the collision-induced dissociation. Although all fractions contained radioactivity, only three phosphopeptides could be identified. A possible explanation is that the concentration of the peptides containing the phosphate group is too low or the peptides containing the phosphate group are not easily ionized with electrospray. MS is a valuable tool, but additional information, such as site-directed mutagenesis in this case, is necessary.

The autoradiogram and the HPLC elution pattern revealed that the phosphorylation was increased approx. ten times. A previous

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study showed that hormone binding results in an increased phosphorylation of existing phosphorylated sites by approx. 1.8 times [15]. This indicates that the apparent extra phosphorylation of approx. five times results from stabilization by hormone binding.

The phosphorylation pattern of the isoforms revealed that Ser-650 is already phosphorylated in the 110 kDa isoform and Ser-94 in the 112 kDa isoform. However, these phosphorylation patterns were studied in the presence of R1881, which raises the question whether Ser-650 is phosphorylated in the 110 kDa isoform and Ser-94 in the 112 kDa isoform in the absence of R1881 as well. However, the RP HPLC elution pattern of the AR in the absence of R1881 also showed the presence of phosphorylation in fractions A, B and D (Ser-94 and Ser-650; Figure 2A). It is therefore probable that the phosphorylation pattern of the 110 and 112 kDa isoforms in absence of R1881 is the same as that in the presence of R1881. Moreover, this suggests that phosphorylation of fraction D in the 110 kDa isoform and phosphorylation of fractions A and B in the 112 kDa isoform are not hormone-induced.

Surprisingly, site-directed mutagenesis of the Ser-515 to an alanine residue revealed dephosphorylation of a totally different site, Ser-650. This type of distal influence on phosphorylation caused by the substitution of another site has been observed by others [16]. Gioeli et al. [16] suggested that substitution of Ser-424 with an alanine residue resulted either in the mobility shift of a phosphorylated peptide or in phosphorylation of new sites. In the present study, the dephosphorylation of Ser-650 induced by the S515A substitution might be due to a conformational change, which resulted in a surface more accessible for phosphatases or less accessible to kinases. Interestingly, both sites are flanking the DNA-binding domain and are located in flexible regions of the AR protein.

The reason why substitution of Ser-515 with an alanine residue does not result in the disappearance of phosphorylation in fraction E might be that other serine residues in this peptide are phosphorylated as well. Although these serine sites are not predicted as potential phosphosites by NetPhos [40], it predicts that Tyr-513 is a phosphorylation consensus site [40]; however, two-dimensional phosphopeptide mapping studies have shown that only serine residues in the AR are phosphorylated [16,17]. Substitution of Ser-94 with an alanine residue resulted in a partial decrease in phosphorylation level (40%) in fractions A and B. This suggests that one phosphopeptide of the two in fraction B as seen on the peptide gel was identical with a peptide with the same mass and charge in fraction A. In addition, this also suggests that there might be another peptide present in both fractions. Alternatively, the presence of a partially digested peptide containing Ser-94 is excluded. The partially digested peptide would consist of amino acids 41–99 and contains the identified phosphosite Ser-81 [16]. However, the peptide will be very large and, therefore, difficult to elute from an SDS/polyacrylamide gel. Furthermore, the presence of two phosphopeptides only in fraction B and not in fraction A is in favour of a complete digestion.

Substitution of Ser-851 with an alanine residue did not result in the disappearance of radioactive phosphate in fraction A. Partial digestion of AR, resulting in a peptide containing Ser-851 and another potential phosphorylation site, is unlikely, since this would result in a very large peptide that cannot be eluted from a gel. Substitution of Ser-851 did not result in a change of phosphorylation in fraction A or other fractions (results not shown), which indicates that Ser-851 is not a phosphosite at all. The presence of another phosphopeptide cannot be excluded.

A change in AR phosphorylation appears to have no prominent function in AR transactivation. Similar findings were reported by Gioeli et al. [16] by using the PSA-Luc reporter construct in CV1 cells. In contrast, Zhou et al. [22] showed a decreased transactivation of the mutant S650A of 10–30% on the MMTV-Luc reporter also in CV1 cells.

It appears that phosphorylation does not play a major role in hormone-induced AR N- and C-terminal interaction or TIF2 coactivation, although it cannot be excluded that the cellular context as well as the reporter construct could influence this activity and that other so far unidentified phosphosites are involved [41].

After AR *de novo* synthesis, the 110 kDa isoform became immediately and predominantly phosphorylated on Ser-650. The 112 kDa isoform displayed an additional phosphorylation of Ser-94 and another peptide in fractions A and B only. The relationship between phosphorylation of Ser-94 and the appearance of the 112 kDa isoform corresponds to an immunoblot study in which S94A caused disappearance of the 112 kDa isoform in the absence of hormone and disappearance of the 114 kDa isoform in the presence of hormone [19].

In contrast with S94A AR mutant, substitution of Ser-650 with an alanine residue does not influence the isoform pattern on SDS/PAGE, which is understandable because Ser-650 phosphorylation occurs already in the 110 kDa isoform and is unchanged in the 112 kDa isoform and slightly changed in the 114 kDa isoform. The Ser-650 site represents basal phosphorylation of the AR and only changes in phosphorylation of other sites could perhaps contribute to the appearance of the isoforms. Substitution of the phosphosite Ser-81 to a glycine residue resulted in the loss of the largest isoform irrespective of the presence of ligand [19]. Similar results were obtained for the double-mutant S81/94A [22]. However, substitution of Ser-81 with an alanine residue resulted in the loss of one isoform only in the presence of hormone (results not shown). The RP HPLC elution pattern of the three isoforms illustrated the correlation between the phosphorylation status of sites other than Ser-650 and the migration pattern of the three AR isoforms during SDS/PAGE. Phosphorylation does not necessarily contribute to the appearance of isoform as shown by the mutant S650A.

The appearance of the 114 kDa isoform induced by R1881 is directly linked to an overall increase in phosphorylation of several sites as compared with the 110 and 112 kDa isoforms. This overall increase in phosphorylation was shown previously by van Laar et al. [15] and Gioeli et al. [16]. However, newly phosphorylated sites could not be identified. It is quite probable that these sites are important for the hormone-regulated transactivation of the AR, because their phosphorylation is linked to DNA-binding and transcription activation. Furthermore, these phosphorylation sites could be target sites for AR activation via crosstalk with other signal transduction pathways as well. Thus identifying the kinase(s) involved in the phosphorylation of these sites and the possible signalling pathway will be useful to elucidate the mechanisms of ligand-independent activation of the AR.

There are several kinases predicted to be involved in the phosphorylation of AR. The identified phosphosites Ser-94 and Ser-650 are so-called Ser-Pro sites, which can be phosphorylated by serine-proline-directed kinases (Ser/Thr-Pro), MAPK (mitogen-activated protein kinase) and cyclin-dependent kinases such as Cdc2 and Cdks. In addition, Ser-650 is a specific consensus site for casein kinases 1 and 2. There are conflicting data concerning which kinases phosphorylate the AR. It has been shown that protein kinase C [16,28], MAPK and AKT kinase [16] have no influence on the phosphorylation of the AR. However, other *in vitro* kinase studies showed that AKT [24,25] is capable of phosphorylating the AR. Furthermore, MAPK was also a candidate protein kinase of the AR [26]. However, in that particular study, the phosphorylation status of the MAPK site S515A AR mutant was not investigated [26].

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Although basal phosphorylation of the AR does not seem to have a function, it is quite probable that a hormone-regulated phosphorylation of the AR is associated with transcriptional activation. Identification of the hormone-induced AR phosphorylation sites and subsequent elucidation of their possible function by mutational analysis *in vivo* could contribute significantly to our understanding of the mechanism of androgen action. Furthermore, it could reveal new targets for intervention in androgen action in prostate cancer.

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CHAPTER 3

A novel mutation F826L in the human androgen receptor in partial androgen insensitivity syndrome; increased NH2-/COOH-terminal domain interaction and TIF2 co-activation

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A novel mutation F826L in the human androgen receptor in partial androgen insensitivity syndrome; increased NH_2-/COOH-terminal domain interaction and TIF2 co-activation

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ABSTRACT

A novel mutation F826L located within the ligand binding domain (LBD) of the human androgen receptor (AR) was investigated. This mutation was found in a boy with severe penoscrotal hypospadias (classified as 46,XY DSD). The AR mutant F826L appeared to be indistinguishable from the wild-type AR, with respect to ligand binding affinity, transcriptional activation of MMTV-luciferase and ARE_2-TATA-luciferase reporter genes, protein level in genital skin fibroblasts (GSFs), and sub-cellular distribution in transfected cells. However, an at least two-fold higher NH_2-/COOH-terminal domain interaction was found in luciferase and GST pull-down assays. A two-fold increase was also observed for TIF2 (transcription intermediary factor 2) co-activation of the AR F826L COOH-terminal domain. This increase could not be explained by a higher stability of the mutant protein, which was within wild-type range. Repression of transactivation by the nuclear receptor co-repressor (N-CoR) was not affected by the AR F826L mutation. The observed properties of AR F826L would be in agreement with an increased activity rather than with a partial defective AR transcriptional activation. It is concluded that the penoscrotal hypospadias in the present case is caused by an as yet unknown mechanism, which still may involve the mutant AR.

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1. Introduction

The androgen receptor (AR) belongs to the superfamily of nuclear receptors (Mangelsdorf et al., 1995; Beato et al., 1995) and is categorized in the subfamily of ligand-inducible steroid hormone receptors. Like the other steroid hormone receptors, the AR consists of an NH_2-terminal transactivation domain (NTD; amino acids 1–557), a DNA binding domain (DBD; aa 558–623), followed by a flexible hinge region (aa 624–670) and a ligand binding domain (LBD; aa 671–919) (Lubahn et al., 1988).

The NTD and the LBD account for the transcriptional activity of the AR (Brinkmann et al., 1999). The NTD is involved in transactivation via the ligand dependent activation function 1 (AF1) region, which consists of aa 51–217 (Jenster et al., 1991). The LBD harbours the AF2, which is involved in ligand-dependent transactivation, and it also takes part in functional interaction with co-factors (Moras and Gronemeyer, 1998; Jenster et al., 1995). Upon ligand binding, a large group of co-factors can interact via the AF2, which is identified in helix 12 between residues 893 and 900 (Wurtz et al., 1996). For example, TIF2 co-activation occurs when TIF2 binds to this AF2 activation domain (AD) core via its LXXLL leucine motif and thereby enhances AR transactivation (Slagsvold et al., 2000; Heery et al., 1997; Berrevoets et al., 1998; Bevan et al., 1999). Besides

Abbreviations: aa, amino acid; AD, activation domain; AF2, activation function 2; DHT, 5α-dihydrotestosterone; DSD, disorders of sex development; GFP, green fluorescent protein; GSF, genital skin fibroblast; LBD, ligand binding domain; LBP, ligand binding pocket; N-CoR, nuclear receptor co-repressor; NC-TDI, NH_2-/COOH-terminal domain interaction; NTD, NH_2-terminal transactivation domain; T, testosterone; TIF2, transcriptional intermediary factor 2; wt, wild-type.

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co-activators, also co-repressors such as nuclear receptor co-repressor (N-CoR) can bind to the AR, to both the NTD and the COOH-terminal domain (Cheng et al., 2002).

Furthermore, once the ligand is bound, an intra- and/or intermolecular interaction takes place between the NH_2-terminal and COOH-terminal domain of the AR, the so-called NH_2-/COOH-terminal domain interaction (Doesburg et al., 1997; Langley et al., 1995). This interaction, herein abbreviated as NC-TDI, occurs through the FXXLF motif in the NTD (He et al., 2000; Steketee et al., 2002), and also the AF2 AD core plays a role (Berrevoets et al., 1998; Langley et al., 1998; Doesburg et al., 1997; He et al., 1999). Recently, experimental evidence was provided for the preference of the AR binding to FXXLF motifs, by structural predictions based on peptide interactions and on the crystal structure of the LBD containing a bound FXXLF motif containing peptide (He et al., 2004; Dubbink et al., 2004).

The influence on AR target gene expression will change dramatically, if the AR is not functioning properly due to mutations. Mutations resulting in decreased ligand affinity, co-activator interaction or DNA binding, ultimately lead to a decreased AR transcriptional activation potential (http://androgendb.mcgill.ca). Such mutations have been described for 46,XY individuals with the androgen insensitivity syndrome (AIS), ranging from partial AIS (PAIS) to complete AIS (CAIS). AIS is classed as a 46,XY disorder of sex development (DSD) (Quigley et al., 1995; Hughes et al., 2006).

In the present study, a novel AR mutation, F826L, was found in a 46,XY DSD boy with severe penoscrotal hypospadias, possibly associated with PAIS. Hypospadias can result from dysregulation of androgen synthesis or its actions, but is also found without a known cause (Hughes et al., 2006). Several aspects of AR functions were studied to determine the effect of the F826L mutation, which is located in the LBD. The studies were focused on protein expression level and hormone binding characteristics of the mutant AR in genital skin fibroblasts (GSFs) from this boy. In addition, the mutant AR was transiently expressed in CHO and Hep3B cells, to compare its functional properties to that of wild-type AR, with respect to sub-cellular distribution, hormone responsivity, transactivation potential, NC-TDI, TIF2 co-activation, and repression by N-CoR.

2. Materials and methods

2.1. Clinical data

The 46,XY boy with the AR mutation F826L was referred to the clinic for a severe penoscrotal hypospadias at the age of 6 months. A mutation in codon 826 of the *AR* was detected (TTC → TTA) resulting in a substitution of a phenylalanine by a leucine residue. The mutation was also identified in the grandmother and the mother of the boy. Sequence analysis of in total 252 alleles (from 82 normal men and from 85 normal women) revealed no alterations at codon 826, indicating that the C → A mutation is not a common AR polymorphism. The poly Gln, Gly and Pro stretches were 22, 17 and 8 residues in length, respectively, and within the normal range. There were no reports on other affected family members. Furthermore, based on a human chorionic gonadotrophin (hCG) stimulation test at the age of 6 months, well-known causes of 46,XY DSD such as Leydig cell hypoplasia, 17β-hydroxysteroid dehydrogenase (HSD) type 3 deficiency, and 5α-reductase type 2 deficiency were excluded (Boehmer et al., 2001). The penoscrotal hypospadias was successfully corrected in 2 stages at the age of 1.5–2 years.

2.2. Site-directed mutagenesis and plasmids

All the AR amino acid numbers used in the present study are based on the National Center for Biotechnology Information accession number AAA51729, which refers to the AR of 919 amino acid residues (Lubahn et al., 1988). The TTC → TTA mutation at codon 826 was introduced into the AR cDNA in the pSG5AR construct using QuikChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA, USA). The mutated AR fragment was excised by EcoR1 digestion and exchanged with the corresponding wild-type fragment in pSG5AR. Ligation was performed with Rapid DNA Ligation Kit (Roche Diagnostics, Basel, Switzerland). This resulted in pAR-F826L. Preparation of the GST-AR.LBD construct was described previously (Steketee et al., 2002). The TTC → TTA mutation was introduced into this construct using the same approach.

The NH_2-terminal domain AR construct pSVAR(TAD 1–494) and the COOH-terminal domain AR construct pSVAR-104 (herein indicated as AR-C; aa 537–919) that were used for the NH_2-/COOH-terminal domain interaction and for the TIF2 activation studies were described previously (Doesburg et al., 1997; Jenster et al., 1995). The pSVAR-104 construct encodes the DBD-hinge-LBD regions of the AR. The mutation was introduced into the pSVAR-104 construct via the EcoR1 restriction fragment of pAR-F826L. This resulted in a construct encoding AR-C-F826L.

Generation of pGFP-AR constructs, coding for NH_2-terminally tagged GFP-AR fusion proteins of which the expression is driven by a CMV promoter, has been described previously (Farla et al., 2004). GFP-AR-F826L was constructed by replacing an EcoRI–PvuI cDNA fragment encoding the LBD of pGFP-AR by the same fragment of pAR-F826L. All constructs were sequenced to check for errors.

The N-CoR expression vector was constructed as described previously (Berrevoets et al., 2004).

2.3. Scatchard plot analysis

A genital skin biopsy was obtained from the boy for the genital skin fibroblasts (GSFs) culture. The GSFs were cultured in minimal essential medium containing 1% (v/v) nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 10% (v/v) fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Verviers, Belgium). For ligand binding characteristics, a whole-cell binding assay was performed as previously described (Bruggenwirth et al., 1996). Briefly, GSFs were cultured to confluency, washed once with PBS, and subsequently incubated overnight in medium without serum. Next, the cells were incubated for 1 h at 37 °C with increasing concentrations of the radiolabeled synthetic androgen ^{3}H-R1881 (NEN Life Science Products, Boston, MA, USA) in the absence or presence of a 200-fold excess of nonradioactive R1881. Cells were then placed on ice, washed 4 times with ice-cold PBS, and subsequently lysed in 0.5 M NaOH. ^{3}H activity in the lysate was measured using a liquid scintillation counter. Scatchard analysis was carried out to determine the equilibrium dissociation constant (K_d) using the Kell software package (Radlig, Biosoft, Ferguson, MO, USA). Protein measurement was performed with the RCDC protein assay according to the instructions from the manufacturer (Bio-Rad, Hercules, CA, USA).

2.4. Luciferase assay

For transcription activation studies, CHO cells were cultured in DMEM/F12 medium (Invitrogen), supplemented with 5% (v/v) fetal calf serum (FCS; Hyclone) that was dextran-coated charcoal-stripped, 100 IU/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Verviers, Belgium). For all transcription activation studies, CHO cells were plated in 24-well plates (Nalge Nunc International, Naperville, IL, USA) at a density of 2×10^4 cells per well. After 24 h, cells were transfected using FuGENE6 reagent (Roche Diagnostics), according to the instructions of the manufacturer, at a DNA:FuGENE ratio of 1:2. The DNA mixture was composed of 50 ng/well of mouse mammary tumour virus (MMTV)-luciferase (LUC) reporter plasmid, 3 ng/well of SV40-Renilla-LUC, increasing concentrations of either wild-type pSG5AR or pAR-F826L (0.1–3 ng/well), and carrier plasmid pTZ19 to adjust to a total amount of 250 ng DNA per well. Five hours after transfection, 1 nM R1881 or vehicle (0.1%, v/v, ethanol) was added to the cells, or in the case of dose–response curves a range of 0.1 pM to 1 μM R1881 was added. Testosterone or 5α-dihydrotestosterone were also added in a concentration range of 0.1 pM to 1 μM as indicated in the figures (Steraloids Inc., Wilton, NH, USA). After overnight incubation, cells were lysed in 50 μl lysis LUC buffer [25 mM Tris-phosphate (pH 7.8), 15%, v/v, glycerol, 1%, v/v, Triton X-100, and 1 mM dithiothreitol], and 25 μl lysate was used to measure LUC activity. At 10 min after addition of 25 μl Dual-Glo the MMTV-LUC activity was measured. The luciferase reaction was stopped with 25 μl "Stop and Glo" and 10 min thereafter the Renilla-LUC activity was measured (Promega, Madison, WI, USA). The data shown are the mean of at least 2 (T) or 3 (R1881 and DHT) independent experiments (mean ± S.E.M.). For this assay also ARE_2-TATA-LUC was used. This luciferase reporter construct contains 2 AREs in front of the E1b TATA sequence as a promoter (Jenster et al., 1997). To perform both LUC assays and immunoblots from the same lysates, 12.5 times the usual amount of CHO cells and DNA constructs were used. Thus 250,000 CHO cells were used for every condition of which 20,000 were used for the LUC assay and the remaining cells for the immunoblot.

2.5. Western blot analysis

For AR Western blot analysis, GSFs containing either the wild-type AR or the F826L AR were cultured in the presence of unstripped FCS for 7 days, as described above. When grown to confluency, medium was replaced by medium containing 10% (v/v) dextran-coated charcoal-stripped FCS in the presence of increasing concentrations of R1881 or vehicle (0.1%, v/v, ethanol) for 24 h, and GSFs were washed with PBS, collected in ice-cold PBS, and centrifuged for 5 min at 800 × g. The cell pellet was resuspended in 100 μl ice-cold RIPA buffer [40 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10%, v/v, glycerol, 10 nM sodium phosphate, 10 mM sodium molybdate, 50 mM NaF, 0.5 mM sodium orthovanadate, 10 mM dithiothreitol, 1%, v/v, Triton X-100, 0.08%, w/v, SDS, and 0.5%, w/v, desoxycholate] containing Complete protease

inhibitors (Roche Diagnostics) and centrifuged for 10 min at 400,000 × g. GSF cell lysates were loaded onto a 7% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Western immunoblotting was performed using monoclonal antibody F39.4.1 and proteins were visualized by Western Lightning chemiluminescence detection (PerkinElmer, Boston, MA, USA).

Western immunoblotting of CHO cells was performed by using lysates from the corresponding LUC assay, by immunoprecipitation with the monoclonal AR antibody F39.4.1 followed by SDS-PAGE, and subsequent detection with polyclonal antibody SP197 targeting the NH_2-terminal domain of the human AR (Kuiper et al., 1993).

2.6. NH_2-/COOH-terminal domain interaction (NC-TDI) assay and TIF2 activation assay

The functional NC-TDI assay and TIF2 activation assay were performed in essentially the same way as the transactivation assay described above, except for the used constructs. For the NC-TDI assay, 100 ng/well of the NH_2-terminal domain AR construct AR-N (pSVAR(TAD 1–494)) (Doesburg et al., 1997) was used in combination with increasing concentrations of the COOH-terminal domain AR construct AR-C (pSVAR-104) (Jenster et al., 1995) or AR-C-F826L (0.3–30 ng/well).

For TIF2 co-activation assay, 100 ng/well of TIF2 expression vector was added in combination with increasing concentrations of constructs encoding AR-C or AR-C-F826L (0.3–30 ng/well).

2.7. N-CoR repression and N-CoR-TIF2 competition assay

The functional N-CoR repression and N-CoR-TIF2 competition assays were performed by essentially the same procedure as the transactivation assay described above. For the N-CoR repression assay, different amounts of N-CoR expression constructs were transfected together with 3 ng of either wild-type pSG5AR or pAR-F826L expression constructs. For the N-CoR-TIF2 competition assay, 30 ng/well of TIF2 and/or N-CoR was used. pSG5 vector was added to obtain equal molar fractions of plasmid in each well for both assays.

2.8. GST pull-down assay

In vitro interaction assays (pull-down assays) were performed as described previously (Steketee et al., 2002). In short, CHO cells were transfected with pSVAR(TAD 1–494) and either GST-AR.LBD-wt or GST-AR.LBD-F826L. After overnight incubation in medium supplemented with 100 nM R1881 or vehicle, cells were lysed and rotated for 5 h at 4 °C with glutathione–agarose beads. Next, beads were washed, subsequently boiled in Laemmli sample buffer, and subjected to SDS-PAGE. After Western blotting, visualization of pSVAR(TAD 1–494) was performed with the AR antibody SP197, and of GST-AR.LBD with the AR antibody SP066 (Jenster et al., 1995). The expression of each protein was semi-quantified by Quantity One® (Bio-Rad). The relative NC-TDI was determined as the ratio between the blotted protein amounts of the NH_2-terminal domain and the corresponding COOH-terminal domain. The ratio of the wild-type AR-LBD expression vector was set at 1 and the ratios for 3 AR F826L isolates were determined relative to that of the wild-type AR-LBD.

2.9. Confocal microscopy of GFP proteins

Hep3B cells were cultured in αMEM (Cambrex, East Rutherford, NJ, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 5% (v/v) FCS (PAN Biotech GmbH, Aidenbach, Germany). Two days before confocal microscopy, cells were seeded on glass coverslips in six-well plates. One day prior to confocal microscopy, medium was substituted by medium supplemented with 5% dextran-coated charcoal-treated FCS. Four hours after medium change cells were transfected with 1 μg/well GFP-AR expression constructs in FuGENE6 (Roche) transfection medium. Four hours after transfection, the medium was replaced by medium containing 5% dextran-coated charcoal-stripped FCS with or without 1 nM R1881. Live-cell imaging was performed using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany) using the 488 nm laser line of a 30 mW Ar laser with tube current set at 6.1 A. Cells were kept at 37 °C. Images were obtained using a 40×/1.3 NA oil immersion lens using 505–530 nm emission filters.

2.10. Protein structure

The three-dimensional (3D) crystal structure of the AR ligand binding pocket complexed with R1881 was obtained from the Protein Data Bank (PDB; accession number 1XOW) deposited in the data bank by He et al. (2004). The diagram showing the LBD and selected residues that were subject to mutation was created using the ViewerLite 5.0 program from Accelrys. The distance between amino acid residues and the amino acid interaction surface were predicted by the ligand–protein contacts (LPC) software (Sobolev et al., 1999).

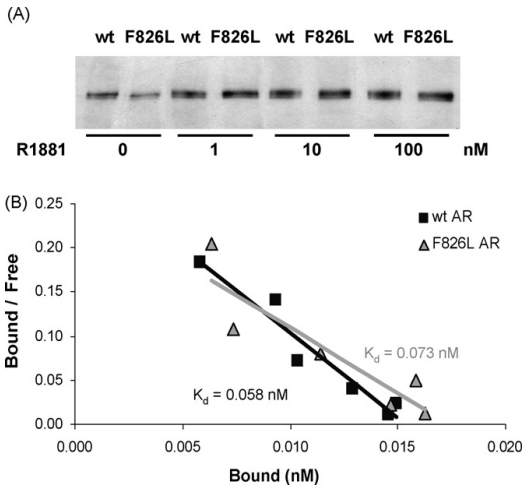

Fig. 1. Expression and ligand binding properties of AR F826L in genital skin fibroblasts (GSFs). (A) AR protein expression levels were analysed in GSFs after incubation for 24 h in the presence of R1881 (1, 10, and 100 nM) or vehicle alone (0.1% ethanol). An amount of 12 μg lysate was subjected to SDS-PAGE and immunoblotting with AR monoclonal antibody F39.4.1. (B) Ligand binding properties of AR F826L were determined by Scatchard analysis, after incubation of GSFs with 1–0.5–0.25–0.1–0.05–0.025 nM ^{3}H-R1881 for 1 h. The dissociation constant (K_d) was determined with the KELL program (Biosoft® KELL).

3. Results

3.1. Ligand binding and expression of AR F826L in genital skin fibroblasts

To determine whether the expression of the F826L AR mutant in GSFs was affected by the mutation, an immunoblot was performed after incubation of GSFs with different concentrations of R1881 or vehicle for 24 h. The protein expression level of AR F826L in GSFs appeared to be similar at every R1881 concentration (Fig. 1A). Scatchard plot analysis revealed a K_d value of 0.073 nM for AR F826L versus a K_d value of 0.058 nM for AR wild-type (normal range: 0.03–0.13 nM) (Fig. 1B). The number of binding sites in the GSFs of AR F826L was 57.2 fmol/mg and for AR wild-type 61.2 fmol/mg (normal range: 39–169 fmol/mg) (Bruggenwirth et al., 1997). In conclusion, the F826L mutation in the AR did not influence the expression level and ligand binding properties of the AR.

3.2. Transcriptional activation and expression of AR mutant F826L

The ligand-induced transactivation activity of the AR mutant F826L appeared to be similar to that of the wild-type AR with the MMTV-LUC reporter construct (Fig. 2A and B). Similar data were found with the minimal promoter construct ARE_2-TATA-LUC (data not shown). To exclude ligand specificity of the mutant the ARE_2-TATA-LUC reporter was also tested in the presence of 5α-dihydrotestosterone (DHT). Also under these conditions the transactivation of the AR mutant F826L was found to be identical to that of the wild-type AR (data not shown).

To study whether the mutation affected AR stability, an immunoblot was performed with cell lysates used in the luciferase assay presented in Fig. 2A. The expression level of AR mutant F826L protein in CHO cells was only slightly higher, compared to the AR wild-type expression level (Fig. 2C). It can be concluded that the

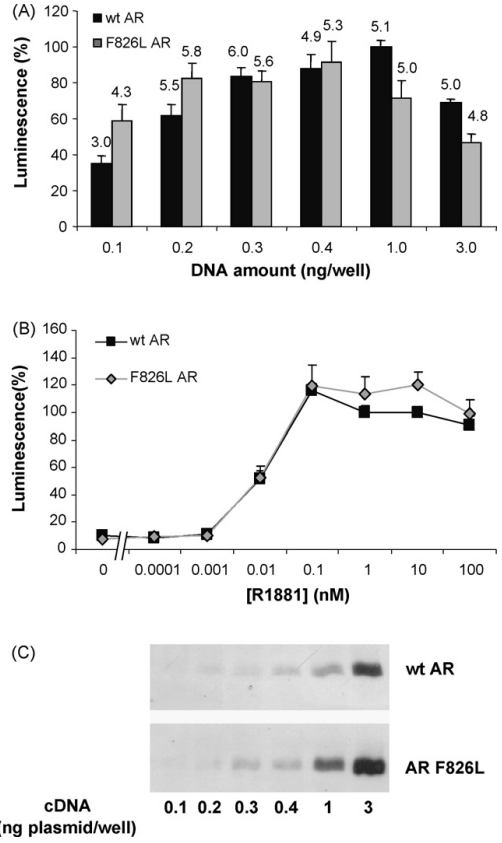

Fig. 2. Transcriptional activation of AR mutant F826L. (A) CHO cells were transfected with increasing amounts (0.1, 0.2, 0.3, 0.4, 1 and 3 ng/well) of DNA plasmids expressing either wild-type (wt) AR or AR mutant F826L, both in combination with 50 ng of the reporter construct MMTV-LUC and 3 ng/well of Renilla luciferase. The cells were harvested 16 h after treatment with 1 nM R1881 or vehicle and luciferase activity was measured. Represented are the means ± S.E.M. for 3 separate experiments. The MMTV-LUC activity was normalized with the Renilla LUC activity and the activity of 1 ng wt AR/well was set at 100%. Fold induction is shown at the top of each bar and represents the mean ratio of activity determined after incubation in the presence or absence of R1881. Statistical significance was calculated using the Student's *t*-test ($p < 0.01$). (B) Dose–response curves of the wt AR and AR F826L constructs (1 ng/well) with increasing concentrations of R1881. LUC activity of the wt AR at 1 nM R1881 was set at 100%. (C) Part of the cell lysates used in the LUC assay were used for immunoblotting. Cell lysates of CHO cells transfected with different amounts of DNA and incubated with 1 nM R1881 were immunoprecipitated with monoclonal AR antibody F39.4.1. The precipitate was thereafter subjected to SDS-PAGE and immunoblotting with polyclonal AR antibody SP197.

mutation F826L did not influence the transactivation function and stability of the AR protein.

3.3. Sub-cellular distribution of AR mutant F826L

The sub-cellular localisation of the AR mutant F826L, was determined in the presence and absence of hormone by confocal microscopy after transfection of GFP-AR and GFP-AR-F826L expressing constructs into Hep3B cells. Both GFP-AR and GFP-AR-F826L were predominantly located in the cytoplasm in the absence of hormone (Fig. 3A and B). In the presence of 1 nM R1881 both GFP-AR and GFP-AR-F826L were translocated in a similar way to the nucleus and displayed a typical punctuate nuclear distribution pattern (Fig. 3C and D). This typical speckled pattern indicates an active transcription of endogenous genes (Farla et al., 2005; van Royen et al., 2007). It can be concluded that the F826L mutation did not influence the sub-cellular distribution of the AR mutant F826L.

Table 1
Quantification of GST pull-down assay

	N/C ratio[a]	Relative to wt[b]
Wild-type AR	0.1	1
AR F826L clone 1	0.6	4.7
AR F826L clone 2	0.3	2.2
AR F826L clone 3	0.3	2.7

[a] The relative NC-TDI from Fig. 6 was determined as the ratio between the blotted protein amounts of the NH_2-terminal domain (N) and the corresponding COOH-terminal domain (C) in the pull-down fraction.

[b] The ratio of the wild-type (wt) AR was arbitrarily set at 1 and the ratios of the 3 AR F826L isolates (clones 1, 2 and 3) were determined relative to the wt AR.

3.4. Functional NH_2-/COOH-terminal domain interaction (NC-TDI) of AR mutant F826L

NH_2-/COOH-terminal domain interaction (NC-TDI) is an important parameter of AR function. The F826L mutant was tested for this interaction. CHO cells were co-transfected with AR-NH_2-terminal domain, MMTV-LUC, and an increasing amount of either wild-type AR-COOH-terminal domain AR-C or AR-C-F826L expression vectors. In the presence of 1 nM R1881, AR-C-F826L displayed an almost two-fold increase in NC-TDI as compared with wild-type AR (Fig. 4A; $p < 0.01$). Immunoblots of cell lysates used in the LUC-assay of Fig. 4A showed that the expression of AR-C-F826L was not different from that of the wild-type AR-C (Fig. 4B).

The effect of different R1881 concentrations on the NC-TDI was compared with that of testosterone (T) and of 5α-dihydrotestosterone (DHT) with the same assay and with an ARE_2-TATA-LUC reporter. At 0.1 nM or higher concentrations of R1881, T as well as DHT, the transactivation by AR-C-F826L was increased, as compared with the transactivation by wild-type AR (Fig. 5A–C). 0.1 nM T induced a smaller increase as compared with DHT and R1881 for both the wild-type and mutant AR, indicating and confirming the relative lower affinity of the mutant and wild-type receptors for T. It can be concluded that all three hormones (R1881, T and DHT) display an at least two-fold increase in the NC-TDI assay for the mutant AR-C-F826L as compared with the wild-type AR-C. A similar increase for the mutant AR was also observed with MMTV-LUC as reporter in the presence of DHT or R1881 (data not shown).

3.5. GST pull-down assay of AR F826L

To confirm the increased NC-TDI of AR mutant F826L, a GST-pull-down assay was performed with lysates from CHO cells transfected with the AR NH_2-terminal domain expression vector and one of the GST-AR.LBD constructs. The experiment was performed in triplicate with 3 different GST-AR.LBD-F826L cDNA isolates. After GST pull-down, SDS-PAGE and immunoblotting, the relative NC-TDI in the presence of 1 nM R1881 was determined as the ratio between the blotted protein amounts of the NH_2-terminal domain and the corresponding COOH-terminal domain. The ratio for the wild-type AR-LBD expression vector was arbitrarily set at 1 and the ratios for the 3 AR F826L isolates (clones 1, 2 and 3) were determined relative to that of the wild-type AR-LBD. The assay revealed that at least twice the amount of the NH_2-terminal domain was pulled down by the 3 GST-AR.LBD-F826L isolates as compared with the wild-type AR-LBD (Fig. 6 and Table 1).

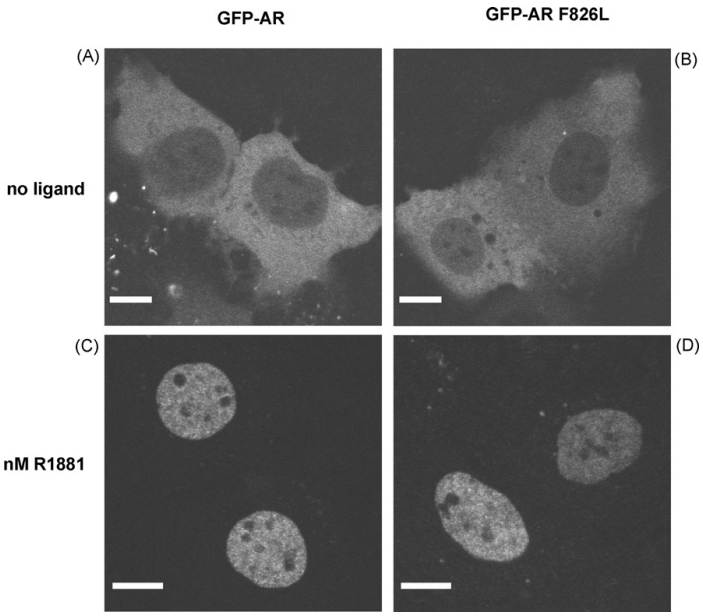

Fig. 3. Sub-cellular distribution of AR mutant F826L. Confocal laser-scanning microscope images of Hep3B cells transfected with 1 μg of GFP-AR or GFP-AR-F826L in the absence of ligand (A and B) or in the presence of 1 nM R1881 (C and D). The bars represent 10 μm.

3.6. TIF2 co-activation of AR mutant F826L

Interaction of the co-activator TIF2 with the COOH-terminal domain of the AR can provide additional information on AR function (Berrevoets et al., 1998). To examine activation of the AR mutant F826L by TIF2, an expression vector encoding this co-activator was co-transfected with AR-C-F826L. The co-activation of AR-C-F826L was significantly higher ($p < 0.01$), up to two-fold compared to the result obtained for the wild-type AR-C (Fig. 7A).

The same assay was performed with increasing concentrations of R1881, testosterone and 5α-dihydrotestosterone. From 0.1 nM onwards the TIF2 co-activation of AR-C-F826L by R1881 and DHT was higher as compared with that of the wild-type AR-C (Fig. 7B and D). TIF2 co-activation in the presence of T was higher from 1 nM onwards for the mutant AR (Fig. 7C). This indicates and confirms again the relative lower affinity of T for both the mutant and wild-type AR. It can be concluded that in the presence of either R1881, T or DHT co-activation by TIF2 of the AR-C-F826L was at least two-fold higher than of the wild-type AR-C. A similar increase for the mutant AR was also observed with MMTV-LUC as reporter in the presence of R1881 (data not shown).

3.7. Repression of AR mutant F826L by N-CoR, in absence and/or presence of TIF2

Since the ligand binding affinity and the transactivation activity of AR F826L were comparable to that of the wild-type AR, and the NC-TDI and the co-activation by TIF2 of mutant F826L were increased, the question arose whether the severe penoscrotal hypospadias of the boy could be explained by preferential binding of co-repressors by the F826L mutant. To answer this question, CHO cells were transfected with either the wild-type AR or AR mutant F826L with increasing concentrations of N-CoR expression vector (0–30 ng/well), in the presence of 1 nM R1881. However, in the presence of N-CoR, the decrease in transactivation of wild-type AR and AR F826L was not different (Fig. 8A).

The present experiments on the effects of TIF2 co-activation and N-CoR repression involved relatively high expression levels of these proteins. Differences between the AR mutant F826L and AR wild-type, regarding differential affinities for TIF2 or N-CoR, may go unnoticed in such an analysis. Therefore, we investigated competition between N-CoR and TIF2, in CHO cells transfected with AR constructs, and with different combinations of expression constructs for N-CoR or TIF2. The transactivation observed in the presence of N-CoR alone decreased with approximately 35%, for both wild-type AR and AR F826L (Fig. 8B). If both N-CoR and TIF2 expression vectors were added, activity could be relieved to 80–85% for both AR wild-type and AR F826L (Fig. 8B). In the presence of TIF2 alone, the activity increased for both AR wild-type and AR F826L to approximately 160% (Fig. 8B).

4. Discussion

The investigated F826L mutation in the AR was found in a 3-year-old boy with severe penoscrotal hypospadias. The grandmother and mother of the boy both were carriers of this genetic alteration. Sequence analysis of 252 alleles (from 82 men and 85 women) and information from the AR gene mutations database (http://androgendb.mcgill.ca/) revealed that alterations at codon 826 do not occur in the general population. Therefore it is highly unlikely that the mutation at codon 826 of the *AR* gene in this individual reflects a common polymorphism in the *AR* gene.

Remarkably, the activity of AR mutant F826L was identical to the wild-type AR with respect to ligand binding, number of ligand binding sites in GSFs, transactivation and repression. The repression by N-CoR of both the wt AR and AR mutant F826L could be relieved by TIF2, although not to 100%. This indicates that repression by N-CoR is stronger than co-activation by TIF2 under these

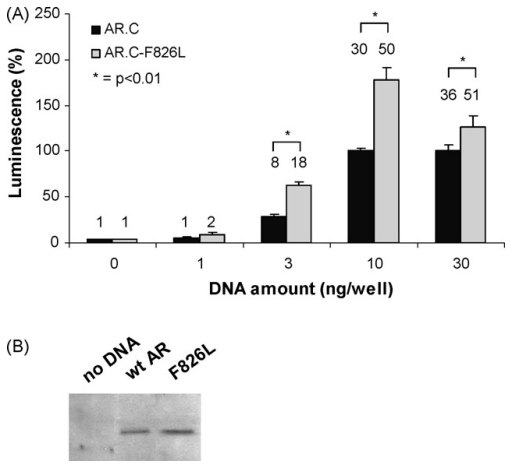

Fig. 4. NH_2-/COOH-terminal domain interaction of AR mutant F826L. (A) CHO cells were transfected with 50 ng of reporter construct MMTV-LUC, 3 ng/well of Renilla luciferase, and different amounts (0, 1, 3, 10, and 30 ng) of DNA plasmid for either wt COOH-terminal domain construct AR-C or the mutant construct AR-C-F826L, both together with 100 ng of AR NH_2-terminal domain construct pSVAR(TAD 1–494) (AR-N). The cells were harvested 16 h after incubation with 1 nM R1881 or vehicle, and luciferase activity was measured. Results represent data of 3 experiments (means ± S.E.M.). Fold induction is shown at the top of each bar and represents the mean ratio of activity determined after incubation in the presence or absence of R1881. The MMTV-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 ng DNA/well was set at 100%. Statistical significance was calculated between wt and mutant AR using the Student's *t*-test ($p < 0.01$) and is indicated with asterisks (*). (B) Part of the cell lysates used in the LUC assay was used for immunoblotting. Lysates of CHO cells transfected with AR COOH-terminal domain constructs (30 ng/well) and incubated with 10 nM R1881 were immunoprecipitated with monoclonal AR antibody F52.24.4 in the presence of 0.3 M NaCl. Subsequently, the immunoprecipitate was subjected to a 10% SDS-PAGE gel electrophoresis and immunoblotted with polyclonal AR antibody SP066 against the COOH-terminal domain.

conditions, which confirms a previous study for only the wild-type AR (Berrevoets et al., 2004).

It was thought that AR F826L was more abundantly present in the nucleus as compared to wild-type AR, resulting in a similar outcome of transcription activity measurements, even if AR F826L would be less active itself. However, this can likely be ruled out, since the sub-cellular distribution was found to be similar for AR F826L and wild-type AR. Furthermore, after addition of R1881, GFP-AR and GFP-AR-F826L displayed a typical punctuate nuclear distribution pattern, as previously shown by Farla et al. (2004, 2005) for the wild-type receptor. This typical speckled pattern overlaps with sites of active transcription (van Royen et al., 2007).

Interestingly, this study showed that the NC-TDI of the AR mutant F826L was increased compared to the wild-type AR and this increase was shown with 2 different assays, 2 different promoters and 3 different ligands. This increase in activity could not be explained by a higher protein stability of the AR mutant. Furthermore, the TIF2 co-activation of AR mutant F826L was also enhanced compared to the wild-type AR. These experiments also confirmed the lesser potency of T versus DHT, because higher T concentrations were needed to achieve the same transcriptional activity as with DHT (Askew et al., 2007). In addition, the NC-TDI might be very sensitive to subtle changes in the AR ligand binding domain. Also for other mutations, information on NC-TDI and TIF2 co-activation can be of much value to detect a possible difference between wild-type and mutant AR. In contrast to our study, several studies showed that AR mutations found in subjects with AIS

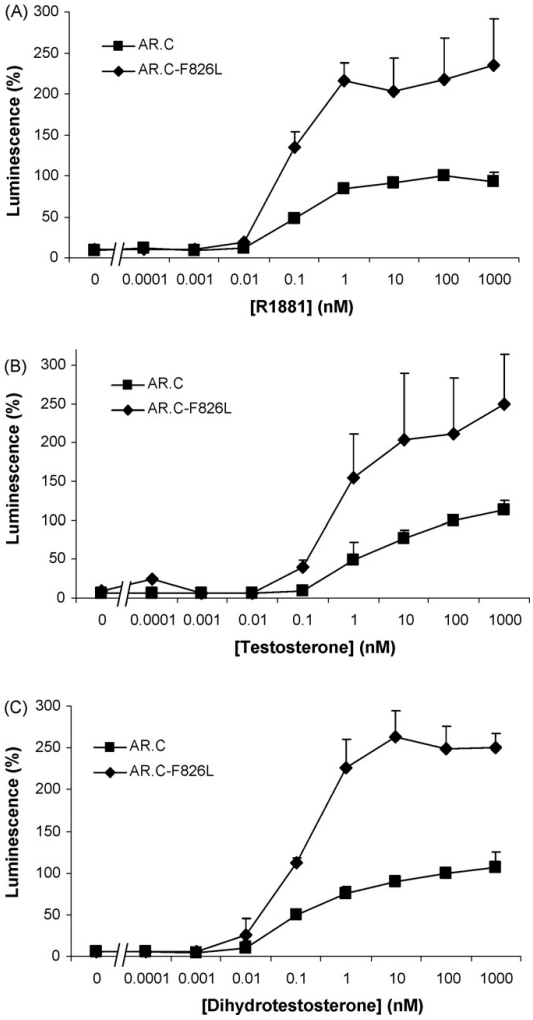

Fig. 5. Dose–response of the NH_2-/COOH-terminal domain interaction of AR mutant F826L. (A) NC-TDI of mutant F826L and wt AR COOH-terminal domain (10 ng/well) with AR NH_2-terminal domain (100 ng/well) in the presence of increasing concentrations of R1881. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM was set at 100%. Results are based on at least 3 experiments (mean ± S.E.M.). (B) NC-TDI of mutant F826L and wt AR COOH-terminal domain (10 ng/well) with AR NH_2-terminal domain (100 ng/well) in the presence of increasing concentrations of testosterone. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM was set at 100%. Results represent data of 2 experiments (mean ± S.E.M.). (C) NC-TDI of mutant F826L and wt AR COOH-terminal domain (10 ng/well) with AR NH_2-terminal domain (100 ng/well) in the presence of increasing concentrations of 5α-dihydrotestosterone. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM was set at 100%. Results are based on at least 3 experiments (mean ± S.E.M.).

and prostate cancer, can result in an altered NH_2-/COOH-terminal domain interaction and co-factor interaction. Five mutations resulting in AIS, L712F, F725L, I737T, Q733H and I898T, respectively, are located in the hydrophobic region of AF2, which all result in a defective NC-TDI and in a defective interaction with TIF2 (Quigley et al.,

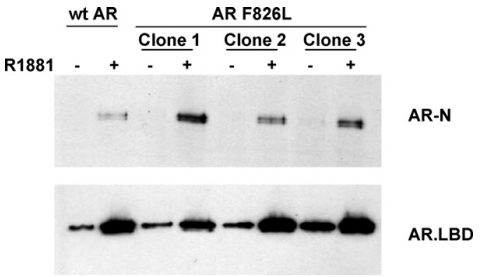

Fig. 6. GST pull-down assay of AR F826L. Proteins were produced in CHO cells by transfection of AR-N (1 μg) and co-transfected with either the wt GST-AR.LBD construct or one of the 3 isolates of the GST-AR.LBD-F826L constructs (3 μg). After overnight incubation in the absence or presence of 100 nM R1881 the cells were lysed. Subsequently, the pull-down assay and SDS-PAGE was performed, followed by immunodetection with AR antibody SP197 against the NH_2-terminal domain and AR antibody SP066 against the COOH-terminal domain.

2004; He et al., 2006). In addition, the AR mutants F725L and I737T have a defective interaction with SRC1 (Quigley et al., 2004). AR mutations L907F and R885H, which both can result in an defective NC-TDI, are also found in close proximity of the AF2 (Jaaskelainen et al., 2006). Another mutation Q902K, also located close to AF2, displays a defective NC-TDI and an increased R1881 equilibrium dissociation constant (Umar et al., 2005). An affected NC-TDI and TIF2 interaction is also found for two other mutations close to AF2, G743V (PAIS) and V889M (CAIS) (He et al., 2006; Thompson et al., 2001). In close proximity of G743, three mutations are reported in AIS subjects, R871G, S814N and V866M, which were found to have a defective NC-TDI (Ghali et al., 2003). The M745I mutated residue from a CAIS subject is part of the ligand binding pocket, and causes a defective NC-TDI and a defective interaction with ARA70 (Bonagura et al., 2007). Remarkably, this mutation does not affect the interaction of AR with TIF2 and SRC1, whereas the R1881 equilibrium dissociation constant is increased 5 times compared to wild-type AR (Bonagura et al., 2007). Mutations D695N, Y763C, E772A, R774H, R774C and Q798E from AIS subjects are all located on the surface of the LBD at a relatively large distance from AF2, but surprisingly all mutants display a defective NC-TDI (Ghali et al., 2003; Jaaskelainen et al., 2006). Three of these residues (D695, Y763 and R774) together with residues R752 and F754 have been suggested to form a new region for protein–protein interactions, although this is not supported by experimental data (Jaaskelainen et al., 2006). Another mutation, R855H, found in an AIS individual is located also at a large distance from the AF2 region, within helices 10/11 which contain residues of the ligand binding pocket (Matias et al., 2000). This mutant displays a decreased NC-TDI, but also an increased androgen equilibrium dissociation constant (Elhaji et al., 2004). For prostate cancer, several mutations (V715M, R726L, H874Y) have been reported displaying either an increased NC-TDI or an increased p160 co-activator activation or both (Thompson et al., 2001; Duff and McEwan, 2005; He et al., 2006; Brooke et al., 2008). The mutation V715M resulted in a small increase of NC-TDI, but in a normal p160 co-activator activation, while for the mutant R726L the reverse was found (Thompson et al., 2001). However, in another study the mutant V715M has been reported to display an increased NC-TDI as well as an increased p160 co-activator interaction (He et al., 2006). The mutant H874Y has been studied extensively and this mutant displayed an increased p160 co-activator interaction (Brooke et al., 2008; Duff and McEwan, 2005; He et al., 2006). Contradictory results were reported with respect to the NC-TDI. Either wild-type activity (Duff and McEwan,

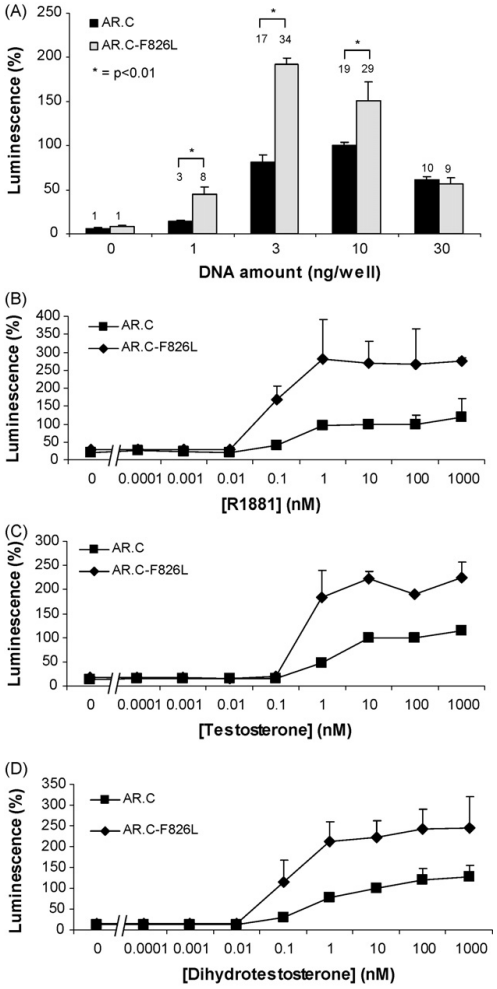

Fig. 7. TIF2 co-activation of AR mutant F826L. (A) CHO cells were transfected with 50 ng of reporter construct MMTV-LUC, 3 ng/well of Renilla luciferase, and different amounts of DNA plasmids from either wt COOH-terminal construct AR-C or mutant AR-C-F826L, both together with 100 ng of a construct encoding TIF2. The cells were harvested 16 h after incubation with either vehicle or 1 nM R1881, and luciferase activity was measured. Results represent data of 3 experiments (means ± S.E.M.). Fold induction is shown at the top of each bar and represents the mean ratio of activity determined after incubation in the presence or absence of R1881. The MMTV-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 ng DNA/well was set at 100%. Statistical significance was calculated between wt and mutant AR using the Student's *t*-test ($p < 0.01$) and is indicated with asterisks (*). (B) Dose–response curve of the 2 AR COOH-terminal constructs (10 ng/well) in the presence of TIF2 expression vectors (100 ng/well) and increasing concentrations of R1881. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM R1881 was set at 100%. Results represent data of at least 3 experiments (means ± S.E.M.). (C) Dose–response curve of the 2 AR COOH-terminal constructs (10 ng/well) in the presence of TIF2 expression vector (100 ng/well) and increasing concentrations of T. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM T was set at 100%. Results represent data of at least 3 experiments (means ± S.E.M.). (D) Dose–response curve of the 2 AR COOH-terminal constructs (10 ng/well) in the presence of TIF2 expression vector (100 ng/well) and increasing concentrations of DHT. The ARE_2-TATA-LUC activity was normalized with the Renilla LUC activity and the activity of wt AR-C at 10 nM DHT was set at 100%. Results represent data of at least 3 experiments (means ± S.E.M.).

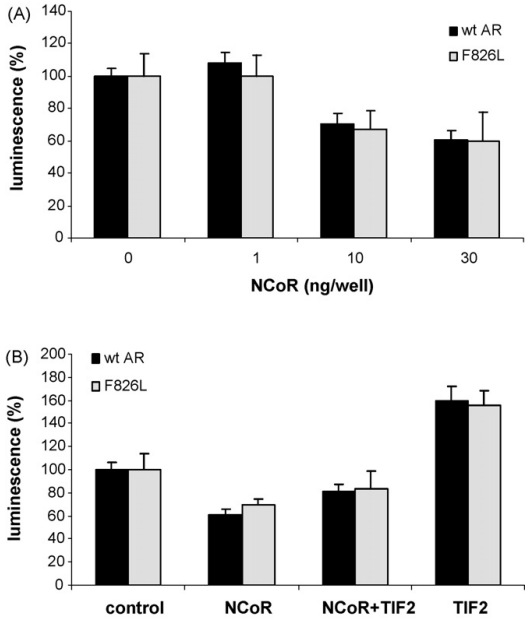

Fig. 8. N-CoR repression of AR mutant F826L. (A) CHO cells were transfected with 50 ng of reporter construct MMTV-LUC, 3 ng/well of Renilla luciferase, and different amounts of N-CoR DNA plasmids together with 3 ng of either wild-type AR or pAR-F826L constructs. In the control situation, equal molar fractions of empty vector (pSG5) were transfected. The cells were harvested 16 h after treatment with vehicle alone or 1 nM R1881, and luciferase was measured. Results represent data of 3 experiments (means ± S.E.M.). The MMTV-LUC activity was normalized with the Renilla LUC activity. The minus N-CoR situation for each AR construct was set to 100% from which the relative repression by N-CoR was calculated. (B) Competition between TIF2 (30 ng/well) and N-CoR (30 ng/well). Except for the different combinations of transfected constructs, the assay was performed as described above (A). In the control situation, equal molar fractions of empty vector (pSG5) were transfected and the outcome was set at 100%. Results represent data of 3 experiments (means ± S.E.M.). The MMTV-LUC activity was normalized with the Renilla LUC activity.

2005) or a significant increase was found with the H874Y mutant (He et al., 2006).

Summarising it can be concluded that mutations in or near the AF2 region predominantly negatively influence the protein interaction with AF2. However, for certain mutations in prostate cancer a positive influence on protein interactions with AF2 is reported. Furthermore, mutations in the LBD at a far more distance from AF2 and not influencing ligand binding affinity, seem to influence the protein interaction with AF2, as well.

What might be causing the increased NC-TDI and the increased activation by TIF2 observed for the mutant AR F826L? Although the 3D model for the AR-LBD predicts that residue F826 is neither part of the ligand binding pocket nor the co-activator binding groove, this mutation might still influence the co-activator binding groove indirectly. The ligand–protein contacts (LPC) software predicts that F826 can have hydrophobic interactions with residues N727 and L728 (Sobolev et al., 1999). The distance to N727 is 3.5 Å and the distance to L728 is 3.8 Å. However, the contact surface area between F826 and N727 is about 4 times larger than between F826 and L728, which would indicate that F826–N727 might give the strongest interaction. Residues N727 and L728 are both located in the loop region between helices 3 and 4 (Fig. 9) (Wurtz et al., 1996). Changes in the interaction between F826 and either N727 or L728 due to the F826L mutation could have consequences for positions of residues in the LBD structure including those of the co-activator groove. Since residues in helix 3 (K717 and K720) and in the loop region between helices 3 and 4 (R726) belong to the co-activator binding groove. A subtle rearrangement in the F826–N727 or F826–L728 interaction might have a considerable impact on the co-factor interaction.

Most importantly, it has been suggested that residue N727 plays a role in AR transcriptional activation, based on the presence of an AR mutant N727K found in an individual with PAIS (Yong et al., 1994). Other data indicated that N727 could influence NC-TDI and TIF2 co-activation (Lim et al., 2000). Similar to the present findings for the AR F826L mutant, Lim et al. (2000) found that, the N727K AR mutant has unaltered transcriptional activation and ligand binding activities, but increased NC-TDI and TIF2 co-activation activities, compared to wild-type AR. However, this was observed using the synthetic androgen mesterolone, (1-α-methyl DHT). Remarkably, when testosterone or DHT was used, the transcriptional activation, NC-TDI and TIF2 co-activation of the AR N727K mutant were found to be decreased, compared to the wild-type AR (Lim et al., 2000). It appears that the effect of the N727K mutant depends on the type of ligand used. But in the present study, this was not the case. The synthetic androgen R1881 generated similar results as testosterone and 5α-dihydrotestosterone.

Recently, Estebanez-Perpina et al. (2007) showed that residue F826 is part of an allosteric regulatory site termed binding function (BF)-3 in the AR LBD. Mutagenesis of residues that form BF-3 modulated AR function (Estebanez-Perpina et al., 2007). The AR F826A mutant showed normal, wild-type level of AR transcriptional activity (Estebanez-Perpina et al., 2007). However, another mutant, F826R displayed a small decrease (15%) in transcriptional activity, probably caused by the change in hydrophobicity (Estebanez-Perpina et al., 2007). The F826L mutation changes the hydrophobic phenylalanine to an even more hydrophobic leucine (hydropathy

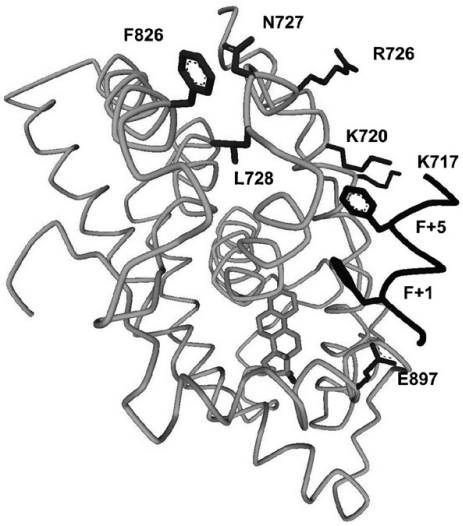

Fig. 9. Position of AR F826 in the AR LBD with respect to the co-activator binding groove. Structural model of the AR LBD with liganded R1881 in the ligand binding pocket. Residue F826 might have hydrophobic interactions with residues N727 and L728; the distances are 3.5 and 3.7 Å, respectively. N727 and L728 are both in the loop region between helices 3 and 4. R726 in the loop region and K717 and K720 in helix 3 belong to the co-activator binding groove. The AR FXXLF peptide is shown to indicate the co-activator binding groove. K720 and E897 represent the charge clamp residues essential for binding of FXXLF and LXXLL motifs.

index changes from 2.8 to 3.8) (Kyte and Doolittle, 1982). The mutant residue F826A has become less hydrophobic (hydropathy index of 1.8) and the mutant residue F826R became even lesser hydrophobic (hydropathy index of −0.8). The hydrophilic arginine has probably caused the 15% decrease in transcriptional activation activity. Such a change in hydrophobicity does not seem to be notable in a functional assay with the full-length mutant AR. For the AR mutant N727K, the change to a very hydrophilic residue decreased the transcriptional activity (Lim et al., 2000).

The increase in hydrophobicity of the F826–N727 interaction appeared to be associated with an enhanced transcriptional activity whereas a decreased hydrophobicity is associated with a reduced transcriptional activity. Consequently, the observed increased NC-TDI and TIF2 co-activation in the present experiments might be caused by the increased hydrophobicity of the leucine residue of AR mutant F826L.

Another question that remains, concerns the factor(s) causing the severe penoscrotal hypospadias phenotype in the boy with the AR mutation F826L. Several reports described AR mutations in helix 9 in the region surrounding F826 that resulted in AIS. For example, Q824L was identified in a boy with a partial androgen insensitivity syndrome. This mutation resulted in a decreased AR activity, depending on the type of ligand used. In the presence of DHT, 62% of wild-type activity was observed, whereas with the synthetic androgen mibolerone, no difference in activity was found (Giwercman et al., 2000). Another mutation close to the position of F826, F827V, found in an individual classified as having CAIS, was reported to result in just 20% less ligand binding affinity in genital skin fibroblasts (Chavez et al., 2001a). The AR mutant L830V was also found in a CAIS individual (Chavez et al., 2001b). These facts suggest that mutations in the region of F826 result in AIS (ranging from partial to complete). It has been shown that different cell types have a cell-specific expression of co-factors (Folkers et al., 1998; Shang and Brown, 2002). The severe penoscrotal hypospadias observed in the boy carrying the F826L AR mutation might be the result of a combination of a different repertoire of tissue specific co-factors and a subtle alteration of the co-activator binding groove. Consequently this can result in less recruitment of important key co-activators, other than TIF2, in genital skin fibroblasts. Alternatively, an altered LBD conformation may enhance a preferential recruitment of co-repressor(s), present in specific androgen target cells.

In conclusion, the mutant F826L displayed an unexpected increased NH_2-/COOH-terminal domain interaction and TIF2 co-activation. These findings cannot directly explain the observed severe penoscrotal hypospadias, but offer indirect indications for an altered AR functioning.

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CHAPTER 4

DNA dependent recruitment of DDX17 and other interacting proteins by the human androgen receptor

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DNA dependent recruitment of DDX17 and other interacting proteins by the human androgen receptor

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ABSTRACT

An oligonucleotide-based assay (OBA) was used to identify novel co-factors that can be recruited by the deoxyribonucleic acid (DNA)-bound androgen receptor (AR). Nuclear extracts obtained from LNCaP cells, after incubation with R1881, were incubated with biotinylated oligonucleotides bound to streptavidin coated beads. The oligonucleotides contain 3 copies in tandem of the androgen responsive element ARE1 from the prostate specific antigen (*PSA*) gene promoter. As control incubation, a scrambled version of the tandem ARE1 was used. Immunoblots of the eluents revealed that the AR was bound to the ARE1 oligonucleotide and to a much lesser extent to the scrambled oligonucleotide. Proteins eluted from the oligonucleotides, were separated on a 5–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel, followed by identification using mass spectrometry. Identified proteins were scored for having one or more of the following known properties: nuclear localization, involved in transcription regulation, involvement in steroid hormone receptor (SHR) function, or specifical involvement in AR function. A total number of 85 nuclear proteins were found in two separate OBAs. Based on peptide counting, we found enrichment of 7 proteins eluted from the ARE1 oligonucleotide, compared to the scrambled oligonucleotide. Taken together with the obtained scores, these proteins are considered putative AR co-factors. One of these proteins, DDX17, is known to be a co-factor for estrogen receptor α (ERα), but has never been associated with AR function. The results indicate that the ARE oligonucleotide-based assay may allow enrichment of new candidate DNA-bound AR interacting proteins.

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1. Introduction

Androgens (testosterone and dihydrotestosterone) are powerful hormones, exerting actions which are essential for male development and functioning [1]. The androgenic steroid hormones exert their functions by activating the androgen receptor (AR) after binding to the ligand binding domain (LBD). Once activated, the AR uses its DNA binding domain (DBD) to bind to specific DNA sequences, the so-called hormone or androgen responsive elements (HRE or ARE), which results in induction or repression of transcription of target genes. However, androgen mediated transcription regulation does not take place without the recruitment of several proteins from a repertoire of co-factors by the AR [2–4]. These co-factors can be divided into 2 groups of chromatin modifying enzymes. One group is the family of histone modifying enzymes (HME) which influence the DNA–histone interaction by (de)acetylation and/or (de)methylation of core histones [5]. The other group of chromatin modifying enzymes is the ATP-dependent chromatin remodeling complexes (CRC) which can displace or remove the histone complexes from the DNA. This group consists of the SWI/SNF, ISWI/hSNF2h, and Mi-2/NURD complexes [6–12]. Besides chromatin modifying enzymes three other groups of co-factors can be recruited by the AR. The first group is the Mediator (MED)-complex (TRAP, DRIP, ARC and SMCC) which is essential for transcription activation by facilitating RNA-Pol II recruitment [13–15]. The second group consists of co-activators, which can enhance transcriptional activation, but do not belong to any of the above-mentioned categories [16,17]. Finally, a last group represses transcription, the co-repressors. Well known co-repressors in the nuclear receptor field are N-CoR and SMRT [18,19]. An overview of co-repressors which are able to inhibit the transactivation of AR and other nuclear receptors has been published [20].

Co-factors have been isolated by different isolation methods, such as yeast two-hybrid, mammalian two-hybrid, glutathione *S*-transferase (GST) pull-down and co-immunoprecipitation (co-IP) assays. The protein interactions in yeast two-hybrid and mammalian two-hybrid assays take place *in vivo*, in yeast or mammalian cells, respectively.

Abbreviations: aa, amino acid; AD, activation domain; AF-2, activation function 2; ARE, androgen responsive element; DHT, dihydrotestosterone; LBD, ligand binding domain; N-CoR, nuclear receptor co-repressor; NTD, NH2-terminal domain; OBA, oligonucleotide-based assay; PSA, prostate specific antigen; T, testosterone; wt, wild-type

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The protein (fragment) of interest is expressed as a fusion protein containing a GAL4–DNA binding domain. If an interaction takes place with a possible partner containing a transactivation domain, a functional transcription factor is formed, which functions as the interaction indicator [21,22]. Interacting proteins in co-immunoprecipitation assays are obtained by using antibodies against the protein of interest. Glutathione–agarose beads are used to capture GST-tagged proteins. The protein of interest and the interacting proteins are either captured as an *in vivo* formed complex, or the complexes are formed *in vitro* when cellular extracts are added to immobilised tagged proteins or antibodies.

In the early nineties, several studies showed the effects of DNA on the conformation of DNA binding transcription regulators [for an overview: [23]]. It was suggested that DNA alone, or together with another DNA binding protein on or near the binding site, has allosteric effects on DNA binding proteins. One study involving ERα and ERβ clearly showed the allosteric effects of different response elements on the conformation of these steroid hormone receptors [24]. Remarkably, these response elements together with the conformational change of the ERα and ERβ resulted in a differential recruitment of co-factors [24]. Recently, it has been shown that different response elements from one or from different AR responsive promoters can recruit a different repertoire of proteins [25,26]. Furthermore, different AR binding response elements modified the hormone response of the AR in the presence of different co-factors [27]. These effects on the AR are probably caused by the response element dependent conformational changes, which in turn influence the co-factor recruitment and finally the transcription of genes.

The above-mentioned assays for co-factor identification (such as yeast and mammalian two-hybrid, GST pull-down and co-IP) lack the involvement of AR DNA binding during or after the procedure to capture the interacting proteins. These assays may miss interacting proteins normally attached to DNA-bound AR. Therefore, in the present study DNA-bound AR interacting proteins are isolated by using biotinylated oligonucleotides containing ARE1, one of the strongest AR binding AREs of the prostate specific antigen (*PSA*) gene promoter. In this oligonucleotide based assay (OBA), AR-co-factor complexes from nuclear extracts were reconstituted *in vitro* and isolated for further identification by mass spectrometry.

2. Materials and methods

2.1. Cell culture and nuclear extract preparation

AR expressing LNCaP cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 7.5% (v/v) dextran-coated charcoal-treated fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker, Vervier, Belgium). This medium was replaced by phenol-free RPMI-1640 containing similar supplements, 3 days before harvesting. The synthetic androgen R1881 was added to a final concentration of 10 nM, 16 h before harvesting. For harvesting, the LNCaP cells were first washed twice with PBS and subsequently scraped with ice-cold Buffer A [40 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10% (v/v) glycerol, 10 mM sodium molybdate, 10 mM dithiothreitol (DTT), Complete protease inhibitors (Roche Diagnostics, Basel, Switzerland) and 0.2 μM R1881] and collected. The cell suspension was frozen for 1 min in liquid nitrogen, thawed at 10 °C and mixed. This procedure was repeated twice followed by centrifugation at 800×*g* at 4 °C for 10 min. The obtained nuclear pellet was resuspended and incubated for 5 min in ice-cold Buffer B [Buffer A supplemented with 0.2% (v/v) Triton X-100], followed by centrifugation at 800×*g* at 4 °C for 10 min and resuspension in Buffer A. This was followed by centrifugation at 800×*g* at 4 °C for 10 min. Extraction was carried out by immediate resuspension of the nuclear pellet in 1 pellet volume of Buffer C [Buffer A with 40 mM Tris–HCl (pH 8.5) supplemented with 1 M sodium chloride] and 2 volumes of Buffer D [Buffer C supplemented with 0.5 M sodium chloride]. After incubation on ice for 1 h, the cell suspension was pelleted at 40,000×*g* for 10 min.

2.2. Oligonucleotide-based assay (OBA)

DNA-protein complexes were purified and analysed for AR content using an adapted streptavidin–biotin complex DNA binding assay [28]. Two oligonucleotides were used; one contains three copies of the ARE1 of the *PSA* promoter [29] and another one with three copies of a scrambled version of the ARE1 (in capitals, bold and italic). It was verified that the scrambled version of ARE1 and the flanking sequences were not similar to an existing steroid hormone responsive element, androgen responsive element, or a predicted responsive element to which the AR can bind [30].

PSA ARE1 oligonucleotide
5′cttaa***AGAACAGCAAGTGCT***attgatttttag***AGAACAGCAAGTGCT***aattca-***AGAACAGCAAGTGCT***taatt-3′
Scrambled ARE1 oligonucleotide
5′cttaa***CACGAGAAGGACTTA***attgatttttag***CACGAGAAGGACTTA***aattca-***CACGAGAAGGACTTA***taatt-3′

The biotinylated oligonucleotides containing three copies of *PSA* ARE1 or the scrambled version and their complementary sequence were mixed in annealing buffer [40 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl], heated to 100 °C, and cooled slowly in boiled water to room temperature. They were then incubated with washed Streptavidin agarose beads (Sigma-Aldrich, St. Louis, MO, USA) overnight, under constant shaking at 4 °C, in oligo binding buffer [20 mM HEPES (pH 7.6), 10% (v/v) glycerol, 0.5 mM EDTA, 2.5 mM Mg Acetate, 0.1% (v/v) Nonidet P-40, 130 mM NaCl, 2.5 mM DTT, Complete protease inhibitors (Roche Diagnostics, Basel, Switzerland) and 0.2 μM R1881]. It was verified that the AR did not bind to the Streptavidin agarose beads. The flow-through fraction containing excess oligonucleotides was removed, and the oligonucleotide-loaded agarose beads were washed with oligo binding buffer. After that, the nuclear extract was diluted to 130 mM NaCl with oligo binding buffer without NaCl and supplemented with 0.2 μM R1881. This solution was mixed with oligonucleotide-loaded agarose supplemented with 14.84 ng/μl of double-stranded poly(dI–dC, dI–dC) and 14.84 ng/μl double-stranded poly(dA–dT, dA–dT). Incubation was for 2 h at 4 °C under constant shaking. After removal of the flow-through fraction, the protein-bound oligo-loaded beads were transferred to Micro Bio-Spin chromatography columns (Bio-Rad Laboratories Inc., Hercules, CA, USA). This was followed by washing with oligo binding buffer supplemented with 0.2 μM R1881. Elution of bound proteins was performed with Laemmli sample buffer for 15 min at 4 °C.

2.3. SDS-PAGE, gel staining and Western immunoblotting

The protein content of the complexes was assessed by gel electrophoresis (5–15% gradient SDS-polyacrylamide gel) and SYPRO Ruby staining (Molecular Probes Inc., Eugene, OR, USA). For determination of AR content, the proteins were transferred from a 7% SDS-PAGE gel to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Western immunoblotting was performed using monoclonal antibody F39.4.1 raised against the human AR NH_2-terminal domain [31] and proteins were visualised by Western Lightning chemiluminescence detection (Perkin-Elmer, Boston, MA, USA).

2.4. Mass spectrometric analysis

SDS-PAGE gel lanes were cut into slices of 1 mm using an automated gel slicer, and 3 consecutive slices were pooled and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with sequencing grade trypsin (Promega

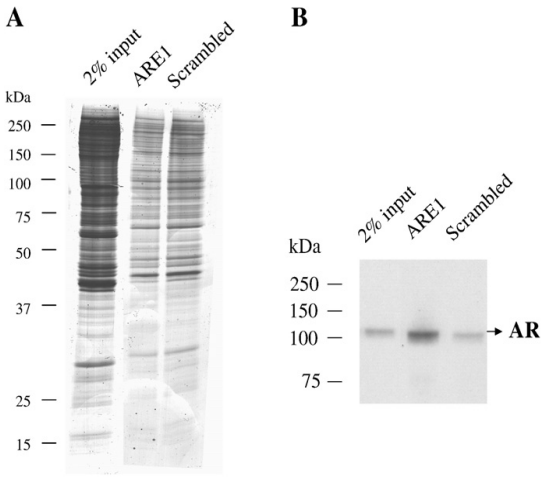

Fig. 1. Isolation of AR complexes. Equal amounts of nuclear extracts obtained from R1881-treated LNCaP cells were incubated with either *PSA* ARE1 or with scrambled biotinylated oligonucleotides bound to streptavidin beads. The 2% input lane contains 2% of the nuclear extract which is used for the oligonucleotide-based assay. The ARE lane and the scrambled lane contain the eluted fractions from the ARE1 oligonucleotide and the scrambled oligonucleotide, respectively. (A) The protein complexes were separated on a 5–15% SDS-polyacrylamide gradient gel and subsequently stained with SYPRO Ruby. (B) Immunoblotting of AR was performed with anti-AR antibody F39.4.1.

Madison, WI, USA), essentially as described by Wilm et al. [32]. NanoLC-MS/MS for the first OBA was performed on a CapLC system (Waters, Manchester, UK) coupled to a Q-ToF Ultima mass spectrometer (Waters, Manchester, UK), operating in the positive mode and equipped with a Z-spray source. The 1100 series capillary LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an LTQ mass spectrometer (Thermo) operating in the positive mode and equipped with a nanospray source was used for the second OBA. Peptide mixtures were trapped on a Jupiter™ C18 reversed phase column (Phenomenex; column dimensions 1.5 cm×100 μm, packed in-house) at a flow rate of 7 μl/min. Peptide separation was performed on Jupiter™ C18 reversed phase column (Phenomenex; column dimensions 15 cm×50 μm, packed in-house) using a linear gradient from 0 to 80% (v/v) acetonitrile in 0.1 M formic acid during 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in the continuum mode; fragmentation of the peptides was performed in the data-dependent mode.

2.5. Data analysis and protein identification

Peak lists were automatically created from raw data files using the ProteinLynx Global Server software (version 2.0; Waters, Manchester, UK) for Q-ToF spectra and the Mascot Distiller software (version 2.0; MatrixScience, London, UK) for LTQ spectra. The Mascot search algorithm (version 2.0, MatrixScience, London, UK) was used for searching against the NCBInr database (release data: 3rd March 2006; taxonomy: *Homo sapiens*). The peptide tolerance was typically set to 150 ppm and MS/MS tolerance to 0.2 Da in case of Q-ToF spectra, and to 2 Da and 0.8 Da, respectively, in case of LTQ spectra. Only doubly and triply charged peptides were searched for. A maximum number of 1 missed cleavage by trypsin was allowed and carbamidomethylated cysteine and oxidised methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 45. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded.

3. Results

3.1. Isolation of DNA-bound AR interacting proteins

Searching for novel AR co-factors, an oligonucleotide based assay (OBA) was used for the isolation and purification of candidate proteins. The oligonucleotides were biotinylated and contained the strong ARE1 of the *PSA* gene in triplicate in tandem [29]. An oligonucleotide containing the scrambled version of the ARE1 was used as a control. Streptavidin-agarose beads were first incubated with one of the oligonucleotides, followed by incubation with the nuclear extract from LNCaP cells. After elution, the proteins were separated in a 5–15% acrylamide gradient on a SDS-polyacrylamide gel and stained with SYPRO Ruby (Fig. 1A). The total protein amount visible on the gels was comparable between the scrambled and the ARE1 lane. Furthermore, the protein banding pattern for the two conditions appeared to be identical, but differed from the input. The eluents were also analysed by Western immunoblotting using an antibody targeting the AR. It was found that the AR is present, both for the ARE1 and the scrambled oligonucleotides, although a larger amount of AR was bound to the ARE1 oligonucleotide (Fig. 1B). In conclusion, with this approach we obtained a protein preparation enriched with the androgen receptor, together with a large number of other proteins. Although the stained protein band pattern did not show a clear difference between the ARE1 oligonucleotide and the scrambled oligo, the immunoblot analysis indicated that the AR was preferentially bound to the ARE1 oligonucleotide. This difference in AR binding may be associated with preferential binding of AR associated proteins to the ARE1 oligonucleotide, as well.

3.2. Protein identification

To identify DNA-bound AR interacting proteins, whole lanes from the SDS-polyacrylamide gel containing proteins from an OBA, were cut into small slices and subjected to trypsin in-gel digestion and followed by mass spectrometric analysis. The mass spectrometric data were edited in several steps (Table 1). First, structural and extra-nuclear proteins were excluded. The second step included sorting of proteins into 3 groups, for binding to either one of the two oligonucleotides (ARE1 or scrambled) or to both. The third step was based on peptide counting which was performed only for proteins that were isolated from both OBAs. Potential AR co-factors were selected based on peptide counts of 1.5-fold or more for proteins from the ARE1 as compared with the scrambled ARE1 oligonucleotide. The fourth step was the scoring of proteins for a known characteristic. A protein property score of 1 was assigned, when the protein was known to be either: a) localized to the nucleus, b) involved in transcription

Table 1
Editing steps of mass spectrometry data

Step	Type of action	Variables
1	Deletion of structural and extra-nuclear proteins	e.g. keratins, fibrinogen, and fibrinopeptide
2	Sorting by oligonucleotide specific associated binding	ARE1 binding; scrambled ARE1 binding; ARE1 and scrambled binding;
3	Peptide counting	Peptide numbers of proteins from: a) ARE1 oligonucleotide b) scrambled ARE1 oligonucleotide
4	Protein scoring	a) nuclear b) involved in transcription regulation c) involved in steroid hormone receptor function d) involved in AR function

The column "type of action" indicates the four steps for editing mass spectrometry data. "Variables" in the last column are the aspects that were checked or scored for.

Table 2
Protein selection by peptide counts

Protein	Function	Protein property score					Mascot score [a]		Peptide counts [b]
		Nucleus	Transcription	SHR	AR	Total	ARE	Scr	
CHD5/chromodomain helicase DNA binding protein 5	Transcription regulation, helicase	1	1			2	75 (2)	*x* (1)	2
							272 (6)	206 (4)	1.5
DDX17/DEAD (Asp–Glu–Ala–Asp) box polypeptide 17	Co-activator, co-repressor of ER	1	1	1		3	280 (5)	*x* (1)	5
							252 (4)	*x* (1)	4
DDX18/DEAD (Asp–Glu–Ala–Asp) box polypeptide 18	Unknown					0	313 (5)	*x* (1)	5
							471 (7)	153 (2)	3.5
MYBBP1A/MYB binding protein (P160) 1a	Co-activator, co-repressor	1	1			2	822 (16)	395 (7)	2.3
							2254 (31)	1716 (21)	1.5
RECQL/RecQ protein-like /DNA helicase Q1-like	DNA repair, helicase	1				1	95 (2)	*x* (1)	2
							486 (7)	252 (4)	1.75
RSL1D1/ribosomal L1 domain containing 1	Regulation of replicative senescence	1				1	285 (5)	46 (1)	5
							443 (6)	244 (3)	2
ZNF384/Zinc finger protein 384	Transcription regulation	1	1			2	64 (2)	*x* (1)	2
							484 (4)	205 (2)	2

The names of the proteins and their abbreviations are from the HUGO Gene Nomenclature Committee (HGNC) database. Furthermore, the protein property scores are shown for presence in the nucleus, involvement in transcription regulation, involvement in steroid hormone receptor function, and whether the respective protein is known to act together with the AR. Some alternative names, synonyms, aliases, and some common used names are also given.

[a] ARE = ARE1; Scr = scrambled ARE1. The first and second rows with the Mascot score for each protein represent the first and second OBA, respectively. The number of identified peptides is given in brackets. *x* = Mascot score below the threshold of 45.

[b] Peptide counts are calculated by the ratio between the number of peptides found for the ARE1 and the scrambled ARE1 oligonucleotide.

activation/repression, c) functioning together with a steroid hormone receptor or d) functioning specifically together with the AR. The maximal protein property score for a protein could be 4. Three databases were used for scoring the proteins: the Human Protein Reference Database (www.hprd.org), GeneCards (www.genecards.org) and PubMed (www.pubmed.com). Basically, when the protein property scores were summed up for each protein, a protein property score of 0 means that no information is available in the literature for the protein. A protein property score of 1 indicates that the protein has been found in the nucleus, but is not known to be involved in transcription regulation of genes. A protein property score of 2 indicates a nuclear protein which is known to be involved in transcription regulation of genes. A protein with protein property score of 3 is a nuclear protein involved in transcription regulation of genes, and also known to be involved in modulation of steroid hormone receptor (SHR) signalling. These proteins have a high potential to be AR interacting proteins, because of the high homology between AR and the other SHRs. Proteins with a protein property score of 4 have been described to act together with the AR in transcription regulation of genes.

3.3. Identified proteins

After the second step, the number of proteins which were found in both OBAs on at least 1 ARE1 oligonucleotide is 85. These 85 proteins could be grouped into 7 categories: co-activating proteins, co-repressing proteins, proteins possessing both co-activating and co-repressing capabilities, other transcription regulating proteins, proteins with a function related to RNA, nuclear proteins not involved in transcription regulation, and proteins with an unknown function (see Supplementary Tables 1–7).

These 85 proteins were checked for peptide counts. Peptide counts of 1.5-fold or more for proteins bound to the ARE1 as compared with the scrambled ARE1, revealed 7 proteins. These proteins were: chromodomain helicase DNA binding protein 5 (CHD5), DEAD (Asp–Glu–Ala–Asp) box polypeptide 17 (DDX17), DDX18, MYB binding protein (P160) 1a (MYBBP1A), RecQ protein-like (RECQL), ribosomal L1 domain containing 1 (RSL1D1), and zinc finger protein 384 (ZNF384; Table 2). All 7 proteins can be found in the nucleus. RECQL and RSL1D1 are not known to be involved in transcription regulation, but rather in DNA repair and regulation of replicative senescence, respectively. CHD5, MYBBP1A and ZNF384 are involved in transcription regulation and have a property score of 2. Studies have shown that MYBBP1A can act as a co-activator as well as a co-repressor. A function of DDX18 is not known yet, but from the same protein family DDX17 is known to be a co-activator and a co-repressor of ERα [33].

DDX17 seems to have the highest potential to be a co-factor of the AR, compared to CHD5, DDX18, MYBBP1A, RECQL, RSL1D1, and ZNF384. DDX17 has a protein property score of 3, and was found enriched with only the ARE1 and not with the scrambled version in both OBAs. DDX17 is an RNA helicase and has been shown to be involved in both pre-mRNA and pre-rRNA processing [33]. But DDX17 possesses also both co-activating and co-repressing capabilities. The repressing capabilities of DDX17 appear to be promoter-specific and are achieved by recruiting histone deacetylase 1 (HDAC1) [33]. Co-activation by DDX17 takes place with or without the recruitment of any member of the SRC-1/TIF2 protein family [34]. These co-activating and co-repressing capabilities of DDX17 are reported for the ERα, but never for the AR [34].

4. Discussion

To identify possible novel AR co-factors which associate with the AR in a hormone and DNA dependent fashion, an oligonucleotide-based assay (OBA) was set up and applied in this study. The AR together with interacting proteins was isolated via binding to oligonucleotides containing either three ARE1s from the *PSA* gene promoter in tandem or a scrambled version of the ARE1s. After careful selection of the data obtained from two OBAs, in total 85 proteins were identified, of which 7 proteins were shown to be enriched after selection by peptide counting. DDX17 has been found to be a potential AR co-factor based upon its function in presence of ERα and its exclusive binding to the ARE1 oligonucleotide.

LNCaP cells were used in this study as a source for potential AR interacting proteins, although these cells originate from a lymph node carcinoma of prostate, and expresses the AR mutant *T877A*, the AR mutant functions normally in the presence of androgens [35,36].

The difference between proteins isolated from the ARE1 oligonucleotide and scrambled ARE1 oligonucleotide could not be shown by SYPRO Ruby staining. Similarly in a study a difference could not be shown by Coomassie Blue staining [37]. Most likely, DNA-bound AR specific interacting proteins are in such a low concentration that they were not visible by either staining.

The AR was detected with immunoblotting in the eluents of both the ARE1 oligonucleotide and, to a much lesser extent, of the scrambled oligonucleotide. The AR protein itself was only detected with mass spectrometry, unexpectedly, in the first OBA and on the ARE1 oligonucleotide, but not in the second OBA. However, the identification of the AR for the first OBA was based on just one peptide with a Mascot score of 52. This indicates that the identification of AR by mass spectrometry might be very difficult. One possibility could be that the abundance and the numbers of other proteins are too high as compared to the AR protein level, which resulted in masking the AR.

Analysing the results, none of the 7 proteins which appear in both OBAs are known to interact with DNA-bound AR. Available data indicate that as many as 169 proteins can interact with the AR [5]. However, it has to be taken into account that these 169 AR interacting proteins were identified by other methods, such as yeast two-hybrid screening, mammalian two-hybrid screening, glutathione *S*-transferase (GST) pull-down assays, and co-immunoprecipitation assays. Most of these AR interacting proteins are identified after an abundant overexpression of proteins of interest, which might sometimes lead to false positive results [38]. Therefore, additional experiments in physiologically relevant cells lines are required with physiological expression levels of these co-factors. In addition, AR DNA-binding was not involved in these assays, whereas the oligonucleotides used in the present experiments are part of an androgen responsive promoter. Binding to response elements can cause conformational changes in the AR, which in turn can influence co-factor recruitment and finally transcription of genes. This might also explain that not all known AR interacting proteins were identified in the present study.

In conclusion, with the oligonucleotide based assay (OBA) as described herein, putative AR interacting proteins can be isolated. Based on two selection methods, DDX17 appears to be the most interesting putative AR co-factor identified in the present study. To obtain more conclusive evidence that DDX17 is a functional DNA-bound AR interacting protein, additional interaction and functional assays need to follow. Interaction assays that would be informative include pull-down, co-immunoprecipitation, and specific ChIP assays. Functional reporter assays should preferably be performed for DDX17 in the presence of the AR and DNA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2008.11.001.

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Supplementary Table 1 Co-activating proteins

protein	function	protein property score					Mascot score[#]		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
SMARCe1/ SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily e member 1/ BAF-57	co-activator of AR	1	1	1	1	4	75 (1) 45 (1)	149 (4) 45 (1)	(Link et al., 2005)
CREB1/ cAMP responsive element binding protein 1	co-activator of AR	1	1	1	1	4	104 (2) 251 (3)	176 (3) 265 (3)	(Kim et al., 2005; Magee et al., 2006)
FOXA1/ forkhead box A1/ hepatocyte nuclear factor-3alpha/ HNF-3	co-activator of AR	1	1	1	1	4	51 (1) 789 (8)	47 (1) 267 (3)	(Gao et al., 2003)
SFPQ/ splicing factor proline/glutamine rich/ polypyrimidine tract binding protein associated/ PSF/ protein-associated splicing factor	co-activator of AR	1	1	1	1	4	130 (3) 200 (3)	302 (5) 79 (1)	(Kuwahara et al., 2006)
USF2/ upstream transcription factor 2	co-activator of AR	1	1	1	1	4	117 (2) x	144 (2) 71 (2)	(Kivinen et al., 2004)
XRCC5/ X-ray repair complementing defective repair in Chinese hamster cells 5/ double-strand-break rejoining; Ku80	co-activator of AR, helicase	1	1	1	1	4	x 862 (12)	50 (1) 498 (7)	(Mayeur et al., 2005)
RBM39/ RNA binding motif protein 39/CAPERα	co-activator of ER and PR	1	1	1		3	x 104 (1)	71 (2) 91 (1)	(Dowhan et al., 2005; Jung et al., 2002)
PSIP1/ PC4 and SFRS1 interacting protein 1/ transcriptional coactivator p75/p52	co-activator	1	1			2	306 (6) 168 (3)	236 (5) 50 (1)	

The names of the proteins and their abbreviations are from the HUGO Gene Nomenclature Committee (HGNC) database. Furthermore, the protein property scores are shown for presence in the nucleus, involvement in transcription regulation, involvement in steroid hormone receptor function, and whether the respective protein is known to act together with the AR. Some alternative names, synonyms, aliases, and some common used names are also given. Proteins are sorted by total score and thereafter by number of appearance.

[#] ARE = ARE1; Scr = scrambled ARE1. The first and second rows with the Mascot score for each protein represent the first and second OBA, respectively. The number of identified peptides is given in brackets. x = Mascot score below the threshold of 45.

* A reference is given only for a protein property score of 3 or higher.

Supplementary Table 2 Co-repressing proteins

protein	function	protein property score					Mascot score[#]		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
HDAC2/ histone deacetylase 2	co-repressor of AR, chromatin remodeling	1	1	1	1	4	119 (4) 226 (3)	134 (4) 189 (3)	(Gross et al., 2004; Tao et al., 2006)
HNRPA1/ Heterogeneous nuclear ribonucleoprotein A1	co-repressor of AR	1	1	1	1	4	508 (7) 225 (4)	551 (8) 632 (8)	(Yang et al., 2006)
RREB1/RAS responsive element binding protein 1	transcription factor negatively regulating AR	1	1	1	1	4	61 (2) 507 (8)	54 (2) 353 (4)	(Mukhopadhyay et al., 2007)
HDAC1/ histone deacetylase 1	co-repressor of AR, chromatin remodeling	1	1	1	1	4	67 (2) 354 (5)	144 (3) 234 (3)	(Gaughan et al., 2002; Fu et al., 2002)
CUTL1/ cut-like 1 CCAAT displacement protein/ CDP	co-repressor of GR	1	1	1		3	237 (6) 2585 (33)	515 (9) 970 (12)	(Zhu and Dudley, 2002)
HNRPU/ heterogeneous nuclear ribonucleoprotein U/ scaffold attachment factor A	co-repressor of GR	1	1	1		3	683 (10) 798 (11)	634 (10) 1161 (16)	(Eggert et al., 2001; Eggert et al., 1997)
MECP2/ methyl-CpG-binding protein 2	co-repressor	1	1			2	448 (6) 1463 (21)	520 (9) 1558 (24)	
SMARCA5/ SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily a member 5	co-repressor	1	1			2	x 1382 (25)	135 (4) 1372 (23)	
SIAHBP1/ fuse-binding protein-interacting repressor	co-repressor	1	1			2	x 486 (6)	272 (5) 216 (3)	

Legend: see Supplementary Table 1

Supplementary Table 3 Proteins possessing both co-activating and co-repressing capabilities

protein	function	protein property score					Mascot score[#]		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
JUN/ JUN oncogene	co-activator and co-repressor of AR	1	1	1	1	4	x	145 (2)	(Young et al., 1994; Wise et al., 1998)
							377 (5)	333 (4)	
DDX17/DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	co-activator, co-repressor of ER	1	1	1		3	280 (5)	x	(Watanabe et al., 2001)
							252 (4)	x	(Wilson et al., 2004)
ACTL6A/ Actin-like 6A/ ArpNbeta/ BAF53	co-repressor, co-activator, chromatin remodeling	1	1			2	131 (3)	196 (4)	
							203 (2)	252 (3)	
MYBBP1A/ MYB binding protein (P160) 1a	co-activator, co-repressor	1	1			2	822 (16)	395 (7)	
							2254 (31)	1716 (21)	

Legend: see Supplementary Table 1

Supplementary Table 4 Other transcription regulating proteins

protein	function	protein property score					Mascot score[#]		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
NCL/ nucleolin	chromatin remodeling, pre-rRNA transcription, interacting with GR	1	1	1		3	233 (4) 403 (8)	557 (9) 496 (7)	(Schulz et al., 2001)]
RUVBL1/ RuvB-like 1	chromatin remodeling, ATPase, transcription activation	1	1			2	89 (2) 431 (6)	347 6) 514 (6)	
RUVBL2/ RuvB-like 2	chromatin remodeling, ATPase, transcription activation	1	1			2	57 (1) 213 (3)	389 (8) 100 (1)	
PARP1/ poly (ADP-ribose) polymerase family, member 1	transcription regulation, DNA repair	1	1			2	255 (4) 2123 (31)	1644 (29) 473 (9)	
NFIX/ nuclear factor I/X /CCAAT-binding transcription factor	transcription regulation	1	1			2	312 (5) 529 (7)	229 (4) 454 (7)	
DDX3X/ DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	transcription regulation	1	1			2	141 (3) 208 (3)	123 (2) 191 (3)	
ILF2/ interleukin enhancer binding factor 2, 45kDa	transcription regulation of ILF2 gene	1	1			2	490 (9) 601 (7)	439 (7) 750 (9)	
GRHL2/ grainyhead-like 2/ transcription factor CP2-like 3	transcription factor	1	1			2	252 (5) 1146 (19)	388 (10) 1248 (16)	
PURA/ purine-rich element binding protein A	transcription regulation	1	1			2	255 (3) 562 (8)	162 (2) 704 (7)	

Supplementary Table 4 continued

CHD3/ chromodomain helicase DNA binding protein 3	transcription regulation, helicase	1	1	2	186 (5) 123 (2)	51 (1) 119 (2)
SMARCC2/ SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2/ BAF 170	chromatin remodeling, subunit SWI/SNF complex	1	1	2	63 (2) 241 (3)	122 (3) 149 (3)
HNRPDL/ heterogeneous nuclear ribonucleoprotein D-like	transcription regulation	1	1	2	304 (5) 471 (7)	219 (4) 567 (8)
ZNF384/ Zinc finger protein 384	transcription regulation	1	1	2	64 (2) 484 (4)	x 205 (2)
YY1/ YY1 transcription factor/ transcription activator/repressor protein delta	transcription factor	1	1	2	121 (2) 60 (1)	x 52 (1)
ATBF1/ AT-binding transcription factor 1	transcription factor	1	1	2	109 (2) 684 (9)	x 803 (11)
AHCTF1/ AT hook containing transcription factor 1/ transcription factor ELYS	transcription factor	1	1	2	113 (3) 1564 (22)	x 1563 (20)
CIC/ capicua protein	transcription regulation	1	1	2	52 (2) 57 (2)	x 270 (6)
CHD5/ chromodomain helicase DNA binding protein 5	transcription regulation, helicase	1	1	2	75 (2) 272 (6)	x 206 (4)
ILF3/ interleukin enhancer binding factor 3, 90kDa/ translational control protein 80	transcription regulation of ILF2 gene	1	1	2	756 (13) x	471 (7) 128(2)
MATR3/ matrin 3	transcription regulation	1	1	2	x 302 (5)	141 (2) 613 (9)

Supplementary Table 4 continued							
BAZ1A/bromodomain adjacent to zinc finger domain 1A	chromatin remodeling, ATPase	1	1	2	x 833 (12)	135 (3) 877 (12)	
ATF2/ activating transcription factor 2	transcription factor	1	1	2	x 645 (8)	67 (1) 657 (8)	
JUND/ jun D proto-oncogene	transcription factor	1	1	2	x 434 (5)	61 (1) 532 (5)	
RBBP7/ retinoblastoma binding protein 7	transcription regulation, chromatin remodeling	1	1	2	168 (4) x	x 155 (2)	
UBP1/ upstream binding protein 1 /LBP-1a	transcription regulation	1	1	2	x 114 (2)	89 (1) x	
TAF15/ TAF15 RNA polymerase II TATA box binding protein (TBP)-associated factor	preinitiation complex	1	1	2	x 76 (1)	135 (2) x	

Legend: see Supplementary Table 1

Supplementary Table 5 Proteins with functions related to RNA

protein	function	protein property score					Mascot score[#]		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
DDX21/ DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	RNA helicase	1				1	585 (11)	145 (5)	
							733 (9)	289 (11)	
HNRPA3/ heterogeneous nuclear ribonucleoprotein A3	RNA cytoplasmic shuttling	1				1	376 (6)	212 (2)	
							184 (4)	692 (11)	
HNRPL/ heterogeneous nuclear ribonucleoprotein L	RNA binding	1				1	213 (4)	269 (4)	
							459 (6)	715 (9)	
HNRPA2B1/ heterogeneous nuclear ribonucleoprotein A2/B1 protein	RNA binding	1				1	70 (1)	302 (4)	
							156 (2)	394 (6)	
HNRPAB/ Heterogeneous nuclear ribonucleoprotein A/B	RNA binding	1				1	508 (7)	551 (8)	
							993 (15)	869 (14)	
HNRPC/ heterogeneous nuclear ribonucleoprotein C (C1/C2)	RNA binding	1				1	522 (9)	445 (6)	
							1049 (15)	1014 (13)	
HNRPD/ heterogeneous nuclear ribonucleoprotein D/ AU-rich element RNA binding protein 1, 37kDa	RNA binding	1				1	521 (9)	471 (9)	
							1215 (18)	1095 (16)	
HNRPA0/ heterogeneous nuclear ribonucleoprotein A0	part of ribonucleosome	1				1	235 (4)	193 (3)	
							216 (2)	344 (3)	
Nucleolar protein NOP5/NOP58	RNA binding	1				1	100 (2)	165 (4)	
							628 (8)	755 (8)	
SYNCRIP protein/ synaptotagmin binding, cytoplasmic RNA interacting protein	RNA binding	1				1	677 (12)	513 (9)	
							165 (4)	518 (7)	
POLR1B/ DNA-directed RNA polymerase I largest subunit	rRNA transcription	1				1	237 (6)	124 (3)	
							88 (1)	105 (1)	
PRPF4/ PRP4 pre-mRNA processing factor 4 homolog	pre-mRNA splicing	1				1	241 (4)	420 (8)	
							217 (3)	268 (3)	
FBL/ fibrillarin	RNA binding	1				1	472 (9)	398 (8)	
							73 (1)	91 (2)	

Supplementary Table 5 continued

SF3B1/ splicing factor 3b, subunit 1, 155kDa	RNA splicing	1		1	50 (2) 369 (7)	78 (2) 628 (10)
SF3B2/ splicing factor 3b, subunit 2, 145kDa	RNA splicing	1		1	103 (2) 208 (3)	184 (3) 285 (5)
SF3B3/ splicing factor 3b, subunit 3, 130kDa	RNA splicing	1		1	62 (1) 239 (3)	372 (8) 345 (5)
STRBP/ spermatid perinuclear RNA binding protein	RNA binding	1		1	244 (7) 65 (1)	306 (7) 58 (1)
U2AF2/ U2 small nuclear RNA auxiliary factor 2	Spliceosomal catalysis	1		1	x 324 (4)	176 (5) 243 (4)
KHSRP/ KH-type splicing regulatory protein /FUSE binding protein 2	splicing	1		1	x 486 (6)	272 (5) 216 (3)
SF3A1/splicing factor 3a, subunit 1, 120kDa	splicing	1		1	x 161 (3)	72 (2) 317 (5)
NPM1/ Nucleophosmin/ nucleolar phosphoprotein B23, numatrin	ribosome binding	1		1	x 310 (4)	50 (1) 205 (3)
RBMX/ RNA binding motif protein, X-linked	RNA binding	1		1	93 (1) 83 (1)	101 (2) x
ZFR/ zinc finger RNA binding protein	RNA binding	1		1	457 (6) x	554 (9) 159 (2)
HNRPH1/ heterogeneous nuclear ribonucleoprotein H1	RNA processing	1		1	x 292 (4)	101 (2) 396 (5)
CDKN2AIP/CDKN2A interacting protein/ Collaborates/cooperates with ARF	RNA binding	1		1	118 (1) x	x 243 (2)

Legend: see Supplementary Table 1

Supplementary Table 6 Nuclear proteins not involved in transcription regulation

protein	function	protein property score					Mascot score #		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
DDB1/ Damage-specific DNA binding protein 1, 127kDa	DNA repair	1				1	x	104 (3)	
							405 (6)	466 (6)	
GNL3/ guanine nucleotide binding protein-like 3	Cell cycle control protein, GTP binding	1				1	x	135 (4)	
							116 (1)	430 (6)	
FUS/ fusion (involved in t(12;16) in malignant liposarcoma)	Nucleocyto-plasmic transporter activity	1				1	x	108 (2)	
							128 (2)	60 (1)	
RECQL/ RecQ protein-like /DNA helicase Q1-like	DNA repair	1				1	95 (2)	x	
							486 (7)	252 (4)	
MDC1/ mediator of DNA damage checkpoint 1	DNA repair	1				1	189 (4)	51 (1)	
							52 (1)	55 (1)	
RPA1/ Replication protein A1, 70kDa	DNA replication	1				1	89 (2)	66 (2)	
							x	83 (1)	
HSPA1A/ heat shock 70kDa protein 1A	chaperone, shuttling, interaction with GR			1		1	64 (2)	120 (2)	
							53 (1)	x	
SDAD1/ SDA1 domain containing 1	pre-ribosomal subunits export to cytoplasm	1				1	69 (1)	x	
							76 (1)	x	
PKP3/ Plakophilin 3	associated with desmosomes	1				1	60 (2)	x	
							69 (1)	x	

Legend: see Supplementary Table 1

Supplementary Table 7 Proteins with an unknown function

protein	function	protein property score					Mascot score[#]		references*
		nucleus	transcription	SHR	AR	total	ARE	Scr	
RBM28/ RNA binding motif protein 28	unknown	1				1	61 (1)	54 (1)	
							359 (5)	169 (2)	
RSL1D1/ ribosomal L1 domain containing 1	unknown	1				1	285 (5)	46 (1)	
							443 (6)	244 (3)	
RALY/ RNA binding protein, autoantigenic/ hnRNP-associated with lethal yellow homolog (mouse)	unknown					0	179 (4)	76 (1)1)	
							408 (4)	513 (4)	
DDX18/ DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	unknown					0	313 (5)	x	
							471 (7)	153 (2)	

Legend: see Supplementary Table 1

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION AND CONCLUSIONS

The main theme of this thesis is the study of factors and processes which can modulate androgen receptor (AR) transcriptional activity. To this end, three main questions have been addressed (see Fig. 5-1). In the following paragraph the conclusions regarding these questions are formulated.

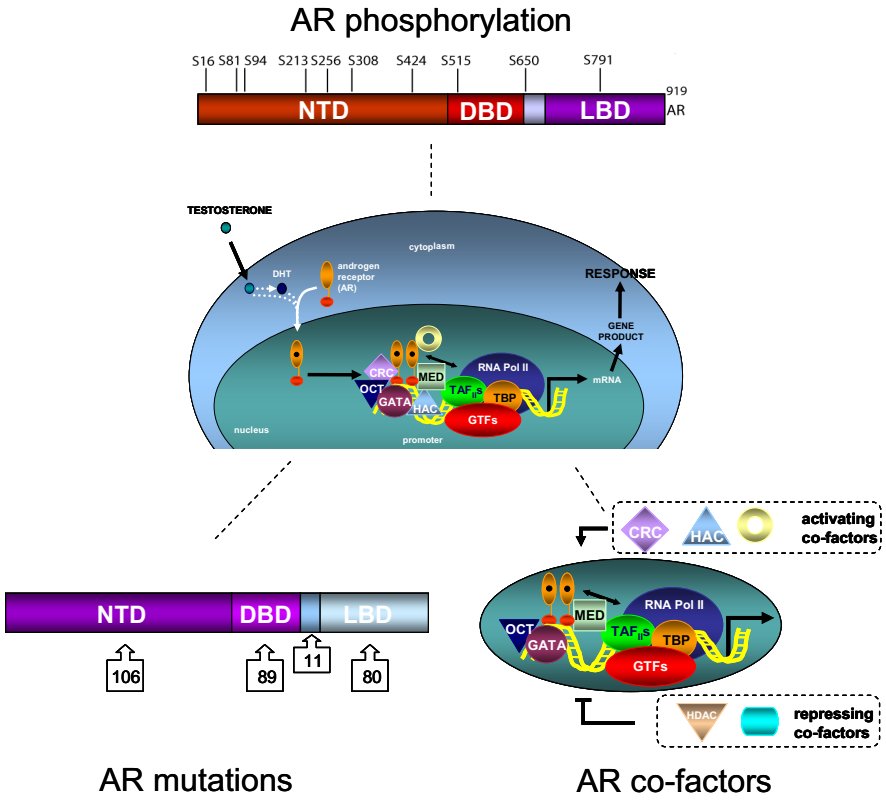

Figure 5-1
A schematic overview of factors and processes which can modulate AR transcriptional activity, studied in this thesis: AR phosphorylation, AR mutations, and AR co-factors. “AR phosphorylation” shows the known serine phosphorylation sites of the human AR. “AR mutations” shows the number of AIS mutations found for each domain. “AR co-factors” shows a part of the activating and repressing co-factors of the AR.

Question 1: *What is the influence of AR phosphorylation on AR isoforms and AR transactivation?*

When the present study of AR phosphorylation was started, the influence of phosphorylation on AR transactivation was largely unknown. According to the present study it became clear that phosphorylation of serine 94 is increased in a ligand dependent way, and that serine 650 is being constitutively phosphorylated (Chapter 2). With respect to the AR isoform pattern appearance, each isoform appears to be differentially phosphorylated at a distinct set of phosphosites. However, not every phosphorylation site necessarily contributes to the isoform pattern, as was shown by mutational analysis (Chapter 2). Furthermore, AR phosphorylation at serine residues does not appear to influence AR function directly, e.g. transactivation, NH_2-/COOH-terminal domain interaction, and co-activation by transcriptional intermediary factor 2 (TIF2) are not affected. Interestingly, phosphorylation at Ser-650 can be modulated by mutating Ser-515, another predicted phosphorylation site (Chapter 2).

Question 2: *What is the effect of the novel mutation F826L on AR transactivation?*

Initially, the AR LBD mutation F826L was found in the AR of a 46,XY DSD individual with a severe penoscrotal hypospadias, which suggested that this phenotype could be caused by this mutation. However, the LBD mutation did not affect AR transactivation, ligand binding, hormone response, protein stability, cellular localisation, and N-CoR co-repression. In contrast, this mutation resulted in a two-fold higher NH_2-/COOH-terminal domain interaction and TIF2 co-activation (Chapter 3). These effects are unlike the traditional way in which a detected AR mutation from an AIS subject results in complete or partial loss of AR transcriptional activation. The F826L mutation might affect AR functioning in a way that is not detected with the currently used functional assays. An alternative explanation for the severe penoscrotal hypospadias could be that the AR mutant recruits, in genital skin fibroblasts, a different repertoire of co-activators, not including TIF2. Alternatively, an altered LBD conformation may enhance a preferential recruitment of co-repressor(s) other than N-CoR, present in specific androgen target cells.

Question 3: *Can existing and novel AR co-factors be isolated when complexed with the AR in an oligonucleotide-based in vitro assay?*

Many AR co-factors have been isolated via isolation procedures in the absence of AR binding to specific DNA sequences. However, there is ample evidence that specific binding to DNA is essential for co-factor recruitment. Therefore, we used oligonucleotides containing the ARE1 of the PSA promoter to study co-factor recruitment by the AR. Identified proteins were scored for having one or more of the following known properties: nuclear localisation, involvement in transcription regulation, involvement in steroid hormone receptor (SHR) function, or specific involvement in AR function. In the so-called oligonucleotide-based assay (OBA), in total 85 proteins were recruited by the AR in nuclear extracts obtained from LNCaP cells (Chapter 4). Of the isolated proteins 11 were known to interact with the AR and to influence AR transactivation. Five other bound proteins are known to influence transactivation by other SHRs. In total 32 proteins with less AR interacting potential were also found. These proteins are known to be involved in transcription regulation of different genes. Furthermore, 25 proteins with an RNA binding or processing function, were isolated. Finally, for 4 proteins the function is still unknown. Based on peptide counting, a more strict slection criterion for specificity, we found enrichment of 7 proteins eluted from the ARE1 oligonucleotide, compared to a scrambled oligonucleotide control. One of these proteins, DDX17, is known to be a co-factor for estrogen receptor α (ERα), but has never been reported to be associated with an AR function. In conclusion, the results indicate that the ARE1 of the oligonucleotide-based assay allows the isolation of known AR interacting proteins and of new potential DNA-bound AR interacting proteins.

5.2 PHOSPHORYLATION

In total, 7 serine residues in the human AR have now been identified as potential *in vivo* phosphorylation sites (Gioeli et al., 2002; Wong et al., 2004). These serine residues, Ser-16, Ser-81, Ser-94, Ser-256, Ser-308, Ser-424, and Ser-650, were all identified and confirmed as phosphosites by mutagenesis, peptide mapping and mass spectrometry. Unfortunately, a clear function for these phosphorylated serine residues has not been found. Phosphorylation of other steroid hormone receptors, like the ER, GR, and PR, has an influence on transcriptional activation of these receptors (see Chapter 1). Some of these phosphosites are newly phosphorylated upon hormone binding, but such a change in phosphorylation has surprisingly not been detected for the AR. If such a site would be present, a hormone-induced AR phosphosite could influence the transactivation capacity of the AR.

There are several explanations for the fact that a possible hormone-induced phosphosite as well as other potential AR phosphosites could not be identified by peptide mapping and mass spectrometry. First: the phosphate

group can be lost during the AR phosphopeptide isolation procedure. Second: phosphorylated peptides can be masked by unphosphorylated peptides during mass spectrometry analysis. Third: elution of large in-gel trypsinised peptides containing one or more potential phosphosites may face considerable difficulties.

Until recently, the identification of phosphosites was complicated, because phosphoserine and phosphotyrosine residues can easily lose the phosphate group through β-elimination reactions (Cantin et al., 2006; DeGnore and Qin, 1998). However, newly developed methodology has solved the problem of phosphate group loss, for example by using biotinylation. Phosphoserine residues are chemically modified using strong alkaline conditions. The peptides undergo a β-elimination reaction, resulting in a reactive dehydroalanine moiety (Oda et al., 2001; Garcia et al., 2005). The next steps include biotinylation enrichment after the above transformation of the phosphate group to a thiol derivative with DTT or EDT. This procedure does not only substitute for the loss of phosphate, but also enables selective purification of the modified proteins resulting in enrichment of phosphoproteins or phosphopeptides. The most effective way to substitute for the loss of phosphate groups is to use the procedure on the phosphoproteins as soon as possible after protein extraction from cell lysates, rather than after several other purification procedures (Oda et al., 2001; Tao et al., 2005).

The second possible reason why some phosphosites might not be detected, is the fact that phosphorylation of a specific site *in vivo* is seldom stoichiometric. Consequently, most of the potential phosphopeptides are unphosphorylated, which results in only a small proportion of phosphorylated peptides (frequently less than 5%). In this way, a relatively large amount of unphosphorylated peptides can mask the phosphorylated peptides during mass spectrometry analyses. A solution to this problem is the enrichment of phosphopeptides, which can be achieved by selective isolation via the commonly used Immobilised Metal ion Affinity Chromatography (IMAC). Phosphoproteins or -peptides can be bound to immobilised metal ions via their phosphate moiety and thereafter eluted from the metal ion-affinity columns (Neville et al., 1997). However, due to an easy loss of the phosphate group, IMAC alone is not the preferred method. In contrast, IMAC in combination with the use of a previously mentioned phosphate group replacement by biotin is a more appropriate method to prevent the masking. Such enrichment would ease identification of phosphosites. A disadvantage of biotinylation is that this also involves a general modification of other proteins, which can result in a considerable contamination. Therefore, other more specific chemical modifications are more appropriate.

Concerning the third explanation for unidentified/missed phosphosites, in some phosphorylation studies antibodies were used for the precipitation of the

phosphoprotein of interest (see also Chapter 2). SDS-PAGE purification is usually performed to avoid the overload of antibodies and to selectively isolate the protein of interest, facilitating the mass spectrometric identification of phosphosites. However, the SDS-PAGE step is necessarily followed by an in-gel digestion by a protease, which might become problematic when large peptides have to be extracted from the SDS-polyacrylamide gel. But a SDS-PAGE purification step can possibly be eliminated, because nowadays mass spectrometers are more sensitive, have an optimal liquid chromatography separation system and an optimal peptide fragmentation, thus sequencing possibilities. These features facilitate protein identification markedly and especially from a large mixture of proteins which in combination with alternatives of in-gel digestion can result in an even better protein identification. Some alternatives of in-gel digestion are just recently being developed. For instance, proteases immobilised on beads to which the protein sample can be added, thus eliminating the use of SDS-PAGE (Lim et al., 2006; Wu et al., 2006). However, such methods have not been tested on a complex mixture of proteins. Another alternative, but still being studied, is the addition of a protease to a purified mixture of proteins which are still attached in some way to beads, the so-called on-bead digestion. This method is likely to work well with proteins which are not isolated via antibody precipitation, because protein identification will likely be more difficult in the presence of an antibody overload. Although the excess of antibodies can be problematic, the amount of antibodies might be decreased by competition between the bound proteins and an excess of an exogenous peptide to which the antibody binds. If necessary, when the excess of peptide interferes with the protein identification, it can be removed by the use of a tag or an isotope to discern the competing peptide from the original peptide of the protein of interest.

Most likely, an effective way to identify AR phosphosites is by using a combination of the following methods: 1) replacement of the phosphate group by biotin or by another kind of group, followed by 2) enrichment of phosphopeptides. Even more or additional other phosphosites will probably be found if in the last method the SDS-PAGE-step is eliminated. In conclusion, more AR phosphosites can likely be identified by using a combination of the three above-mentioned methods.

As discussed above, one possible cause which leaves the function of phosphorylation undetected could be a less optimal isolation and identification of phosphosites. Besides this, the detection methodology for a possible function for phosphosites could also be rather limiting. Detection of a possible function for phosphosites is generally performed by the use of functional assays in which one or more phosphosites in transcription factors and also in nuclear receptors, like the AR, are mutated and tested for the ability to activate transcription of a reporter gene via interaction with an enhancer

and/or promoter. As concluded in Chapter 2, AR phosphorylation of the sites studied does not seem to have a function. Different gene promoters and cell lines have been used to study the AR in a more or less natural environment. In most functional assays the AR with one mutated phosphosite has been investigated. In addition, double phospho-mutants have been studied, but no function for AR phosphorylation could be revealed (Zhou et al., 1995; Gioeli et al., 2002). Combinatorial phosphorylation could probably play a role in keeping the AR function normal. It has been shown that mutation of certain AR phosphosites results in phosphorylation or dephosphorylation of another site (Gioeli et al., 2002; Wong et al., 2004). Perhaps combinatorial phosphorylation can preserve AR function by phosphorylation of another amino acid residue. However, more research has to be performed to investigate the process of combinatorial phosphorylation. The effect of mutagenesis of more than 2 known AR phosphosites needs to be investigated too, as well as different combinations of mutated phosphosites. In this way, combinatorial phosphorylation might be prevented. Interestingly, acetylation of the AR seems to be important for the appearance of the hormone-induced and highly phosphorylated AR 114 kDa isoform (Fu et al., 2004). In addition, it has been shown that phosphorylation of ER Ser-305 blocks acetylation of Lys-303, which results in an enhanced transactivation (Cui et al., 2004). Perhaps the phosphorylation status of one or more sites in the AR can also influence other types of AR modifications, or vice versa, which will make the specific influence of phosphorylation even more complex.

A second explanation why functional assays do not reveal the function of phosphorylation, is a possible involvement in non-genomic actions of AR phosphorylation. Apart from the known AR signaling pathway that results in transcription regulation, there is evidence that the AR can exert a rapid, non-genomic effect. In prostate cancer cells, it has been shown that androgens can induce the association of both the AR and ERβ with Src and thereby stimulating the Src/RAF1/ERK signal transduction pathway (Migliaccio et al., 2002; Migliaccio et al., 2000). If AR phosphorylation influences non-genomic effects via this signal transduction pathway, then the use of the mentioned functional assays would not be suitable. Therefore, more research has to be done also on possible non-genomic effects of AR phosphorylation.

An interesting approach is the use of antibodies for the study of protein phosphorylation. Nowadays, antibodies against certain serine phosphorylation sites have proven to be quite specific. Phosphosite-specific AR antibodies have not yet been used for elucidation of a function of AR phosphorylation. However, two phosphosite-specific antibodies, each acting against one particular phosphosite of the GR, revealed that there are different GR phosphorylated isoforms and that each isoform is located at different regions within the cell at the same time point (Lin et al., 2002). If this also occurs for

the AR, each phosphosite-specific antibody might be used to isolate a specific set of interacting proteins from which a certain function can be derived for one or more phosphosites. Phosphosite-specific antibodies might also be useful as semiquantitive indicator for the phosphorylation level of a certain phosphosite.

Not until recently, tyrosine phosphorylation was thought not to occur for the AR (Kuiper et al., 1993; Gioeli et al., 2002; Kuiper and Brinkmann, 1995). However, this has been disapproved by the use of phospho-tyrosine specific antibodies. It has been shown that AR tyrosine residue 534 is highly phosphorylated (Guo et al., 2006; Kraus et al., 2006). Phosphorylation of AR Y534 is mainly induced by EGF via the activation of Src (Guo et al., 2006; Kraus et al., 2006). Remarkably, Src has also been shown to be important for the R1881-induced tyrosine phosphorylation (Kraus et al., 2006). The tyrosine phosphorylation level is increasing from 10 min to 30 min after addition of 1 ng/ml EGF. However, when 10 or 100 ng/ml EGF was used, the highest level of phosphorylation was already reached within 5 min, but decreased between 10 min and 30 min (Guo et al., 2006; Kraus et al., 2006). The same high level of tyrosine phosphorylation is reached only after 30 min of R1881 incubation, which thereafter decreases (Kraus et al., 2006). In contrast to serine phosphorylation, tyrosine phosphorylation is involved in the EGF- and DHT-induced transactivation of the AR, as was shown by the use of tyrosine mutants (Guo et al., 2006). The AR mutant Y534F has a 50% reduction in transcription activity. Remarkably however, a mutated tyrosine 534 AR can still be fully active when a high concentration (10 nM) of DHT is used (Guo et al., 2006). In contrast, one research group has found that the transcriptional activity of AR mutant Y534F was minimally impaired (Mahajan et al., 2007). Furthermore, phosphorylation of Tyr-534 is involved in the translocation of the AR to the nucleus (Guo et al., 2006). However, a weak point in this study is the large discrepancy between the incubation times (16 hours with EGF, which is needed for the functional assays) and the time point for phosphorylation (which occurs already within 10 to 30 minutes). Two other tyrosine phosphorylation sites were identified, Tyr-267 and Tyr-363. Mutation of Tyr-267 to phenylalanine abolished the AR transcriptional activity and the binding to AREs (Mahajan et al., 2007). AR mutant Y363F resulted in a 76% decrease of the overall AR tyrosine phosphorylation, a decrease in AR transcriptional activity by 65% and a reduced DNA binding of 50% (Mahajan et al., 2007). However, these experiments were performed in the presence of a constitutive active ACK-1 kinase and the phosphorylation status was not verified in the presence of hormone alone (Mahajan et al., 2007).

Like tyrosine phosphorylation, it was thought that threonine phosphorylation does not occur for the AR (Kuiper et al., 1993; Gioeli et al., 2002; Kuiper and Brinkmann, 1995). Recently, it was reported that threonine

phosphorylation of the AR in human sperm cells could be demonstrated with the use of a phosphothreonine specific antibody (Aquila et al., 2007). However, the investigators have not determined the exact location of the threonine residues involved.

The role of AR phosphorylation still remains unclear. Mutations of individual phosphosites, or of five phosphosites in one AR protein, did not reveal an indication for function, nor does the phosphorylation mimicking mutation of six serine residues to aspartic acid (Yang et al., 2007). In many studies the transcriptional activity of the AR has not been studied with androgens in a dose-dependent manner, whereas it has been shown that a high dose of androgens can result in a normal AR activity (Guo et al., 2006).

In conclusion, there might be still opportunities for finding a function for AR phosphorylation with the suggested experimental approaches.

5.3 AR MUTATIONS AND AIS

It is generally accepted that defects in the androgen receptor can prevent the normal development of both internal and external male structures and properties in 46,XY individuals, and information on the structure of the human *AR* gene has facilitated the study of molecular defects associated with androgen insensitivity. Naturally occurring mutations in the *AR* gene are an interesting source for the investigation of receptor structure-function relationships. In addition, the variation in clinical phenotypes provides the opportunity to find a possible correlation between a mutation in the AR structure and an impairment of a specific physiological function.

A mutation in the AR protein can result in an increased AR transcriptional activity or a broadened ligand specificity, which is most times coupled to prostate cancer. A mutation also can result in a decreased AR transcriptional activity or loss of ligand binding, which is coupled to AIS. The mutation F826L, found in a boy with severe penoscrotal hypospadias, did not show a clear functional difference compared with the wild-type (wt) AR. The full-length mutant F826L showed a similar transcriptional activity as the wt AR. Furthermore, this mutant appeared to be indistinguishable from the wild-type with respect to ligand binding affinity and expression of an isoform pattern in genital skin fibroblasts (GSF), hormone responsivity, and sub-cellular distribution in transfected Chinese Hamster Ovary (CHO) cells. However, the NH_2-/COOH-terminal domain interaction and the TIF2 co-activation were increased two-fold as compared with the wt AR (see Chapter 3). These experiments included luciferase assays performed in CHO cells. An ideal situation would be a functional study of the F826L AR mutant in a natural environment; such as GSFs from the AIS subject, because the recruitment of tissue specific co-factors might be different from that in CHO cells. Recently,

Holterhus *et al.* (2005) managed to study the transactivation of wt AR and mutant ARs in AR negative GSFs, by transfecting these cells with either wt AR or mutant AR, together with a reporter construct (Holterhus et al., 2005). The best way to study AR F826L, would be to transfect only a reporter construct in GSFs of the AIS subject and compare it to a panel of GSFs containing a wt AR, to eliminate the possible difference between GSFs populations. However, the AR levels in GSFs is very low, and it might be necessary to enhance the amount of reporter construct to detect any signal, but overexpression has to be prevented, to exclude perturbation of the cells. If a difference is found between the wt and the mutant F826L AR in activating the reporter construct in GSFs, then the OBA can be applied to show a possible difference in the recruited co-factor repertoire between wt and mutant AR.

The lengths of the polyglutamine (poly Gln) and polyglycine (poly Gly) stretches in mutant ARs might also be relevant for AR activity, because the length of the stretches in combination with an AR mutation can have pronounced influences on AR transactivation capacity. In a recent publication it was shown that two 46,XY individuals with undervirilisation and genital malformations, have a short poly Gly repeat of 10 residues and a relatively long poly Gln repeat of 28 or 30 residues within the NH_2-terminal domain of the AR. In addition, the AR harbours in these two individuals a A645D mutation in the hinge region (Werner et al., 2006). Functional assays with different plasmids showed that a shorter poly Gly repeat (10 residues) down-modulated AR activity to approximately 60-65% as compared to a longer poly Gly repeat (16 residues). However, in combination with an A645D mutation, the decrease in activity associated with the short poly Gly repeat was found to be less. On the contrary, a short poly Gly repeat (10 residues) in combination with a long poly Gln repeat (28-30 residues) and the A645D mutation decreased the AR activity to less than 50% (Werner et al., 2006). Interestingly, in the context of a short poly Gln and a short poly Gly repeat, the A645D mutation can even rescue AR activity to almost wild-type levels. It is evident that different lengths of the poly Gly and poly Gln repeats might have an influence on the activity of a mutant AR. However, it can be expected, based on the previous considerations, that the length of the repeats do not play a crucial role in the transcriptional activity of the AR mutant F826L, because the lengths are within the normal range (poly Gln: 22; poly Gly: 17; Chapter 3).

5.4 PROTEIN-PROTEIN INTERACTION

5.4.1 Advantages and disadvantages of the oligonucleotide-based assay

The oligonucleotide-based assay (OBA) was performed to isolate AR co-factors recruited by DNA-bound AR and which could be missed with conventional methods in which specific DNA binding is not an issue. Beside the DNA-bound AR dependent recruitment, the OBA has several other advantages. It is a straightforward, clearly defined method and the preferred responsive elements with their flanking sequences can be selected. Therefore specific binding of recruited proteins to the AR and the flanking sequences of each ARE can be studied. The use of combinations of responsive elements may reveal more proteins involved in a complex which are only recruited by at least two different DNA-bound AR molecules and/or bound co-factors.

Besides the above-mentioned advantages OBA, this approach has also some disadvantages. This method demands a large amount of AR containing cells to obtain sufficient quantities of AR protein. OBA is a clearly defined method to study co-factor recruitment on certain responsive elements, but co-factors are recruited *in vitro* and the isolated proteins by OBA may not represent the total protein complexes which can be found on a regular promoter in its native context in intact cell systems.

5.4.2 Methods to isolate in vivo recruited proteins

After identification of possible AR interacting proteins via the oligonucleotide-based assay, additional experiments have to be performed, to confirm the interaction between the AR and the identified AR interacting protein. One approach which may represent more the *in vivo* situation, uses a DNA construct with tags flanking the promoter of interest to isolate the promoter sequence and the attached proteins after fixation. A research group has isolated complexes, which were assembled *in vivo* and cross-linked to chromatin containing an inserted Tet-O binding site. The isolation takes place by using a Tet R tag and cleavage sites which are located on both sides of the promoter of interest (Grosveld et al., 2005). However, disadvantages of this experimental approach are some non-specific protein-protein and protein-DNA cross-linking. Often the used cross-linking chemical is formaldehyde, and this compound crosslinks proteins and protein-DNA within a radius of approx. 20 Å, which is beyond the closest distances between protein and DNA (5-10 Å).

In conclusion, crosslinking experiments can result in valuable information on complex composition, although this approach has its limitations due to non-specific chemical modifications.

5.4.3 Conventional protein interaction methods

Besides OBAs, yeast two-hybrid, mammalian two-hybrid, GST pull-down and co-immunoprecipitations are often used for searching interacting proteins. Although, these conventional methods are not very suitable for identifying DNA-bound proteins, they have their own advantages.

The yeast two-hybrid system is one of the most commonly used methods to study protein-protein interaction. The protein (fragment) of interest is expressed as a fusion protein containing a GAL4-DNA binding domain. If an interaction takes place with a possible partner containing a transactivation domain, the complex may form a functional transcription factor which can activate transcription of a reporter gene (Chien et al., 1991; Dang et al., 1991). This method is useful for identifying either a large amount of interacting proteins or just a few specific proteins. It is suitable for studying interaction between proteins which are unable to bind DNA. However, it has to be kept in mind that several weak points of this method can lead to false positives. These are summarised below: 1. The number of false positives can be increased by strong transcription activation domains in case of weak protein-protein interactions (Stephens and Banting, 2000); 2. The high number of copies of the upstream activation sequence in a promoter region can also contribute to the increase of false positives; 3. The protein of interest can be sticky or can bind unexpectedly directly to promoters; 4. Overexpression of proteins in yeast can lead to modifications in yeast permeability, which can result in more uptake of substrates (Serebriiskii et al., 2000). With the yeast two-hybrid method, protein-protein interactions also can be missed. These false negatives are due to a lack of cell-specific posttranslational protein modifications or processing (cleavage) prior to interaction (Ito et al., 2000). However, this can be overcome by using mammalian cells, the so-called mammalian two-hybrid method. In addition, it allows the monitoring of modifications in the interaction in response to cell stimulation (Brent and Finley, 1997). This system is frequently used to confirm the interaction of known proteins or proteins found by the yeast two-hybrid method.

Another commonly used method to identify interactions is the glutathione S-transferase (GST) pull-down. Herein the protein (fragment) of interest, the bait, is expressed as a GST fusion protein and captured by a glutathione-agarose column. Interacting proteins (the prey) are obtained by adding cellular extracts to the column. This method is also suitable for capturing a large

number of proteins. However, the following has to be kept in mind. This method involves the usual overexpression of both the bait and the prey and certain proteins are brought together which normally may not co-localise in the cell. Beside these disadvantages, GST pull-down assay can result in a high background due to a-specific binding and in addition it may miss a relatively high number of proteins (Edwards et al., 2002).

Co-immunoprecipitation is also frequently used to identify protein-protein interactions. It has the advantage that protein interactions take place between endogenous proteins from cells in culture (or from a tissue sample). However, co-immunoprecipitation is technically difficult, due to low expression levels of the protein of interest, weak affinity of the interactions, limited availability of specific antibodies, and of suitable target epitopes. In addition, it is often necessary to overexpress proteins in irrelevant cell lines.

Many tags are also fused to “bait” proteins for isolation of interacting proteins as a complex (Chang, 2006; Terpe, 2003). Frequently used tags are FLAG, MYC, hexahis(tidine) and biotin. Proteins containing these tags can easily be isolated by either antibodies or for biotin by (strept)avidin. However, these proteins need to be overexpressed or stably expressed in cells. This might sometimes form a problem. Furthermore, tagged proteins may not act similarly as their native counterparts (Chang, 2006). In addition, during the isolation of biotin tagged recombinant proteins one has to deal with endogenous biotin modified proteins, which compete in the isolation procedure and increase the number of false positives.

In general, most of the interactions in these methods are not between endogenous proteins or do not take place in physiological relevant cell lines. In addition, the usual overexpression of proteins results in abnormally high physiological levels, which may cause loss of regulation that normally is dependent on the cell cycle phase or on other cellular states. These events can result in altered sub-cellular localisation and/or aberrant interactions, leading to false positives. Furthermore, the detected protein interaction can also be indirect via another protein.

5.4.4 In-cell visualisation of protein-protein interactions

Determination of direct interactions between two known proteins as well as studying these interactions in a more natural environment can be very informative and has the preference over the conventional protein interaction identification methods. One of these recently developed methods is the nowadays widely used fluorescence resonance energy transfer (FRET) analysis, in which the change of energy transfer from a fluorescent protein to another fluorescent protein is measured (Wallrabe and Periasamy, 2005).

This method can show the direct interaction between two proteins, since energy transfer only occurs if two fluorescent proteins are in close proximity of each other. This method does not need overexpression of the fluorescent proteins. Only stable expression at physiological levels is sufficient. Beside this advantage, the interaction can be visualised in real-time in living cells. In this way complex formation and dissociation can by studied in time. A disadvantage of this method is related to the fluorescent tag. The tag might negatively influence the interaction and action of a fusion protein as compared with its native counterpart.

A new method based on protein fragmentation complementation, has almost similar advantages as the FRET method. The interaction between two proteins, fused to complementary fragments of a reporter protein, results in a functional complementation and reporter activity (Kerppola, 2006; Fig. 5-2). This method causes minimal cellular perturbation. However, a disadvantage is that the protein fragment association can have a time lag ranging from minutes to 24 h depending on the character of the used protein. Furthermore, the association can also result in extra stabilisation of the complex, which makes this method less suitable for studying protein-protein interaction dynamics. The spatial resolution can be at the sub-cellular or cellular level and within a cellular population (Kerppola, 2006). However, like in the FRET approach, the fusion proteins used for the complementation method may not necessarily interact or behave in the same way as their native counterpart.

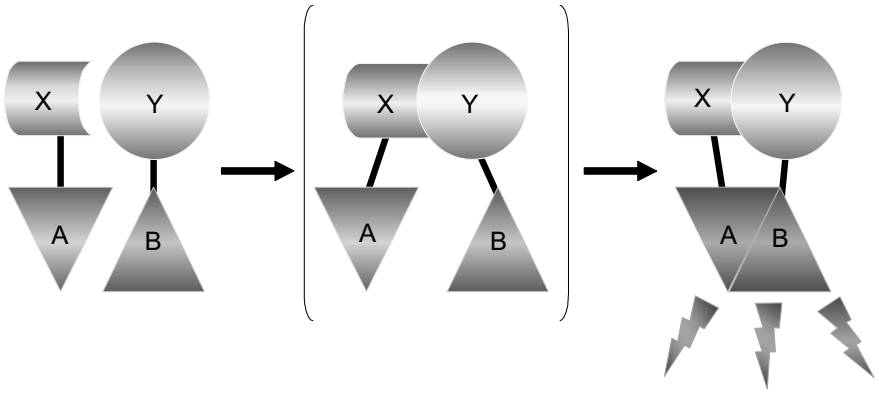

Figure 5-2
Schematic representation of the principle of the protein complementation assay. Upon interaction of proteins (or their fragments) X and Y, the fused fragments A and B are also brought to close proximity, thereby reconstituting the original function (e.g. proteolytic activity or luminescence).

Recently, the proximity ligation *in situ* assay (P-LISA), a method to study direct interactions between endogenous proteins, has been described (Soderberg et al., 2006). Proximity probes are oligonucleotides attached to antibodies against two target proteins and they function by guiding the formation of circular DNA strands if bound in close proximity. The DNA circles in turn serve as templates for localised rolling-circle amplification (RCA), allowing individual interacting pairs of protein molecules to be visualised and counted. With P-LISA it is possible to study multiprotein complexes. However, the applied antibodies have to be very specific and the protein interactions can only be studied in fixed cells and tissues.

In conclusion, FRET, fragmentation complementation and P-LISA are good alternative ways to confirm or study in more detail potential protein-protein interactions. More importantly, these methods are ideal in showing protein-protein interaction between a DNA-bound transcription factor and a co-factor.

5.4.5 AR functional research

Besides confirmation of an interaction between the AR and identified candidate AR interacting partners, another more important aspect is to determine what the functional relationship is between the AR and an AR interacting protein. This can be achieved by studying the role of an AR interacting protein in AR transactivation, first with a reporter construct, if the AR interacting protein can be easily expressed from a construct. The usual procedure is either a gradual increase in protein expression level or knock-down of its expression using si-RNA, which might result in a changed level of AR-induced transcription of a gene, such as the *prostate specific antigen* (*PSA*) gene. Further research may involve the effect on AR sub-cellular localisation, the NH_2-/COOH-terminal domain interaction assay, and the competition between co-repressors and co-activators. However, when performing these experiments, several aspects have to be kept in mind. Often proteins of interest are overexpressed or constitutively expressed in irrelevant cell lines. The overexpression may result in the loss of regulation which may be dependent upon the phase of the cell cycle or other cellular states. It may also result in aberrant interactions with other proteins and/or altered sub-cellular localisation. Kinase overexpression may result in loss of specificity of substrates. Although most assays are performed with overexpressed recombinant proteins, they are rather easy to perform and give at least an indication of a function. Very informative methods are knockout or knock-in approaches to remove or modify genes in mice. In this way proteins can be studied at a more physiological level and in a natural environment. However,

the consequences of the observed changes in mice may not be similar in human situations, but the mouse models may help to decipher mechanisms step by step. However, the most informative indication for a function of a human protein, is the experiment of nature, in which the protein of interest appears to be mutated in a human being with a changed phenotype.

5.4.6 Peptide counting as selection criterion for isolated proteins

For the present OBA, peptide counting was used to determine specific DNA-bound AR interacting proteins. This method provides quantitative estimates of protein abundance and is therefore very useful to determine protein enrichment, without requiring either the tagging of proteins or expensive isotope labelling (Gao et al., 2005). Peptide counting is literally the counting of peptides which are identified by mass spectrometry. Peptide counting is based on the finding that protein abundance is correlated with the total number of identified peptides for any given protein (Gao et al., 2003; Liu et al., 2004; States et al., 2006). The determination of the ratio between counted peptides of the sample of interest and of the control sample, gives a reliable quantitative estimation of protein enrichment. This method is useful as a selection criterion if a large number of identified proteins is present in the sample of interest and in the control sample. In the present investigation, peptide counting revealed enrichment of 7 putative AR co-factors eluted from the ARE1 oligonucleotide, compared to the scrambled oligonucleotide. One of these proteins, DDX17, is known to be a co-factor for estrogen receptor α (ERα), but has never been shown to be associated with AR function. The present results indicate that the ARE oligonucleotide-based assay may allow enrichment of new candidate DNA-bound AR interacting proteins (Chapter 4).

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SUMMARY

Androgens, testosterone (T) and 5α-dihydrotestosterone (DHT), are important for male and female physiology, in particular for male sexual differentiation, development of secondary male characteristics and spermatogenesis. These hormones exert their actions by binding to the androgen receptor (AR), a transcription factor that belongs to the family of steroid hormone receptors (SHRs). After ligand binding, the AR migrates to the nucleus and binds to androgen response elements (AREs), which are present in the promoter and enhancer regions of androgen regulated genes. After DNA binding, chromatin remodelling factors, other co-factors (co-activators or co-repressors) and proteins of the transcription initiation complex, including RNA polymerase II, are recruited by the AR to regulate gene transcription. Several modulating processes and factors can influence AR transactivation. Not only co-factors play a role in AR transactivation, but also post-translational modifications of the AR, such as acetylation, ubiquitylation, sumoylation and phosphorylation, can modify AR transactivation. In addition, mutations in the *AR* gene can have dramatic consequences for AR transactivation. In general, AR mutations result in the androgen insensitivity syndrome, in which the male phenotype is affected. However, the exact influence of these modulating processes and factors is still not clear. In **Chapter 1**, three questions were formulated, which were addressed in detail in this thesis:

Question 1: *What is the influence of AR phosphorylation on AR isoforms and AR transactivation?*

Question 2: *What is the effect of the novel mutation F826L on AR transactivation?*

Question 3: *Can existing and novel AR co-factors be isolated when complexed with the AR in an oligonucleotide-based in vitro assay?*

Phosphorylation has been shown to influence the function of several steroid hormone receptors. The AR is known to be phosphorylated, but the effect of phosphorylation on AR function is unknown. In the experiments described in **Chapter 2**, it became clear that phosphorylation of serine 94 can be increased in a ligand dependent way, and that serine 650 is being constitutively phosphorylated. With respect to the AR isoform pattern, each isoform appears to be differentially phosphorylated at a distinct set of phosphosites. However, not every phosphorylation site necessarily contributes to the isoform pattern, as was shown by mutational analysis. Furthermore, AR phosphorylation at serine residues does not appear to influence AR function directly, e.g. transactivation, NH_2-/COOH-terminal domain interaction, and co-activation by transcriptional intermediary factor 2 (TIF2).

Interestingly, dephosphorylation at Ser-650 occurred by mutating Ser-515, another potential phosphorylation site.

Androgen signalling is important in particular for male development and physiology. A defective signalling in an individual can result in the androgen insensitivity syndrome (AIS), which belongs to the group of disorders of sex development (DSD), and which is predominantly caused by mutations in the *AR* gene. In **Chapter 3**, the study of the newly identified F826L mutation in the AR is described. This mutation was found in the *AR* gene of a boy with a severe penoscrotal hypospadias (classified as 46,XY DSD). Remarkably, this LBD mutation did not affect AR transactivation, ligand binding, hormone response, protein stability, sub-cellular localisation, and N-CoR co-repression. In contrast, this mutation resulted in a two-fold higher NH_2-/COOH-terminal domain interaction, and a two-fold increase in TIF2 co-activation. The F826L mutation might affect AR functioning in a way that is not detected with the currently used functional assays. An alternative explanation for the severe penoscrotal hypospadias could be that the AR mutant recruits, in genital skin fibroblasts, a different repertoire of co-activators, not including TIF2. Alternatively, an altered LBD conformation may enhance a preferential recruitment of co-repressor(s), present in specific androgen target cells, other than N-CoR.

Transcription regulation of genes involves a large numbers of co-factors. Many AR co-factors have been isolated via isolation procedures in the absence of AR binding to specific DNA sequences. However, there is evidence that specific binding to DNA is important for co-factor recruitment. Therefore, we used an oligonucleotide containing the ARE1 of the PSA promoter, and a scrambled version of this oligonucleotide as control, to study co-factor recruitment by the AR (**Chapter 4**). Identified proteins were scored for having one or more of the following known properties: nuclear localisation, involvement in transcription regulation, involvement in steroid hormone receptor (SHR) function, or specific involvement in AR function. With the so-called oligonucleotide-based assay (OBA), a selection of 85 proteins from AR containing nuclear extracts from LNCaP cells were found to be associated with the AR bound to the ARE1. Of the bound proteins, 11 proteins were known to interact with the AR and to influence AR transactivation. 5 other bound proteins are known to influence transactivation of other SHRs. In total 32 proteins with less AR interacting potential were also found. These proteins are known to be involved in transcription regulation of different genes. Furthermore, 25 proteins with an RNA binding or processing function, were isolated. Finally, from 4 proteins the function is still unknown. When peptide counting was applied as a more strict selection criterion for specificity, we found enrichment of 7 proteins eluted from the ARE1 oligonucleotide, compared to the scrambled oligonucleotide. One of these proteins, DDX17, is known to be a co-factor for estrogen receptor α (ERα). The present study is the first report in which DDX17 is being associated with an AR function. In

conclusion, the results indicate that the ARE1 in the OBA allows the isolation of known AR interacting proteins and of new potential AR interacting proteins.

In **Chapter 5**, the results obtained from studies described in Chapters 2, 3 and 4 are discussed in a broader perspective. Future directions for additional studies on a relationship between phosphorylation and AR function, a possible cause of the severe penoscrotal hypospadias of the 46,XY DSD individual with the F826L mutation in the AR and on a possible function for the identified co-factors are also discussed.

SAMENVATTING

De androgenen testosteron (T) en 5α-dihydrotestosteron (DHT) zijn belangrijk voor de mannelijke en vrouwelijke fysiologie, met name voor de mannelijke geslachtsdifferentiatie, voor de ontwikkeling van de secundaire mannelijke geslachtskenmerken en voor de spermatogenese. Deze hormonen oefenen hun werking uit door binding aan de androgeenreceptor (AR), een transcriptie-factor die tot de familie van steroidhormoonreceptoren (SHR) behoort. Na ligand binding is de AR in de celkern aanwezig en bindt daar o.a. aan androgeen responsieve elementen (ARE), die gelokaliseerd zijn in de promoter en zogenaamde enhancer regio's van androgeen gereguleerde genen. Na DNA binding, worden chromatine modulerende factoren, andere co-factoren (co-activatoren en co-repressoren) en eiwitten van het transcriptie initiatie complex, inclusief RNA polymerase II, gerekruteerd door de AR om gentranscriptie te reguleren. Diverse modulerende processen en factoren kunnen de AR transactivatie beïnvloeden. Niet alleen co-factoren spelen een rol in AR transactivatie, ook post-translatie modificaties van de AR, zoals acetylering, ubiquitilering, sumoylering en fosforylering kunnen de transactivatie beïnvloeden. Bovendien kunnen mutaties in het *AR* gen dramatische gevolgen hebben voor de AR transactivatie. In het algemeen, resulteren AR mutaties in het androgeen-ongevoeligheidssyndroom (AIS), waarbij het mannelijke fenotype is aangetast. Echter, de exacte invloed van deze modulerende processen en factoren is nog niet duidelijk. In **Hoofdstuk 1** worden er 3 vragen geformuleerd die in dit proefschrift in detail worden behandeld.

Vraag 1: *Wat is de invloed van AR fosforylering op AR isovormen en op AR transactivatie ?*

Vraag 2: *Wat is het effect van de nieuwe mutatie F826L op AR transactivatie?*

Vraag 3: *Kunnen bestaande en nieuwe AR co-factoren met een oligonucleotide bindingsmethode worden geïsoleerd uit AR complexen.*

Er zijn voldoende aanwijzingen dat fosforylering de functie van enkele steroidhormoonreceptoren beïnvloedt. Bekend is dat de AR wordt gefosforyleerd, maar het effect van fosforylering op AR functies is onbekend. In de experimenten beschreven in **Hoofdstuk 2** werd duidelijk gemaakt dat de fosforylering van serine 94 verhoogd kan worden in een hormoon afhankelijke manier en dat serine 650 constitutief is gefosforyleerd. Met betrekking tot het AR isovorm patroon werd gevonden dat elke isovorm verschillend is gefosforyleerd op een aantal fosforyleringsplaatsen. Echter, niet elk gefosforyleeerd aminozuur draagt noodzakelijkerwijze bij aan het isovorm patroon zoals werd aangetoond met mutatie analyses. Bovendien, modulatie van AR fosforylering van serine residuen lijkt niet

direct de transcriptie activatie, NH_2-/COOH-terminale domein interactie en de co-activatie door transcriptional intermediary factor 2 (TIF2) te beïnvloeden. Interessant is dat de-fosforylering van serine 650 wordt waargenomen na mutatie van een andere potentiële fosforyleringsplaats (serine 515).

Androgeen signaaltransductie is belangrijk voor de mannelijke geslachtsontwikkeling tijdens de embryogenese. Een defecte androgeen signaal transductie in een individu kan leiden tot AIS, dat behoort tot de stoornissen in de geslachtsontwikkeling, en hoofdzakelijk wordt veroorzaakt door mutaties in het *AR* gen. In **Hoofdstuk 3** is de studie van de nieuw geïdentificeerde F826L mutatie in de AR beschreven. Deze mutatie werd gevonden in het *AR* gen van een jongen met een ernstige penoscrotale hypospadie (geclassificeerd als 46,XY DSD). Opmerkelijk is dat de LBD mutatie geen invloed heeft op de AR transactivatie, de ligand binding, de AR eiwit stabiliteit, de kinetiek van de subcellulaire AR localisatie en de N-CoR co-repressie. Echter, deze mutatie resulteerde wel in een tweemaal hogere NH_2-/COOH-terminale domein interactie en ook in een verhoogde TIF2 co-activatie. De F826L mutatie kan mogelijk het AR functioneren negatief beïnvloeden op een manier die niet detecteerbaar is met de gebruikte functionele testen. Een alternatieve verklaring voor de ernstige penoscrotale hypospadie zou kunnen zijn dat de AR mutant, in genitale huidfibroblasten, een ander repertoire van co-activatoren, exclusief TIF2, rekruteert. Een andere mogelijkheid is dat door de veranderde LBD conformatie, een preferentiële binding van co-repressoren, exclusief N-CoR, plaatsvindt in specifieke androgeen doelwitcellen.

Bij de transcriptie regulatie van genen is een groot aantal co-factoren betrokken. Veel AR co-factoren zijn geïsoleerd door middel van isolatie-procedures waarbij AR binding aan specifieke DNA sequenties ontbreekt. Echter, er zijn voldoende aanwijzingen dat specifieke binding aan DNA belangrijk is voor co-factor rekrutering. Om de co-factor rekrutering door de AR te onderzoeken is gebruik gemaakt van oligonucleotiden die de ARE1 van de PSA promoter bevat of een niet-coderende versie van de ARE1 (**Hoofdstuk 4**). Geïdentificeerde eiwitten werden gescoord voor het bezit van één of meer van de volgende bekende eigenschappen: nucleaire localisatie, betrokkenheid bij transcriptie regulatie, betrokkenheid bij steroidhormoon functie of specifieke betrokkenheid bij AR functie. De toepassing van deze zogenaamde “oligonucleotide-based assay” (OBA) resulteerde in de selectie van in totaal 85 eiwitten die met de AR in combinate met DNA werden gebonden uit een kernextract van LNCaP-cellen. Hiertoe behoorden 11 eiwitten, waarvan al bekend was dat ze kunnen interacteren met de AR en tevens AR transactivatie kunnen beïnvloeden. Van 5 andere gebonden eiwitten was bekend dat ze de transactivatie van andere steroidhormoonreceptoren beïnvloeden. Daarnaast werden er 32 eiwitten gevonden waarvan bekend was dat ze betrokken zijn bij transcriptie regulatie van verschillende genen, echter niet specifiek van AR gereguleerde genen. Bovendien zijn er 25 eiwitten geïsoleerd met een RNA bindende of “RNA-processing” functie. Tot slot, van 4 eiwitten is de functie onbekend. Wanneer een

strictere selectie op basis van het aantal unieke peptiden werd toegepast, vonden we, in vergelijking met de scrambled oligonucleotide, een specifieke verrijking van 7 eiwitten met de ARE1 olignucleotide. Eén van deze eiwitten, DDX17, is beschreven als een co-factor voor de oestrogeenreceptor α (ERα). Deze studie is de eerste waarin DDX17 wordt geassocieerd met een AR functie. Geconcludeerd kan worden dat de ARE in de OBA de isolatie van bekende AR interacterende eiwitten en nieuwe potentieel AR interacterende eiwitten mogelijk maakt.

In **Hoofdstuk 5** worden de resultaten van de studies, beschreven in de Hoofdstukken 2, 3 en 4, bediscussieerd in een breder perspectief. Er worden mogelijkheden geopperd voor nieuwe studies naar een relatie tussen fosforylering en AR functie. Nieuwe studies naar de mogelijke oorzaak voor de penoscrotale hypospadie van het 46,XY individu met de F826L mutatie wordt ook bediscussieerd. Tevens wordt aangegeven welke studies er kunnen worden uitgevoerd om de functies van de nieuw geïdentificeerde co-factoren in het werkingsmechanisme van androgenen te onderzoeken.

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CURRICULUM VITAE

Hao Yun Wong was born on the 2nd of October 1977 in Dordrecht in the Netherlands. His secondary education (VWO) started in 1990 at Thuredrecht College in Dordrecht and it was continued in 1991 at Libanon Lyceum in Rotterdam. In 1996 he started his study Biomedical Sciences at the University of Utrecht. In 1999, he performed his first graduation project at the department of Infectious Diseases and Immunology (division of Virology) at the University of Utrecht, entitled “the cloning of feline infectious peritonitis (corona)virus (FIPV)” under the supervision of Dr. B-J Haijema and Prof. Dr. PJM Rottier. His second graduation project was from January 2000 till May 2001 at the departments of Internal Medicine and Pathology at the University of Utrecht, entitled “islet amyloid polypeptide (IAPP) and apoptosis of insulin producing cells in type 2 diabetic IAPP transgenic mice” under the supervision of Dr. JWM Höppener and Prof. Dr. CJM Lips. During the second stage he also did research at the department of Physiological Sciences, Section Neuroendocrine Cell Biology at the Lund University in Sweden, entitled “postnatally disturbed pancreatic islet cell distribution in human islet amyloid polypeptide transgenic mice” under the supervision of Prof. Dr. B Ahrén and Prof. Dr. F Sundler. After graduation in Biomedical Sciences in August 2001, a start was made with the PhD research in September 2001 at the department of Reproduction and Development (formerly known as Endocrinology and Reproduction) at the Erasmus MC, Rotterdam, under the supervision of Dr. AO Brinkmann and Prof. Dr. JA Grootegoed. In 2006 he performed research as a guest worker and remained guest worker until recently at the same department.

PhD PORTFOLIO SUMMARY

Name PhD student:	Hao Yun Wong
Erasmus MC Department:	Reproduction and Development
Research School:	Postgraduate School Molecular Medicine
PhD period:	September 2001 - December 2008
Promotor:	Prof.dr. J.A. Grootegoed
Supervisor:	Dr. A.O. Brinkmann

1. PhD training

	Year	Workload (Hours)
General academic skills		
- Biomedical English Writing and Communication 6 June - 21 November, Rotterdam	2002	85
In-depth courses		
- Advanced Course Molecular Medicine Postgraduate School Molecular Medicine 7 - 14 December, Rotterdam and Leiden	2001	34
- Advanced Course Oncogenesis and Tumor Biology; Postgraduate School Molecular Medicine/Medical Genetic Center, 2 - 5 October, Rotterdam	2001	32
- Experimental Aproach to Molecular and Cell Biology; Master of Science; part I and part II; Postgraduate School Molecular Medicine, 29-10-2001 till 23-01-2002 and 4-02-2002 till 8-07-2002, Rottterdam	2001-2002	35
- Contemporary Research Topics; Master of Science; Postgraduate School Molecular Medicine 15 October till 4 March, Rotterdam	2002-2003	24
- Course Molecular Medicine; Postgraduate School Molecular Medicine 16 - 20 June, Rotterdam	2003	34
-		
Presentations and conferences		
- 7th Molecular Medicine Day, 14 October, Rotterdam (poster)	2002	40
- Androgens 2002 symposium, April, Leuven, Belgium (poster)	2002	30
- 8th Molecular Medicine Day, 21 January, Rotterdam (poster)	2004	30
- Nuclear Receptors: Orphan Brothers and Steroid Sisters, 28 February - 4 March, Keystone, USA (poster)	2004	30
- Second Winter School of the International Graduiertenkolleg from Marburg/Giessen-Rotterdam Universities, 13 - 18 March, Kleinwalsertal, Austria (oral)	2004	40
- Androgens 2004 symposium, 7 - 8 October, Berlin, Germany (poster)	2004	20

Seminars and workshops		
- Progress and Controversies in Oncological Urology VII and the 7th International Congress of the Dutch Urological Association, 10 -12 October, Rotterdam	2002	
- Symposium Organon, 5 November, Oss		
- Seminar Steroiden Receptoren, 12 January, Poelgeest	2003 2004	
- Get out of your lab Days 2005, workshop giving presentations, 10 -12 March, Domburg	2005	
Didactic skills		
- Usage of reversed-phase high performance liquid chromatography	2002	50
-		
2. Teaching activities		
	Year	**Workload (Hours)**
Supervising Bachelor's theses		
- "Functionele analyse van de androgeen receptor F826L en F891L mutaties gevonden in patienten met partiele androgeen ongevoeligheidssyndroom", Kar Lok Pang, MLO student, 15-09-2003 till 15-06-2004	2003-2004	200

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Tot slot, wens ik iedereen het allerbeste en veel succes met allerlei plannen!

Hao Yun
浩雲

“Life is not the amount of breaths you take, it's the moments that take your breath away” (Hitch).