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Molecular Mechanisms of Androgen Receptor Interactions

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ACADEMIC DISSERTATION

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Mum, Dad, Ben and Laura.

CONTENTS

SUMMARY

The androgen receptor (AR) mediates the effects of the male sex-steroid hormones (androgens), testosterone and 5α-dihydrotestosterone. Androgens are critical in the development and maintenance of male sexual characteristics. AR is a member of the steroid receptor ligand-inducible transcription factor family. The steroid receptor family is a subgroup of the nuclear receptor superfamily that also includes receptors for the active forms of vitamin A, vitamin D_3, and thyroid hormones. Like all nuclear receptors, AR has a conserved modular structure consisting of a non-conserved amino-terminal domain (NTD), containing the intrinsic activation function 1, a highly conserved DNA-binding domain, and a conserved ligand-binding domain (LBD) that harbors the activation function 2. Each of these domains plays an important role in receptor function and signaling, either via intra- and inter-receptor interactions, interactions with specific DNA sequences, termed hormone response elements, or via functional interactions with domain-specific proteins, termed coregulators (coactivators and corepressors).

Upon binding androgens, AR acquires a new conformational state, translocates to the nucleus, binds to androgen response elements, homodimerizes and recruits sequence-specific coregulatory factors and the basal transcription machinery. This set of events is required to activate gene transcription (expression). Gene transcription is a strictly modulated process that governs cell growth, cell homeostasis, cell function and cell death. Disruptions of AR transcriptional activity caused by receptor mutations[‡] and/or altered coregulator interactions are linked to a wide spectrum of androgen insensitivity syndromes, and to the pathogenesis of prostate cancer (CaP). The treatment of CaP usually involves androgen depletion therapy (ADT). ADT achieves significant clinical responses during the early stages of the disease. However, under the selective pressure of androgen withdrawal, androgen-dependent CaP can progress to an androgen-independent CaP. Androgen-independent CaP is invariably a more aggressive and untreatable form of the disease. Advancing our understanding of the molecular mechanisms behind the switch in androgen-dependency would improve our success of treating CaP and other AR related illnesses.

[‡] Mutation: a DNA alteration that occurs in less than 1% of the population (Harris, 1969).

This study evaluates how clinically identified AR mutations affect the receptor's transcriptional activity. We reveal that a potential molecular abnormality in androgen insensitivity syndrome and CaP patients is caused by disruptions of the important intra-receptor NTD/LBD interaction. We demonstrate that the same AR LBD mutations can also disrupt the recruitment of the p160 coactivator protein GRIP1.

Our investigations reveal that 30% of patients with advanced, untreated local CaP have somatic mutations that may lead to increases in AR activity. We report that somatic mutations that activate AR may lead to early relapse in ADT. Our results demonstrate that the types of ADT a CaP patient receives may cause a clustering of mutations to a particular region of the receptor. Furthermore, the mutations that arise before and during ADT do not always result in a receptor that is more active, indicating that coregulator interactions play a pivotal role in the progression of androgen-independent CaP.

To improve CaP therapy, it is necessary to identify critical coregulators of AR. We screened a HeLa cell cDNA library and identified small carboxyl-terminal domain phosphatase 2 (SCP2). SCP2 is a protein phosphatase that directly interacts with the AR NTD and represses AR activity. We demonstrated that reducing the endogenous cellular levels of SCP2 causes more AR to load on to the prostate specific antigen (*PSA*) gene promoter and enhancer regions. Additionally, under the same conditions, more RNA polymerase II was recruited to the *PSA* promoter region and overall there was an increase in androgen-dependent transcription of the *PSA* gene, revealing that SCP2 could play a role in the pathogenesis of CaP.

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles that are referred to in the text by their Roman numeral.

I Thompson J, Saatcioglu F, Jänne OA, Palvimo, JJ (2001) Disrupted Amino- and Carboxyl-Terminal Interactions of the Androgen Receptor Are Linked to Androgen Insensitivity. Mol Endocrinol. 15: 923-935

II Thompson J*, Hyytinen ER*, Haapala K, Rantala I, Helin HJ, Jänne OA, Palvimo JJ, Koivisto PA (2003) Androgen Receptor Mutations in High-Grade Prostate Cancer before Hormonal Therapy. Lab Invest. 83: 1709-1716

III Hyytinen ER, Haapala K*, Thompson J*, Lappalainen I, Roiha M, Helin HJ, Jänne OA, Vihinen M, Palvimo JJ, Koivisto PA (2002) Pattern of Somatic Androgen Receptor Gene Mutations in Patients with Hormone-Refractory Prostate Cancer. Lab Invest. 82: 1591-1598

IV Thompson J*, Lepikhova T*, Teixido-Travesa N, Whitehead MA, Palvimo JJ, Jänne OA (2006) Small Carboxyl-Terminal Domain Phosphatase 2 Attenuates Androgen-Dependent Transcription. EMBO J. 25: 2757-2767

* Equal contribution by authors.

Additional unpublished material is also presented.

ABBREVIATIONS

AD	activation domain
ADT	androgen deprivation therapy
AF	activation function
AIS	androgen insensitivity syndrome
AKT/PKB	AKT/protein kinase B
AR	androgen receptor
ARE	androgen response element
bp	base pair
BPH	benign prostatic hyperplasia
CAIS	complete androgen insensitivity syndrome
CaP	prostate cancer
CBP	CREB-binding protein
cDNA	complementary deoxyribonucleic acid
CTD	carboxyl-terminal domain
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
DRIP	VDR-interacting protein
E	estrogen/estradiol/estrone
EMP	estramustine phosphate
ER	estrogen receptor
FCP1	TFIIF-associating CTD phosphatase 1
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein 1
HAT	histone acetylase
HDAC	histone deacetylase
HMT	histone methyltransferase
hnRNA	heterogeneous nuclear RNA
HR	hormone-refractory
HRE	hormone response element
HSP	heat shock protein
LBD	ligand-binding domain
LBP	ligand-binding pocket

MAIS	mild androgen insensitivity syndrome
MAPK	mitogen activated protein kinase
MB	mibolerone
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NF	nuclear factor
NR	nuclear receptor
NTD	amino-terminal domain
PAIS	partial androgen insensitivity syndrome
PI3K	phosphatidylinositol 3-OH kinase
PIC	preinitiation complex
Pol II	RNA polymerase II
PR	progesterone receptor
PSA	prostate specific antigen
SBMA	spinal and bulbar muscular atrophy
SCP	small carboxyl-terminal domain phosphatase
Slp	sex-limited protein
Sp	specificity protein
SR	steroid receptor
SRC	steroid receptor coactivator
SRY	sex determining region of Y chromosome
SUMO	small ubiquitin-related modifer
SWI/SNF	switch/sucrose non-fermentable
T	testosterone
TAF	TBP-associated factor
TAU	transactivation unit
TBP	TATA-box binding protein
TF	transcription factor
TR	thyroid hormone receptor
TRAP	TR-associated protein
VDR	vitamin D_3 receptor
WT	wild-type

REVIEW OF THE LITERATURE

1 Gene transcription and nuclear receptors

1.1 Overview of gene transcription

Gene transcription is the coordinated process of getting RNA polymerase II (Pol II) to the right place in the right gene in response to the correct signal. A failure in any of these conditions will invariably lead to disease and/or death. Genes are transcribed in a spatio-temporal and tissue-specific fashion that regulates normal growth, differentiation, metabolism, reproduction and morphogenesis in humans. The overall product of Pol II activity is messenger RNA (mRNA), which is the blueprint of the proteins that are subsequently expressed in the various cells of the body. Nuclear receptors (NR) relay the extracellular messages/signals (hormones) to the nucleus of cells, in order to regulate target gene expression. Hormone-bound NRs are usually found in the nucleus, residing on *cis*-acting DNA elements called hormone-response elements (HRE). HREs are DNA sequences in the vicinity of a gene that are required for gene expression. *Cis*-acting DNA elements recruit a menagerie of *trans*-acting factors. *Trans*-acting factors are usually proteins that bind to the *cis*-acting DNA elements to control gene expression. *Trans*-acting factors include NRs and their associated coregulatory proteins and the basal transcription machinery. The basal transcription machinery is defined as the proteins, including Pol II, that are the minimal essential transcription factors (TF) required for transcription *in vitro* from an isolated gene promoter.

Since the cloning of the first NR, human glucocorticoid receptor (GR) (Hollenberg et al., 1985), over 20 years ago by Evans and coworkers, there has been a huge accumulation of data on NR-dependent transcriptional regulation (Mangelsdorf et al., 1995; Aranda & Pascual, 2001; McKenna & O'Malley, 2002a, b; Nagy & Schwabe, 2004). Therefore a comprehensive analysis/review of all the NR signaling pathways is beyond the scope of this literature review. This work reviews how the signals of the androgens, the male sex-steroid hormones, result in androgen receptor (AR)-dependent transcription.

1.2 Overview of nuclear receptor superfamily

The NR superfamily of the human endocrine system regulates a complex network of genes that coordinate nearly all the activities of homeostasis, growth, and reproduction (Novac & Heinzel, 2004; Margolis et al., 2005). The human genome harbors 48 NR genes. Alternative splicing and promoter usage of these 48 genes give rise to 75 currently known NR proteins (Lander et al., 2001; Venter et al., 2001; Robinson-Rechavi et al., 2001, 2003a; Escriva et al., 2004). NRs are ligand (hormone) inducible transcription factors that, with the assistance of auxiliary proteins (coregulators), regulate the expression of their target genes in a temporal and tissue-specific manner (Aranda & Pascual, 2001; McKenna & O'Malley, 2002a, b; Novac & Heinzel, 2004). NRs are characterized by their modular structure (**Fig. 1**). This consists of a hypervariable amino-terminal domain (NTD). The NTD region can range in size from being 6% of the total protein, as for the vitamin D receptor (VDR), to over 50% of the total protein, as for AR. NRs also have a highly conserved central DNA-binding domain (DBD) and conserved ligand-binding domain (LBD). The conservation between NRs suggests that they all come from a common ancestor by gene duplication and divergence (Escriva et al., 2004; Thornton & Kelly 1998).

2 Nuclear receptor nomenclature

2.1 Colloquial nomenclature

In colloquial NR nomenclature, the NRs are divided into 3 types of receptors. Type I receptors are the steroid hormone receptors (SR), including AR, estrogen receptor (ER), GR, mineralcorticoid receptor (MR) and progesterone receptor (PR) (see **Table 1**). Type I receptors, upon binding steroid hormones, translocate from the cytoplasm to the nucleus, where they bind as homodimers to HREs. Type II NRs bind thyroid hormones, retinoids (the active forms of vitamin A), and vitamin D_3. Type II NRs reside within the nucleus, irrespective of the presence of ligand and bind HREs, typically as heterodimers, with the retinoic X receptor. Type III NRs have close sequence and structural homology to known NRs, but lack an identifiable ligand. Type III receptors are affectionately referred to as

orphan receptors (Manglesdorf et al., 1995). Currently there are 24 orphan receptors for which no ligand has yet been discovered (Gronemeyer et al., 2004).

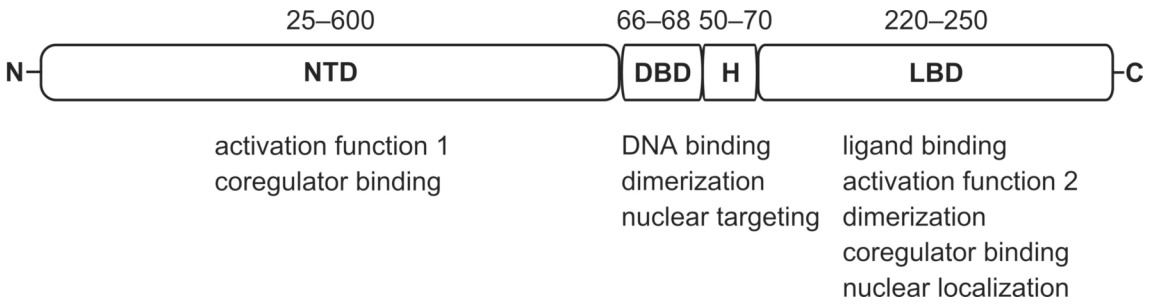

Fig. 1. The modular structure of the NRs. NTD, amino-terminal domain; DBD, DNA-binding domain; H, hinge; LBD, ligand-binding domain. The main functional domains are shown. The numbers indicate the numbers of amino acids in each domain.

Table 1. Colloquial and phylogenetic nomenclature of steroid receptors.

Colloquial nomenclature	**Receptor variant**	**Phylogenetic nomenclature**
Estrogen receptor (ER)	ER α	NR3A1
	ER β	NR3A2
Glucocorticoid receptor (GR)		NR3C1
Mineralcorticoid receptor (MR)		NR3C2
Progesterone receptor (PR)		NR3C3
Androgen receptor (AR)		NR3C4

2.2 Phylogenetic nomenclature

Completion of the genome sequences for human, mouse and other organisms has led to the identification of new NR variants and the colloquial NR classification is becoming inefficient (Robinson-Rechavi et al., 2003b). The Nuclear Receptors Nomenclature Committee 1999 organized a phylogeny-based cataloging system. The phylogeny-based system classifies NRs on the similarity between their DBDs and their LBDs (Robinson-Rechavi et al., 2003b). NRs are now named using the format NR*xyz*; where *x* is the subfamily, *y* is the group and *z* is the

gene. The phylogeny-based system parallels receptor function, so SRs now belong to subfamily 3. This is because the SRs recognize HREs that are partially palindromic and bind to them as homodimers. Subfamily NR3 comprises three groups, the ERs (ERα, ERβ), the estrogen-related receptors α, β, γ, that are orphan receptors and the third group consists of AR, GR, MR and PR (see **Table 1**). The three groups are well defined and there are no problems of relationship between the individual genes of this subfamily. ERs and estrogen-related receptors are separate groups because of their clear functional differences (Giguere, 2002; Horard & Vanacker, 2003). In the phylogeny-based system AR is known as NR3C4. For a detailed and updated classification of NRs see Nurebase (http://www.ens-lyon.fr/LBMC/laudet/nurebase/nurebase.html). In total there are 6 subfamilies of NRs. Subfamilies 1-5 have the usual modular structure. Subfamily NR0 is for NRs that lack domains such as a DBD or LBD. DAX1 and SHP are classified as NR0 (Robinson-Rechavi et al., 2003b). Although the phylogeny-based system gives a 'metric flavor' to the naming of the NRs, it does provide flexibility and enables the precise classification of all NRs.

3 Genes

A gene can be defined as a region of DNA that controls a discrete hereditary characteristic, usually corresponding to a single protein or RNA. This definition includes the entire functional unit, encompassing coding DNA sequences, non-coding regulatory DNA sequences and introns (Alberts et al., 2002). There are several basic elements that are present in most, if not all, eukaryotic genes. The regulatory region of a eukaryotic gene consists of a promoter region in addition to regulatory DNA sequences, such as HREs and enhancer regions.

3.1 Gene promoter regions

The basal transcription machinery and Pol II are recruited to the promoter region of genes in order to initiate transcription. The core promoter is the minimal DNA region required for the assembly of the basal transcription machinery. The core promoter is usually located between -35 to +35 base pairs (bp) from the transcription start site (+1) that is recognized by Pol II. The core promoter may contain elements such as a TATA-box, which is found in about a

third of human genes, and a TFIIB recognition element. The promoter may also contain an initiator element and a downstream core promoter (Smale & Kadonaga, 2003). There are DNA regions located between -100 and -200 bp, called proximal promoter regions. Proximal promoters usually contain motifs between 6 to 20 bp in length such as the CCAAT box, and specificity protein (Sp) 1 box (Smale & Kadonaga, 2003). These regions typically contribute to the efficiency of the transcription initiation. As mentioned above, some genes have enhancer elements located several kbp up or downstream from the transcription start site. These elements coordinate with the HRE of hormone responsive genes to recruit the binding of NR and the subsequent acquisition of the proteins to transcribe the gene (Acevedo & Kraus, 2004; Lee & Chang, 2003).

3.2 Hormone response elements

Our understanding of how NRs selectively recognize and bind their HREs is still incomplete. HREs originate from the consensus sequence 5'-AGAACAnnnTGTACC-3' (see **Table 2**). The consensus HRE is a partial-palindrome of inverted repeats of two hexameric core DNA sequences spaced by 3 bp. All NR3Cs recognize the same consensus, non-selective, high affinity HRE separated by 3 bp. The NR3As (ERα and ERβ) recognize a slightly different consensus sequence 5'-AGGTCAnnnTGACCT-3'. The two half-sites of an HRE can either be partial palindromic, inverted or direct repeats. The half-sites can be spaced by 1 to 5 bp (Truss & Beato, 1993; Claessens & Gewirth, 2004). In various half-site orientations and spacing almost all the other NRs (except NR3Cs) can bind to the NR3A HRE (Glass, 1994). All NR3Cs recognize the same consensus HRE, but the two half-sites of the element are not equal (Haelens et al., 2001; Schoenmakers et al., 1999). The first half-site is less susceptible to sequence variation and is likely to be involved in the high-affinity binding of all the NR3Cs to the HRE. Binding affinity does not, therefore, predict specificity. However the second half-site can diverge from the consensus sequence quite dramatically and small sequence variations of the second half-site can influence the interaction of the DBD with the DNA and influence receptor dimerization (Verrijdt et al., 2003). AR only binds 3 bp spaced half-sites. The binding of AR to the first half-site induces a conformation that influences the receptor's ability to homodimerize (Shaffer et al., 2004; Geserick et al., 2005). In addition, the regions flanking the HRE have also been shown to be important for selectivity by the C-

terminal extension region of the AR DBD (Nelson et al., 1999; Schoenmakers et al., 1999). Therefore the HRE sequence, the spacing and the flanking regions can either be conducive to harmonious homodimer/DNA binding or not. This could, in part, drive selectivity. Unlike the other NR3Cs, AR can recognize and homodimerize to direct repeats (Zhou et al., 1997; Haelens et al., 2003). The conformation of the DNA bound receptor may also influence the interaction of the NTD with the coregulators (Brodie & McEwan, 2005). *In vitro*, isolated HREs may not show specificity, but in the context and dynamics of chromatin, the multiple mechanisms discussed above may impart specificity and androgen responsiveness to genes (Robins, 2004, 2005).

Table 2. Comparison of AR specific and non-specific HREs (adapted from Monge et al., 2006).

Name	**Sequence**	**Specificity**
NR3C consensus	5'-AGAACAnnnTGTACC-3'	Non-specific
GRE consensus	5'-TGTACAggaTGTTCT-3'	Non-specific
ARE consensus	5'-GGTACAgggTGTTCT-3'	Specific
PSA-ARE I	5'-AGAACAgcaAGTGCT-3'	Specific
Slp-HRE	5'-TGGTCAgccAGTTCT-3'	Specific
C3(1) ARE	5'-AGTACGtgaTGTTCT-3'	Non-specific

3.3 Enhancers

Transcriptional enhancer elements are regulatory DNA sequences located at distances between a few kbp up to 1 Mbp away from their target gene. Their effect is to increase the usage of their associated promoters. Enhancers have similar organizational properties to promoters. Using the capturing chromosome conformation technique, it has been demonstrated that there is a physical looping between the enhancer and promoter regions of a gene (Dean, 2006; West & Fraser, 2005). The genes activated by AR often contain enhancer and promoter elements in their regulatory regions. The best-characterized androgen-responsive gene is the human prostate-specific antigen (*PSA*)/human kallikrein 3 gene (Clements et al., 2004). The *PSA* gene encodes for a globular protein that is secreted into the

blood, the level of which is the current test for prostate cancer (CaP) (Edwards & Bartlett, 2005). The enhancer and promoter regions of the *PSA* gene can individually drive gene expression in the presence of androgen, however, maximal transcriptional activity requires the presence of both. The *PSA* promoter region has a TATA box and two consensus androgen response elements (ARE), termed ARE I (–170), and ARE II (–394) (Cleutjens et al., 1996). The *PSA* enhancer element termed ARE III consists of several low affinity AREs found at –4.8 to –3.8 kbp away from the transcription start site (Cleutjens et al., 1997; Huang et al., 1999). Although the core enhancer region is 4 kbp away from the promoter region, in the context of chromatin, these two regions are physically close (**Fig. 2**). This means that the distal AREs could regulate the recruitment of the coregulators to the promoter region (Shang et al., 2002). Interestingly, upon androgen stimulation, 20 times more AR is recruited to the *PSA* enhancer region than to the promoter region (Kang et al., 2004). These data suggest that the *PSA* promoter and enhancer regions have distinct roles in the recruitment of AR into an active transcription complex.

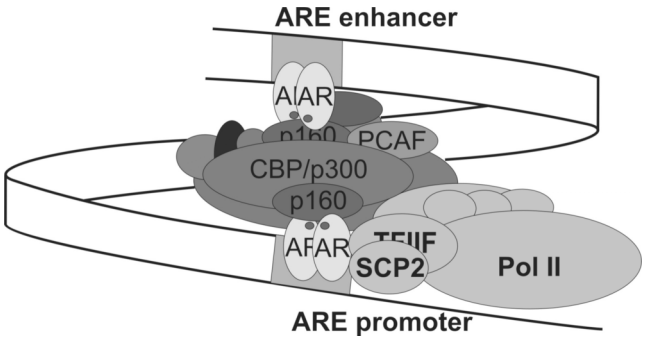

Fig. 2. Androgen bound AR is recruited to the promoter and enhancer regions of the *PSA* gene, which in the context of chromatin are in close proximity (adapted by A Domanskyi from Shang et al., 2002).

4 Basal transcription machinery

NRs relay extracellular signals that ultimately stimulate or suppress Pol II activity. In addition to the binding of the NRs to their HREs of the enhancer and promoter regions and the recruitment of Pol II to the promoter, there is a complex network of proteins that transduces the signals brought by the NR to Pol II. Between the NR and Pol II there can be

six basal TFs plus additional coregulatory proteins (Lemon & Tjian, 2000; Lee & Chang, 2003; Acevedo & Kraus, 2004; Malik & Roeder, 2005).

4.1 RNA polymerase II

Eukaryotic cells have three RNA polymerases; I, II and III. Pol III and I synthesize RNAs that have structural or catalytic roles, mainly as part of the protein synthetic machinery. Pol I synthesizes the large ribosomal RNAs, whilst Pol III synthesizes small and stable RNA, such as the transfer RNAs (Alberts et al., 2002). Pol II is responsible for the transcription of genes that are translated into proteins via mRNA. Pol II is a large and species conserved protein (Cramer et al., 2001). The largest subunit of Pol II contains a carboxyl-terminal domain (CTD) that in humans consists of 52 repeats of the heptapeptide sequence $Y^1S^2P^3T^4S^5P^6S^7$ (Meinhart et al., 2005). The phosphorylation status of the CTD plays a central role in regulating the five main phases of the Pol II transcription cycle: preinitiation, initiation, promoter clearance, elongation and termination. The CTD also plays a central role in mRNA processing (Orphanides et al., 2002). Phosphorylation occurs mainly on serine^2 and serine^5 although serine^7 and tyrosine^1 have also been suggested to be important (Palancade & Bensaude, 2003; Sims et al., 2004; Zorio & Bentley, 2004). In order to achieve transcription initiation and promoter clearance, the CTD is hyperphosphorylated on serine^5 by TFIIH (Komarnitsky et al., 2000; Morris et al., 2005). During the elongation phase of transcription, serine^2 is phosphorylated by positive transcription elongation factor b (Shim et al., 2002). However it is not currently clear if serine^5 remains phosphorylated throughout the elongation phase. At transcription termination both serine^2 and serine^5 are dephosphoryalted so that Pol II can be reloaded onto the promoter region via the CTD's interaction with the Mediator complex (Hausmann & Shuman, 2002; Sims et al., 2004; Malik & Roeder, 2005). The dephosphorylation of serine^2 and serine^5 is performed by the protein phosphatase TFIIF-associating CTD phosphatase (FCP1) (Archambault et al., 1997; Kobor et al., 1999).

4.2 Basal transcription factors

The components of the basal transcription machinery include TFIID, -B, -E, -F, -H, -A, TATA-box binding protein (TBP), and the TBP-associated factors (TAF). The formation of

the preinitiation complex (PIC) is a multi-step process and each promoter has its own composition of factors (Muller & Tora, 2004). The first step in the PIC assembly is the recruitment of TBP to the TATA-box of the core promoter. TBP is able to bind the TATA-box. TFIID, is a complex harboring several TAFs, and is required at promoters that do not have a TATA-box (Burke & Kadonaga, 1996; Smale & Kadonaga, 2003). The binding of TBP bends the DNA and forms a platform for the recruitment of other TFs. TFIIB recruits TFIIF together with Pol II. The NTD of AR also facilitates the recruitment of TFIIF into the PIC (McEwan & Gustafsson, 1997; Reid et al., 2002a; Lee & Chang, 2003). The interaction between the PIC and the binding of Pol II to the promoter is thought to be bridged by the Mediator complex (Malik & Roeder, 2005). Upon hyperphosphorylation of the Pol II CTD by TFIIH, Pol II clears the promoter and enters the transcription elongation phase (Sims et al., 2004; Zoiro & Bentley, 2004; Svejstrup, 2004; Orpanides & Reinberg, 2002). Upon Pol II clearance, the remaining PIC complex (TFIID-TFIIA) and/or the Mediator complex still stays on the promoter, ready to initiate a second PIC. During the Pol II elongation phase mRNA is synthesized. The synthesized mRNA is further processed by 5' capping, intron splicing, and 3' end maturation. The termination of the Pol II elongation phase is accompanied by the total dephosphorylation of the Pol II CTD, a step required for the reloading of Pol II back onto the promoter region (Sims et al., 2004).

5 Coregulatory proteins

5.1 Introduction to nuclear receptor coregulatory proteins

NR transcriptional activity is mediated by an auxiliary set of ligand-dependent and ligand-independent receptor-interacting proteins termed coregulators. There are two types of coregulators, Type I and Type II. Type I coregulators function primarily with the NR at the target gene promoter to facilitate DNA occupancy, chromatin remodeling, or recruitment of the basal transcription machinery. Type II coregulators function primarily to enable the NR to be competent to direct target gene expression. Type II coregulators may also contribute to the stability of the protein in the absence of ligand or in the presence of antagonists. The coregulators that induce transcription are termed coactivators and those that suppress transcription are termed corepressors (McKenna & O'Malley, 2002a, b; Smith & O'Malley,

2004; Lonard & O'Malley, 2006). Coregulators generally cannot direct themselves to bind to the various *cis*-acting DNA elements, unlike the NRs. They are recruited to the various DNA elements by the NR and by other coregulators as and when needed (Lee et al., 2001; Perssi & Rosenfeld, 2005; Lee & Chang, 2003). A comprehensive list of putative AR coregulators is available (http://www.androgendb.mcgill.ca/ARinteract.pdf).

5.2 NR boxes and CoRNR boxes

The identification and cloning of coregulators has furthered our understanding of the mechanisms by which members of the NR family regulate gene expression. X-ray crystal structures of ligand-activated NR bound by peptides corresponding to the receptor-interacting motifs of coactivators have given significant insights into the nature of the transcriptionally active complex (Hur et al., 2004). In these structures, the consensus amphipathic helical LXXLL sequence, where X denotes any amino acid, specifically contacts a hydrophobic surface of the NRs. Coactivators and corepressors both contain LXXLL based motifs. The LXXLL motif is also known as the nuclear receptor interaction box (NR box) and is found within the amino acid sequence of the p160 coactivators (Darimont et al., 1998; Westin et al., 2000; White et al., 2004). Corepressors have LXXXIXXX(I/L) motifs. These are also known as corepressor-nuclear-receptor (CoRNR) box (Hu & Lazar, 1999; Periss & Rosenfeld, 2005; Wang et al., 2005). The interaction between coregulators and NRs is by no means exclusively mediated by NR- and CoRNR boxes. In addition, coregulators can covalently add or remove acetyl-, methyl-, phospho-, ubiquitin-, and small ubiquitin-like modifier (SUMO)-groups to proteins involved in transcriptional regulation (Fu et al., 2003; Kotaja et al., 2002; Gill, 2004). Recruitment of coregulators can be either nuclear receptor-specific or receptor complex-specific. They are often found in large dynamic multiprotein complexes. Many of these multiprotein complexes share common subunits (Muller & Tora, 2004; Perssi & Rosenfeld, 2005). The coregulators integrate signals from multiple regulatory pathways to produce a very controlled rate of transcription in response to hormone signals (Robyr et al., 2000). There are four categories of coregulators (Robyr et al., 2000, Acevedo & Kraus, 2004; McKenna & O'Malley, 2002; Smith & O'Malley, 2004; Baek & Rosenfeld, 2004; Kumar et al., 2004a; Perissi & Rosenfeld. 2005; Malik & Roeder, 2005);

- Mediator complexes such as TRAP/DRIP/ARC,
- histone modifiers such as histone acetyltransferases (HAT), histone arginine methyltransferases (HMT), and histone deacetylases (HDAC). There are also histone kinases and phosphastases,
- ATP-dependent chromatin-modelers, such as switch/sucrose non-fermentable (SWI/SNF) proteins, and
- bridging factors or unknown function.

6 Type I coregulators

6.1 Mediator

Mediator is an important multiprotein component of the basal transcription machinery. It plays an active part in the activation and suppression of gene transcription (Myers & Kornberg, 2000). Mediator complex contains about 20 protein components and its structure and function are conserved from yeast to humans. Bacteria do not have Mediators (Chadick & Asturias, 2005). Mediator is required as an adapter that supports essential communication from transcription factors bound to the enhancer and upstream promoter elements (Myers & Kornberg, 2000). The mechanism by which Mediator influences transcriptional regulation has not been fully established. Mediator subunits seem to be targets for NR transcriptional activation domains. The composition of mammalian Mediator varies, but there is a set of consensus subunits present in most Mediator complexes (Conaway et al., 2005). Due to their considerable size and subunit composition, it initially seemed that Mediators were independent complexes (Blazek et al., 2005). The first Mediator isolated was associated with liganded thyroid hormone receptor (TR) and was termed thyroid hormone receptor-associated proteins (TRAP) (Fondell et al., 1996, 1999). Subsequently, VDR–interacting proteins (DRIP) (Rachez et al., 1998), activator-recruited cofactor (Näär et al., 1999) /cofactor required for Sp1 activation (Ryu et al., 1999), positive cofactor 2 (Malik et al., 2000), mammalian Mediator (Jiang et al., 1998), negative regulator of activated transcription and suppressor of RNA polymerase B mediator-containing cofactor complex (Gu et al., 1999; Ito et al., 1999) were isolated and characterized. The knocking out of the *TRAP220* and *TRAP100* gene subunits of the TRAP/DRIP/ARC complex is either embryonically lethal or

results in birth defects. This is due to impaired TR-regulated gene transcription (Ito et al., 2000, 2002). The Mediator complexes can contact both NRs via the LXXLL motif of TRAP220/DRIP205 (Yuan et al., 1998; Wang et al., 2002) and Pol II via an interaction with the Pol II CTD and the cofactor required for Sp1 activation complex (Näär et al., 2002). Both contacts stimulate transcriptional activity (Näär et al., 2002; Wang et al., 2002; Malik & Roeder, 2005). Mediators only mediate Pol II directed transcription by stimulating or inhibiting TFIIH activity (Blazek et al., 2005). After promoter clearance, it has been shown that Mediator remains bound at the promoter region. This accelerates PIC reinitiation (Rani et al., 2004; Acevedo & Kraus, 2003, 2004). The Mediator complexes can interact with other transcription factors and therefore may be involved in modulating signals of non-NR pathways (Perissi & Rosenfeld, 2005).

6.2 Chromatin and histone modifying coregulators

The 3.2 billion DNA bp in a cell are not floating around free, but are packaged into a protein/DNA structure termed chromatin (Hsieh & Fischer, 2005). Chromatin is the higher-ordered form of a repeating array of highly conserved proteins called histones. Histones bind to 146 bp of DNA to form the building blocks of chromatin, which are called nucleosomes. Regions of chromatin can either be in a compact closed form, which is transcriptionally inaccessible (inactive) (heterochromatin) or a more open form that is transcriptionally accessible (active) (euchromatin). Therefore coordinated positioning and moving of nucleosomes can regulate gene transcription. The histone proteins have N-terminal tails that can be covalently modified on lysine, arginine and serine residues by acetylation (Verdone et al., 2005), methylation (Martin & Zhang, 2005; Wysocka et al., 2005), ubiquitintation (Kinyamu et al., 2005), SUMOylation (Nathan et al., 2003) or phosphorylation (Fischle et al., 2003b). These modifications change the properties of the nucleosomes and by doing so create/abolish binding sites for transcription factors. The coordinated histone tail modifications lead to the promoter region 'histone code' (Santos-Rosa & Caldas, 2002, 2005; Cosgrove & Wolberger, 2005). The histone code creates local structural and functional diversity (Cosgrove et al., 2004; Santos-Rosa & Caldas, 2002, 2005). Hormone induced histone tail modifications are performed by a number of well-characterized proteins (Kang et al., 2004). To review the different modifications is beyond the scope of this review. Briefly,

the HAT cAMP-response-element-binding protein (CREB)-binding protein (CBP) and its homologue p300 have been shown to hyperacetylate histones in the presence of hormone (Chen et al., 1999). It synergistically interacts with Mediator and chromatin templates during ERα-dependent transcription (Avevedo & Kraus, 2003). Furthermore, CBP/p300 is linked to NRs by an interaction with the activation domain (AD) 1 of p160 coactivator family members via a C-terminal p160 coactivator-binding domain (Stallcup et al., 2003). p160 coactivators directly bind NRs. Thus, CBP/p300 (and other HATs) can regulate transcription in two ways, by histone acetylation, which contributes to chromatin accession and by recruitment stabilization of other coregulators and basal transcription machinery proteins (Stallcup, et al., 2003). In addition, CARM1/PRMT4 is an arginine HMT. CARM1 is a coactivator for NR, but is active only in the presence of CBP/p300 and p160 coactivators, demonstrating the interrelations between all the components of the transcription machinery (Koh et al., 2001). The best-characterized NR corepressors are silencing mediator for retinoid acid receptor and TR and nuclear receptor corepressor (Chen & Evans, 1995; Horlein et al., 1995).

6.3 ATP-dependent chromatin-modelers

Chromatin structure is a dynamic entity that undergoes cell cycle dependent folding and unfolding during DNA replication and repair and coordinated gene expression. The folding of nucleosomes into chromatin creates a barrier that prevents the access of transcription factors and other regulatory proteins, which transcribe the genes encoded. Chromatin modeling complexes are directed by the histone code to increase nucleosome mobility in tightly packed chromatin that makes the DNA accessible to the transcription machinery. The nucleosomes on DNA can be disrupted and reconfigured with a set of ATP-dependent SWI/SNF chromatin remodeling proteins. Furthermore the SWI/SNF chromatin remodeling proteins have been shown to be NR coregulators (Dilworth & Chambon, 2001). The NRs can bind to the chromatin template with high affinity to their HRE. However the assembly of the transcription complexes to the target promoter is hindered. Therefore liganded NR recruits chromatin-remodeling proteins to promote the formation of an open chromatin structure. Using the energy from ATP, the chromatin remodeling protein complexes mobilize or structurally alter nucleosomes enabling the rest of the transcription complex access to the

promoter region DNA binding sites (Becker & Horz, 2002). A stepwise model has been proposed for the relationship between NR chromatin binding, chromatin remodeling, and histone acetylation. After ligand-dependent binding, the NR recruits chromatin remodeling protein complexes, which then recruit coactivators that possses HAT activity. Once the chromatin has been loosened and the DNA is open, the basal transcription machinery, with the help of Mediator, recruits and forms the PIC and Pol II (Kumar et al., 2004b; Xu, 2005).

7 Type II coregulators

7.1 The p160 coactivator family

The most comprehensively studied Type II NR coactivators are the closely related p160 coactivator family. As they were first identified in humans and rodents, the three homologous members have several names. These are steroid receptor coactivator-1 (SRC-1)/nuclear receptor coactivator-1, SRC-2/glucocorticoid receptor-interacting protein 1 (GRIP1)/transcriptional intermediary factor 2/nuclear receptor coactivator-2, and SRC-3/amplified in breast cancer 1/activator of thyroid and retinoic acid receptors /receptor-associated coactivator 3/p300/CBP interacting protein (p/CIP)/thyroid hormone receptor activator molecule 1 (Xu & Li, 2003). The SRCs are highly homologous transcription factors and are all about 160 kDa in size. They share 43-55% sequence identity. The SRCs harbor several conserved functional domains, including an N-terminal basic helix-loop-helix–Per–Ah receptor nuclear transolactor (ARNT)–Sim (PAS) domain, a central nuclear receptor interaction domain that contains three LXXLL motifs; and two intrinsic ADs, AD1 and AD2 in the C-terminal part of the protein. In addition there are serine/threonine and glutamine rich regions (**Fig. 3**).

The three LXXLL motifs form an amphiphatic α-helix. A common characteristic shared by the SRC members is their hormone-dependent interaction with the activation fuction (AF)-2 region of NRs (McKenna et al., 1999a, b; Glass & Rosenfeld, 2000; Xu et al., 1999; Freedman, 1999). Under liganded conditions the conformational change of the receptors reveals a hydrophobic groove formed by helices 3, 4 and 12 with which, via amphipathic helices formed by the SRCs, LXXLL motifs can interact (He et al., 2000; He et al., 1999). In

addition to the interaction with the AF2 region, p160 coactivators have been shown to interact with the N-terminally located AF1 region via the regions flanking the LXXLL motifs SRCs, thereby potentially bridging the NTD/LBD interaction of AR (Darimont et al., 1998; Ikonen et al., 1997; Ma et al., 1999; Heery et al., 1997; Ding et al., 1998).

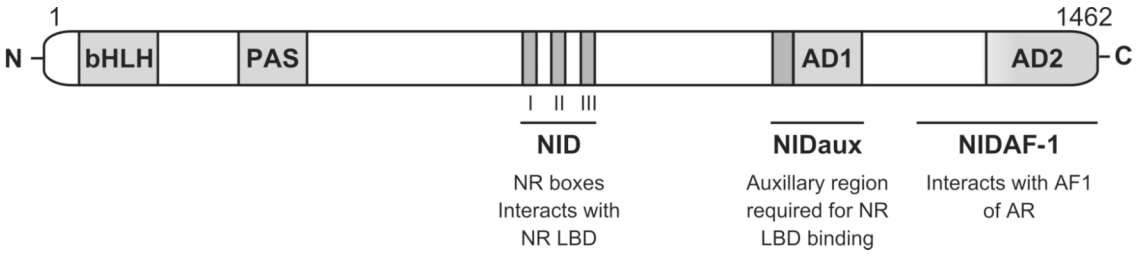

Fig. 3. Schematic structure of the p160 coactivator GRIP1. NID, nuclear receptor-interaction domain; AD, activation domain; I, LXXLL motif I; II LXXLL motif II; III, LXXLL motif III; PAS Per-Arnt-Sim; bHLH, basic helix-loop-helix. Shown are the regions of GRIP1 that interact with AR and other transcription factors. The numbers indicate the number of amino acids present (adapted from Ma et al., 1999).

Under antagonist bound conditions, the hydrophobic groove is not exposed, preventing the binding of p160 coactivators (Shiau et al., 1998). The different LXXLL motifs have different affinities for each NR. This suggests that each NR can select for one motif over another in the same coactivator. Mutation of any one of the LXXLL motifs does not abolish the receptor/p160 coactivator interaction, suggesting that all three motifs cooperate in high-affinity binding to the NR. Interestingly, the AF2 region of AR has a higher affinity for the FXXLF motif found in the NTD of the receptor and the coactivators ARA54 and ARA70 than for the LXXLL motif (Estebanez-Perpina et al., 2005, He et al., 2004a; Heinlein & Chang, 2002a; Culig et al., 2004). The two intrinsic AD1 and AD2 of the p160 coactivators function to recruit HATs and HMTs respectively. AD1 has three LXXLL/LXXLL like motifs and interacts with CBP/p300 and p300/CBP-associated factor. Mutation of these motifs impairs the coactivation function of the SRC and the interaction with the HATs, indicating that the SRCs also orchestrate chromatin remodeling. AD2 recruits the HMTs CARM-1 and PRMT1 to the enhancer and promoter regions of target genes. This indicates that SRCs can influence the local promoter histone code. The SRC members are expressed in a variety of tissues. However there are differences in certain cell types. Mouse models suggest that SRC-1 and SRC-2 have overlapping functions and both need to be knocked out to see a lethal

phenotype (Mark et al., 2004). Although the SRCs have overlapping functions (functional redundancy), they may still play a role in human diseases. The family members amplified in breast cancer 1 and ASC-2 (amplified in breast cancer 3) are amplified or overexpressed in a significant proportion of human mammary and ovarian tumors (Anzick et al., 1997; Lee et al., 1999).

8 Steroid hormones

8.1 Steroid hormones overview

Considering the enormous differences in physiological effects the steroid hormones have (**Table 3**) they are remarkably similar in structure (**Fig. 4**).

Table 3. Diversity of steroid hormone actions in humans (adapted from Bolander, 2004).

Steroid Hormone	Main Source	Main Targets	Action
Androgens	Testis, adrenal cortex	Reproductive tract, etc.	Sexual characteristics/ reproduction/anabolic effects
Estrogens	Ovary, placenta	Reproductive tract, etc.	Sexual characteristics/ reproduction
Glucocorticoids	Adrenal cortex	Muscle, liver	Energy metabolism, gluconeogenesis
Mineralcorticoids	Adrenal cortex	Kidney	Sodium and water maintenance
Progestins	Ovary, placenta	Reproductive tract, etc.	Maintenance of pregnancy

All steroid hormones are small lipophilic molecules derived from cholesterol and contain the four-ring structure of the sterol nucleus (**Fig. 4**) (Bolander, 2004; Alberts et al., 2002).

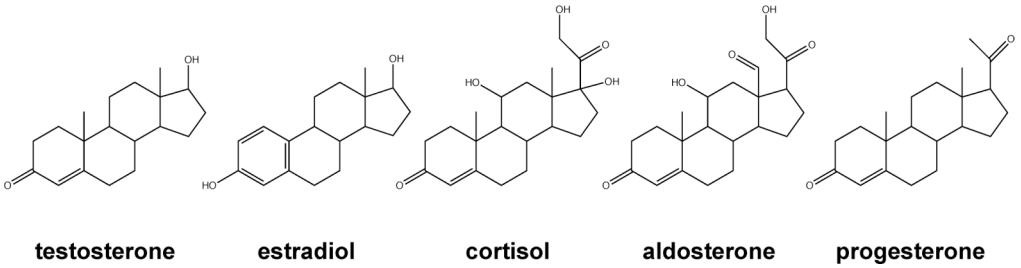

Fig. 4. Chemical structures of five steroid hormones.

8.2 Transport of steroid hormones

Steroid hormones are hydrophobic and therefore are transported in serum bound to carrier proteins that also protect them from degradation. There are two types of carrier proteins, general and specific.

General carriers have several low affinity hydrophobic binding pockets for the steroids. The most important general carrier is albumin. Although it has a low affinity for steroids, the concentration of albumin in the blood is so high that 68% of T and 60% of estrogens (estrone and estradiol) (E) are transported in the serum this way. Specific carrier proteins usually have a single, high affinity-binding site per molecule. 30% of T and 38% of E in humans is transported by sex hormone binding globulin (Bolander, 2004; Hammond & Bocchinfuso, 1995; Hammond et al., 2003).

According to the classic free diffusion theory, only the unbound, free or "bioavailable" fraction of the total steroid is thought to be able to gain access to target cells (Adams, 2005). The bioavailable fraction of T and E is about 2% (Jarow et al., 2005). Due to the lipophillic nature of steroids, the free diffusion theory suggested that free steroid just diffused across cell membranes into target cells where it activated its receptor (Mendel, 1989). Recently, this model has been challenged, suggesting that T and E bound sex hormone binding globulin is actively recruited and internalized by the cell surface lipoprotein receptor-related protein megalin (Hammes et al., 2005). However megalin knockout mice are not phenocopies of mice lacking AR or ER, suggesting that there is also a megalin-independent T and E uptake system (Hammes et al., 2005).

9 Androgens

9.1 Physiological androgens

T serves as a substrate for two metabolic pathways that produce antagonistic sex steroids. T can either be reduced by 5α-reductase to produce DHT or be aromatized to generate estrogens. Androgens and estrogens have opposite effects. Androgens masculinize whilst estrogens feminize. Female differentiation occurs irrespectively of the genetic sex in the absence of T or DHT (Nef & Parada, 2000). Sex determination is a complex process, which, in the early stages, is not hormone-dependent. During embryo development, the genital ridge is unusual in that it can either differentiate into male or female sexual organs (Nef & Parada, 2000; Brennan & Capel, 2004). Genetic sexual determination (in mammals) is in part directed by the presence or absence of the sex-determining region of the Y chromosome (*SRY*) gene. SRY initiates the development of the testes and the external genitalia. The testes start to produce T that promotes the development and stabilization of the Wolffian structures into epididymides, vas deferentia and seminal vesicles. DHT is essential for the development of the penis, scrotum and prostate (Nef and Parada, 2000). T production in early fetal life is controlled by placental chorionic gonadotropin secretion and later by the pituitary luteinizing hormone (Wilson et al., 1981). In the absence of T production or in the presence of estrogens, these male determining structures regress and female sexual organs form. Therefore in the absence of androgens or faulty AR function genetically male embryos develop a female phenotype. Therefore the synthesis of each of these steroids in developing male and female embryos must be subjected to a regulation that maintains the delicate balance between Leydig cell derived androgens and estrogens (Nef and Parada, 2000).

T and DHT control the development, differentiation and function of the male reproductive and accessory sex tissues, such as seminal vesicles, epididymides and prostate. Other organs influenced by androgens include skin, skeletal muscle, bone marrow, hair follicles and behavioral centers of the brain (Quigley et al., 1995; Gelman, 2002).

The synthesis of T occurs within the testicular Leydig cells. T in the testis can act locally or is released into the blood (see **Table 4**). T can be converted to the more potent DHT within

target cells of the peripheral tissues by two types of 5α-reductase. Type I 5α-reductase is expressed mainly in the sebaceous glands of skin and also in the liver. Type II 5α-reductase is found mainly in the hair follicles of skin, the prostate, and also in the liver. T is the main androgen in men and the testes produce about 80-95% of circulating T, whilst the adrenal glands produce the remaining 5-20% (Shen & Coetzee, 2005). In human target tissues the concentration of T can range from 100 nM up to 1 μM as found in the intratesticular fluids, but the percentage that is active remains unknown (Jarow et al., 2005). In women the major source of androgens is not from the adrenal glands, but is from ovary derived estrogens converted to T (Shen & Coetzee, 2005).

Table 4. Helsinki University Central Hospital reference ranges of androgen concentrations in male and female serum.

Hormone	**Male**	**Female**
Androstenedione	1.4 – 7.0 nM	1.2 – 7.0 nM
Testosterone	10.0 –38.0 nM	0.9 – 2.8 nM
5α-Dihydrotestosterone	1.0 – 10.0 nM	0.3 – 1.2 nM

9.2 Introduction to the androgen receptor

Throughout the life of an individual, androgens regulate the development and maintenance of the male phenotype. The signals of androgens are relayed to the basal transciption machinery in the nucleus by the AR (Quigley et al., 1995; Gelman, 2002; Lee & Chang, 2003). Like all NRs, AR has a conserved modular structure, with each domain playing an important role in AR function and signaling. This is either via intra-receptor interactions or via functional interactions with AREs and/or coregulatory proteins (Heinlein & Chang, 2002; Glass & Rosenfeld, 2000; McKenna et al, 1999a, b; McKenna & O'Malley, 2002a, b).

Disturbances in AR functionality caused by receptor mutation, disrupted DNA interactions, or altered coregulator interactions appear to be linked to a range of syndromes including androgen insensitivity syndrome (AIS) and CaP (McPhaul, 1999, 2002; Arnold & Isaacs, 2002; Abate-Shen & Shen, 2000; Parkin et al., 2005).

9.3 The androgen receptor gene

The genomic structure/organization of the *AR* gene is conserved in the mammalian kingdom from mouse to man (Gelmann, 2002) (**Fig. 5**). Human AR is encoded by a single copy gene found on the long arm of chromosome X at Xq11-12 (Lubahn et al., 1988; Brown et al., 1989). The gene spans some 180 kbp and is orientated with the 5' end towards the centromere (www.ensembl.org). The mRNA transcript is 10.6 kb long and has an open reading frame of 2757 bp, which codes for the eight exons of *AR* termed A-H or 1-8. Between 1988 and 1989 several groups cloned the human *AR* complementary DNA (cDNA) (Chang et al., 1988; Lubahn et al, 1988b; Trapmann et al., 1988; Tilley et al., 1989).

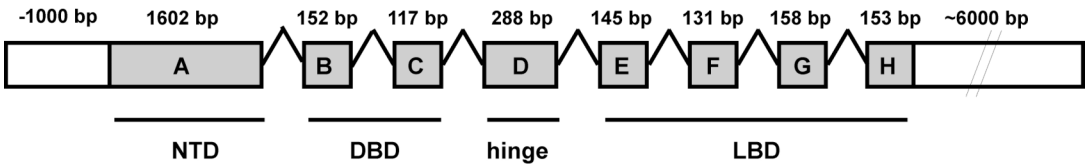

Fig. 5. Structural organization of the human *AR* gene. The exons are shaded and the relationship to the functional domains they encode are shown. NTD, amino-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain. The numbers indicate size of exons in base pairs (bp).

Other important species including rat (Chang et al., 1990; Tan et al., 1988) and mouse (He et al., 1990; Faber et al., 1991) were also cloned at this time. There are two possible transcription start sites for the *AR* gene located 1.1 kbp upstream of the translation start codon in the 5' untranslated region. The two transcription start sites are only 10 bp apart and therefore code for the same protein (Faber et al., 1993). Which transcription start site is used and the mechanism behind selection probably depends on the different cellular milieux where AR is expressed (Chang et al., 1995). The AR protein of human, rat and mouse are all approximately 99 kDa (unphosphorylated) or 110-kDa (post-transcriptionally phosphorylated). The DBD and LBD are 100% conserved, whilst the hinge and NTD are about 70-80% conserved. Each exon encodes for distinct regions of the receptor. Exon 1 encodes the NTD, exons 2 and 3 encode the DBD and exons 4-8 encode the LBD. *ER* and *PR* genes have additional untranslated exons upstream of exon 1 or exons in regions that were previously considered introns ('intronic exons'). They yield truly functionally distinct mRNA splice variants of the receptors in different human tissues (ERα, PR-A, PR-B) but this does

not occur with AR (Hirata et al., 2003). There is, however, one AR isoform, AR-A. AR-A is an 87-kDa protein that is found alongside full-length AR in human genital skin fibroblasts (Wilson & McPhaul, 1994). AR-A lacks approximately 190 amino acids within the NTD and is produced from an alternative translation-initiation methionine codon in exon 1. However, because AR-A is transcribed from the same mRNA as full-length AR, it cannot be considered a true splice variant (Hirata et al., 2003). AR-A represents about 10-26% of the total AR in some tissues (Wilson & McPhaul, 1994; Wilson & McPhaul, 1996), but its physiological role remains contested (Gao & McPhaul, 1998; Liegibel et al., 2003). Some have suggested that rather than being a true cell-directed isoform, AR-A results from *in vitro* proteolysis cleavage of the NTD or the LBD and does not exist *in vivo* (Gregory et al., 2001a). Therefore, despite there being two principal androgens, it seems that only one *AR* gene exists.

9.4 Transcription of the androgen receptor gene

AR expression is widespread and not just confined to the primary and secondary sex organs. AR expression can be found in most tissues including the brain, liver and kidneys (Quigley et al., 1995). The transcription of the *AR* gene to make AR protein is a highly regulated, but not very clear process. Transcription factors that up-regulate *AR* expression are Sp1, CREB and c-myc. Nuclear factor (NF)-κB and NF-1 down-regulate the expression of the *AR* gene (Chen et al., 1997; Mizokami et al., 1994; Grad et al., 1999; Supakar et al., 1995; Song et al., 1999). Regulation of AR expression occurs at all levels from gene transcription to translation of the mRNA into protein (Chang et al., 1995; Ing, 2005). AR regulation is cell type-specific (Quigely et al., 1995; Lindzey et al., 1994) and in some cases, age-specific (Supakar & Roy, 1996). The 5' untranslated region of the *AR* gene promoter lacks the usual TATA and CCAAT motifs but has a series of G/C rich regions indicative of Sp1 sites (Tilley et al., 1990; Baarends et al., 1990; Faber et al., 1991, 1993; Song et al., 1993; Grossmann et al., 1994a; Kumar et al., 1994; Chen 1997; Suske, 1999). In addition, there are several DNA elements, such as an HRE, that is recognized by AR, GR and PR. Also there is a RARE, an ERE and a cyclic AMP response element which is thought to be controlled by gonadotropin follicle-simulating hormone induced cyclic AMP (Varriale & Esposito, 2005; Blok et al., 1992; Lindezy et al., 1993; Mizokami et al., 1994). To some extent AR regulation is an autoregulatory process; androgens can up- or down-regulate AR mRNA or protein (Chang et

al., 1995; Gelmann, 2002; Tan et al., 1988; Quarmby et al., 1990; Takeda et al., 1991). The regulatory elements found within the AR promoter suggest that other hormones can regulate AR expression. This would make the control of AR expression very dependent on cell type and time (Quarmby et al, 1990; Takane et al., 1991; Song et al., 1993; Grossmann et al., 1994b; Mizokami et al., 1994).

9.5 Posttranslational modifications of AR and cross-talk with other signaling pathways

Upon synthesis AR undergoes several different covalent posttranslational modifications including, amongst others, phosphorylation, sumoylation and ubiquitination (Brinkmann et al., 1999; Gioeli et al., 2002, 2005; Poukka et al., 2000; Dehm & Tindall, 2005; Gill 2004, 2005). These covalent modifications are necessary for receptor function. How these modifications affect receptor function is not always clear due to a phenomenon termed cross-talk. Cross-talk is the communication/interaction between different signaling pathways. Cross-talk between signaling pathways may provide regulatory processes occurring in different parts of the cell and increase control over cell homeostasis to the plethora of extra/inter/intra cellular signals a cell receives (Gioeli, 2005; Dehm & Tindall, 2005, Ing, 2005). To review all the possible covalent modifications of AR and the implicated cross-talk cascades goes beyond the scope of this thesis, but brief examples, characteristic of the complexity of these modifications, are given below.

9.6 Phosphorylation of AR

Phosphorylation of AR is one of the most studied covalent modifications. Within 10 min of synthesis AR undergoes posttranslational hormone-independent phosphorylation. This is important for the acquisition of the hormone binding properties of the receptor. Upon hormone binding, the receptor undergoes further androgen-dependent phosphorylation, a step that protects AR from proteolytic degradation and that is required for nuclear import/export and DNA binding (Brinkmann et al., 1999; Edwards & Bartlett, 2005; Gioeli, 2005). Phosphorylation occurs throughout the receptor in over 10 positions. The majority of these sites are located in the NTD (Gioeli et al., 2002). Therefore phosphorylation is linked to the activation and stabilization of AR. Secondly, phosphorylation is an important AR regulatory

mechanism which may provide cross-talk links to the numerous cytoplasmic kinase signaling cascades of a cell, such as the epidermal growth factor receptor-2/Her2, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-OH kinase (PI3K)/AKT/Protein kinase B (PKB)/phosphatase and tensin homologue pathways (Dehm & Tindall, 2005; Linja & Visakorpi, 2004; Gioeli, 2005; Edwards & Bartlett, 2005; Mulholland et al., 2006). It is proposed that these kinase cascades regulate AR function in part by activating AR in the absence of hormone or sensitizing AR to reduced levels of androgens (Gioeli, 2005). It is the activation of AR in reduced levels of androgens that have linked these multiple kinase cascades during CaP development. Many of the kinase pathway proteins frequently have aberrant expression levels in recurrent CaP (Gioeli, 2005; Mulholland et al., 2006; Shand & Gelman, 2006). However, there are several conflicting studies on the effects of the kinase cascades on AR activity. For example, some studies have shown AKT to increase (Manin et al., 2002; Wen et al., 2000) or decrease (Lin et al., 2002; Thompson et al., 2003) AR activity. Furthermore it is still unclear whether AR is directly phosphorylated by AKT. Lin et al. proposed that AR was phosphorylated by AKT on Ser 213 and Ser 791 (Lin et al., 2002), however in agreement with Gioeli et al. we did not observe direct phosphorylation of AR by AKT (Gioeli, 2005; Thompson et al., 2003). Therefore it has to be considered that AKT regulates AR function in an indirect fashion, possibly by phosphorylating (a) coregulatory protein(s). The discrepancies observed in AR activity may then be due to cell specific expression of coregulators.

9.7 Ubiquitination and sumoylation of AR

Most proteins, including AR, are ubiquitinated (McKenna et al., 1999b). Ubiquitin is an 8.5 kDa (76 amino acids) polypeptide tag that is covalently attached to lysine residues of target proteins. Most often, ubiquitin is a signal to degrade the protein via the 26S proteasome. The targeted degradation of proteins serves a critical role in the regulation of cell function (Glickman & Ciechanover, 2002). However, most proteins including AR can be sumoylated (Poukka et al., 2000) on possibly the same lysines that may also be targets of ubiquitination (Muller et al., 2001). SUMOs, of which there are four different types in humans, are structurally related to ubiquitin. However the surface charge of the SUMOs is very different to ubiquitin (Muller et al., 2001; Gill, 2005). Sumoylation does not mark proteins for

degradation, but regulates other things, such as the activity of transcription factors, formation of subnuclear structures and nuclear distribution of target proteins (Muller et al., 2001; Gill, 2004, 2005). Furthermore, the signaling cascades mentioned above are also subjected to cross-talk regulation by phosphorylation, ubiquitination and sumoylation. It is therefore not surprising that covalent modifications of AR have been linked to CaP biology (Gill 2004, 2005; Mo & Moschos, 2005).

9.8 Overview of androgen-dependent transcriptional regulation

In the absence of androgens, AR resides in the cell cytoplasm as a heteroprotein complex with heat-shock proteins (HSP) 90, 70 and immunophillin FSKB (Pratt et al., 2004; Pratt & Toff, 1997). Upon T entry into the cell and the possible cell-specific conversion of T to DHT, AR binds the presented androgen. This induces a conformational change in which the HSPs are released and allows AR to be translocated to the nucleus (Heinlein & Chang, 2001; Pemberton & Paschal, 2005). It is possible though that endogenous AR *in vivo* may reside more or less constantly in the nucleus (Gelmann, 2002). Once inside the nucleus, androgen-bound AR locates and binds to target AREs (Claessens & Gewirth, 2004). The binding of AR to the ARE is a necessary step for transcriptional activity. It initiates the formation of the PIC at the promoter regions of androgen responsive genes that include TFII A-H and Pol II (Lee & Chang, 2003) (see **Fig. 6**).

10 Androgen receptor structure-function relationship

10.1 The structure and function of the androgen receptor domains

As previously mentioned, AR has a conserved modular structure (Beato et al., 1995; Aranda & Pascual, 2001; Gelman, 2002) consisting of the NTD, the DBD, the hinge region and the LBD. Each of the domains has its own particular properties and characteristics (Quigley et al., 1995; Gelmann, 2002). The domains do not function independently, but synergize or antagonize with each other to produce a receptor function that exquisitely regulates the genomic actions of androgens in target tissues. The initial cloning of the AR cDNA resulted in AR being traditionally described as consisting of 910 or 919 amino acids (Trapman et al.,

1988; Lubahn et al., 1988b). This is due to there being two polymorphic[§], homopolymeric amino acid tracts in the NTD that can vary in length so AR can therefore be shorter than 910 or longer than 919 amino acids.

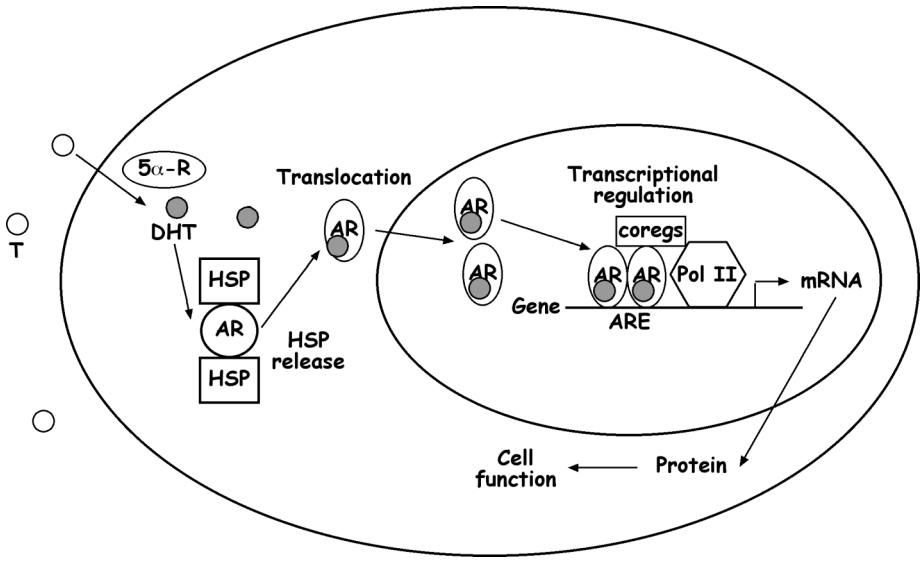

Fig. 6. A simple overview of AR nuclear translocation and transcriptional regulation. AR, androgen receptor; ARE, androgen response element; DHT, 5α-dihydrotestosterone; 5α-R, 5α-reductase; T, testosterone; HSP, heat shock protein; Pol II, RNA polymerase II. Testosterone enters the cell and dependening on cell type can be converted to DHT. T or DHT binds to AR, which is then able to activate transcription.

10.2 The androgen receptor amino-terminal domain

The AR NTD is encoded by exon 1 and covers amino acid residues 1-557 of the 919 amino acids of the total protein. The NTD functions to regulate the recruitment of the PIC (Beato & Sanchez-Pacheco, 1996; Lee & Chang, 2003) to androgen responsive genes. It does so by directly recruiting/contacting the basal transcription factors such as TFIIF (McEwan & Gustafsson, 1997; Reid et al., 2002a; Kumar et al., 2004b). To date, little is known about the structure and folding of the AR NTD as the crystal structure has not been resolved. This may be due in part to the "flexible" nature of the NTD (Reid et al., 2003; McEwan, 2004). Secondary structure prediction analysis and limited proteolysis studies suggest that 13% of

[§] Polymorphism: a DNA alteration that occurs in at least 1% of the population (Harris, 1969).

the NTD is involved in four regions of α-helix and 20% is β-sheet (Reid et al., 2002b; Kumar, et al., 2004b; McEwan, 2004). Reid et al. reported that upon interaction with TFIIF, the NTD adopts a more folded and compact structure. They therefore predicted an "induced fold" model for the structure of the NTD. This means that the NTD is an open structure that, when presented with different factors, can form a structure that is constant but unique to that factor (Reid et al 2002a, b, Kumar et al., 2004b). An induced fold model has also been reported for GR (Dahlman-Wright et al., 1995), ER (Wärnmark et al., 2001) and PPARγ (Hi et al., 1999). Induced fold has the advantage of being able to support different protein-protein interaction platforms, whilst being specific in the range of different interactions at given situations (Dyson & Wright, 2005).

10.3 Activation function 1 of the AR NTD

The AR NTD has several features that make it unique amongst NRs (**Fig. 7**). The AR NTD harbors a powerful and hormone-independent AF 1. The AF1 has been loosely defined as spanning amino acids 142-485 (Rundlett et al., 1990; Palvimo et al., 1993; McEwan, 2004, Shen & Coetzee, 2005). Brinkman and coworkers defined two discrete transactivation units (TAU) of the AF1 and termed them TAU1 (amino acids 100-370) and TAU5 (amino acids 360-529) (Jenster et al., 1995). Callewaert and coworkers further defined the core TAU1 as amino acids 173-196 (Callewaert et al., 2006). In the absence of the LBD, the hormone-independent AF1 is constitutively active to levels comparable with full-length androgen bound AR (Jenster et al., 1995; Ikonen, et al., 1997). The two TAU regions have different functions. p160 coactivators are recruited by TAU5, however this recruitment is attenuated by TAU1 (Callewaert et al., 2006).

10.4 The conserved amino acid stretches of the AR NTD-ANTS

The AR NTD has three conserved amino acid stretches. The first consists of 14 amino acids and resides in the AF1 region from amino acids 233-246 (AKELCKAVSVSMGL) (He et al., 2004). This sequence is absolutely conserved throughout all vertebrates so far sequenced. It is only found in AR so it has been termed AR NTD signature sequence-ANTS. Through an

interaction screen, this signature sequence was proposed as an HSP interaction domain that limits AR transcriptional activity (He et al., 2004b).

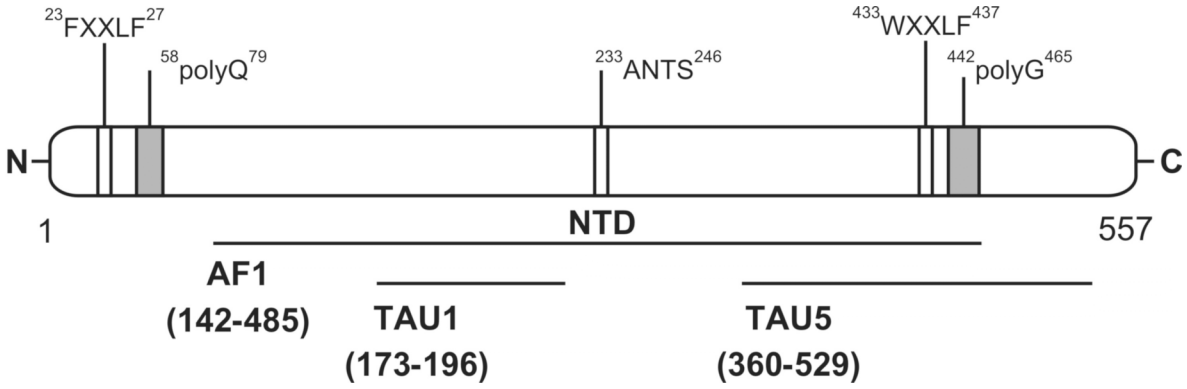

Fig. 7. Location of the AR NTD interaction motifs (adapted from Shen & Coetzee, 2005). AF1, activation function 1; TAU, transactivation unit; ANTS, AR NTD signature sequence; poly, homopolymeric amino acid tract; X, any amino acid.

10.5 FXXLF and WXXLF motifs

There are two other conserved motifs found within the NTD, the FXXLF (amino acids 23-27) and the WXXLF (amino acids 433-437), where X is any amino acid. In humans the motif sequences are FQNLF and WHTLF motifs (He et al., 2000). These motifs direct and stabilize the interaction of the AF1 with the LBD AF2 region (Moilanen et al., 1997; He et al., 2000; Dubbink et al., 2004). The AR LBD has a well-defined structure of 12 α-helices and an anti-parallel β-sheet (Matias et al., 2000). The extensive secondary and tertiary structure creates a hydrophobic cleft in the AF2 that forms the binding surfaces/sites for the NTD FXXLF and WXXLF motifs and several LXXLL motif-harboring coactivators (Heery et al., 1997). FXXLF motif has a greater affinity for the AF2 hydrophobic cleft than the LXXLL motif (He et al., 2002). The reason why the AF2 region has a greater affinity for the bulky aromatic side chain of the phenylalanine residue over the smaller leucine residue may be a combination of electrostatic clamping and induced fit (He & Wilson, 2003; Hur et al., 2004). Furthermore, the regions flanking the FXXLF motif (amino acids 3-13 and 30-34) have also been shown to be important for the AF1/AF2 interaction (Steketee et al., 2002). The amino acid region 3-13 does not directly contact the AF2 hydrophobic cleft, but an adjacent region and stabilizes the AF1/AF2 interaction. Conversely the 30-34 region appears to destabilize the AF1/AF2

interaction (Steketee et al., 2002). The role of the AF1 in contacting the AF2 is not clear, but it is thought that it is connected to the changes in receptor conformation upon hormone binding. The WXXLF motif is involved with the NTD/LBD interaction, but is not involved in the direct contacting of the AF2 region. The role of the WXXLF motif may be to recruit coregulators to the TAU5. Mutation of the WXXLF residues causes a loss of coactivator recruitment and the motif may even inhibit the NTD/LBD interaction (He et al., 2000; Shen & Coetzee, 2005).

10.6 The homopolymeric amino acid tracts of the AR NTD

The third feature of the AR NTD is that exon 1 codes for two uninterrupted, polymorphic, homopolymeric amino acid tracts (Gelman, 2002). The first is a polyglutamine tract starting at codon 58 (in humans) (DNA sequence CAG). It extends for an average of 21±2 repeats (Chamberlain et al., 1994; Ding et al., 2004). The second is a polyglycine tract starting at codon 442 (DNA sequence GGN, (where N is A, C, G, T) the majority of the codons being GGC). On average it extends for 23±1 repeats (Ding et al., 2005). The exact functions of these tracts are not established. However CaP and the X-linked neurodegenerative disease spinal and bulbar muscular atrophy (SBMA) (aka Kennedy's syndrome) (Nelson & Witte, 2002; Zeegers et al., 2004; Beitel et al., 2005; La Spada et al., 1991) have been linked to these tracts, suggesting that they play a major functional role.

Most investigations into the functional effects of the homopolymeric amino acid stretches have concentrated on the polyglutamine tract (Shen & Coetzee, 2005). The CAG polymorphism was first uncovered when patients with SBMA were discovered to have >40 glutamine repeats (La Spada et al., 1991). Expanded polyglutamine tracts result in a receptor that has lower transacitvation ability and has been linked to male infertility and AIS (La Spada et al., 1991). *In vitro* it has been shown that shortening of the polyglutamine tract increases AR activity on simple promoters. Therefore it is thought that the polyglutamine tract provides inhibitory control of the NTD (Irvine et al., 2000; Callewaert et al., 2003; Ding et al., 2004). The polyglutamine tract may function in the correct alignment of the NTD with the LBD. Mutational analysis changing two glutamine residues to leucine reduces the NTD/LBD interaction. However the mutation leads to higher receptor activity due to the

increased recruitment of coactivators to the AF2 region (Buchanan et al., 2004). There are very few investigations into the length of the polyglycine tract on AR function. Keeping the polyglutamine tract a constant length and varying the length of the polyglycine tract, Ding et al. showed *in vitro* that the length of the polyglycine (19-23 glycines) tract affected AR protein expression levels. The longer the polyglycine tract, the less AR protein produced (Ding et al., 2005). They proposed that the reduced levels of AR expression were due to hairpin structures in the mRNA caused by the GGC expansion. The hairpin structures would lead to less efficient translation. Taking polyglycine tract length and protein expression levels into consideration, they concluded that the length of the tract does not affect the transcriptional activity of the receptor. But having a shorter polyglycine tract would result in more AR protein. Having more AR protein would theoretically lead to more transactivation (Ding et al., 2005). The polyglycine tract may function as a flexible hinge between the NTD and the DBD. Removal of the polyglycine tract reduces the activity of the receptor by 90% (Goa et al., 1996). Males having 16 or less glycine residues are more susceptible to hereditary and sporadic CaP (Chang et al., 2002). This is probably as a result of higher AR protein levels. There are currently no known syndromes related to deleted or abnormally long polyglycine tracts. There are also no known cases of the polyglycine tract mutations and AIS, but this is probably because the GGC repeat length is not usually genotyped (Quigley et al., 1995).

The lengths of the polyglutamine and polyglycine repeats are independent of each other and as yet there has been no study investigating whether there is interplay between these two tracts and the *in vivo* effect they will have on AR activity. Currently, conflicting data still remains about the role and mechanisms that results from modest variations in polyglutamine and polyglycine length and the associated increased risk of CaP, infertility and baldness (Edwards et al., 1999; Zitzmann & Nieschlag, 2003; Zeegers et al., 2004).

10.7 The androgen receptor DNA-binding domain

To activate the transcription of hormone-regulated genes, NRs have to recognize and bind to the appropriate HRE and they do so via their DBD. The DBD is the most conserved region of the receptor. There is 100% homology between DBDs from human and rat AR. The DBDs of

PR, GR and ERα share 79%, 76% and 56% amino acid sequence homology to the AR DBD respectively (Gelmann, 2002). Yet again, as with the paradox of the steroid hormones, regulation of the diverse range of physiological processes is directed by highly similar DBD structures. The AR DBD consists of 66-68 amino acids encoded by exons 2 and 3 (Lubahn et al., 1988b, Tan et al., 1988). The AR DBD binds to AREs to regulate the expression of androgen responsive genes (Claessens & Gewirth, 2004; Robins, 2004, 2005). The DBD consists of two cysteine rich zinc fingers, which regulate the recognition and binding of the AREs. The two zinc fingers are encoded separately by each exon and harbor four cysteine residues that are tetrahedrally coordinated to two Zn^{2+} ions (Freeman, 1992) (**Fig. 8**). According to crystal structure data, the two zinc fingers form a single compact structure. The compact structure is quite different from the independently folded zinc fingers of many other transcription factors. Both zinc-coordinating complexes initiate an α-helix starting at the third conserved zinc-coordinating cysteine residue. The two helices are packed at right angles and cross near their midpoints. The structure is stabilized by several hydrophobic interactions between the amino acid residues. The two zinc fingers form a structure that binds to the major groove of DNA (Härd et al., 1990). The fingers interact with the DNA in a distinct and complementary fashion (Luisi et al., 1991; Schwabe et al., 1993). Both fingers make contacts with the nucleotides and backbone phosphates. The first finger defines the specificity of the DNA interaction. The second finger is involved with half-site spacing determination and receptor dimerization. The first zinc finger contains a 5 amino acid peptide stretch termed the 'P-box'. The P-box residues make base-specific contacts with the nucelotides in the DNA major groove. All the SRs except ER have the same P-box amino acid sequence of GSKV and bind specifically to the consensus half site DNA element 5'-TGTTCT-3' (Beato, 1989; Tsai & O'Malley, 1994). The second finger contains the D-box, a region that stabilizes the binding complex by hydrophobic interactions with the first finger and determines the spacing requirements of a receptor's HRE (Schoenmakers et al., 1999). The second finger is also required for receptor dimerization that occurs during DNA binding (Luisi et al., 1991; Dahlman-Wright et al., 1991). The binding of the first receptor to one half of the HRE supposedly creates an optimal DNA-protein surface for cooperative binding of the dimer partner (Tsai et al., 1988). SRs bind HREs as head-to-head homodimers that are invariably spaced by 3 bp (Verrijdt et al, 2003; Claessens & Gewirth, 2004). The structures and mechanisms mentioned above still do not describe how SRs selectively bind to their HREs.

To date several androgen responsive promoters and enhancers have been described (Robins et al., 2004, 2005). Most are promiscuous in that they can also be activated by the other SR and are termed 'non-selective' AREs. However there are some promoters that are only activated by androgens, e.g. the rat probasin promoter (Rennie et al., 1993) and as such are called 'selective' AREs. Selective AREs seem to be composed of partial direct repeats with a 3 bp spacer. The direct repeats rather than the classic palindromic repeats, would then 'force' AR to assume a head-to-tail dimerization rather than the classical head-to-head dimerization (Verrijdt et al., 2003). It has been proposed that AR is the only NR3C able to head-to-tail dimerize (Verrijdt et al., 2003). However, crystal structures show that when AR binds to a selective direct repeat ARE, it does so in a classical head-to-head fashion. This 'non-standard' binding to the direct repeat could also be a mechanism of the ARE selection process (Shaffer et al., 2004). It is possible that both head-to-head and head-to-tail homodimeriztion exists. *In vitro* evidence demonstrates that AR can bind in a head-to-tail fashion (Langley et al., 1995). Recently it has been shown that AR binding to 'selective' and 'non-selective' AREs causes a conformational change in the receptor's NTD. It was proposed that interdomain communication between the NTD and DBD also aid ARE selectivity (Brodie & McEwan, 2005). Taken together *in vivo* ARE selectivity is a combination of multiple mechanisms working in tandem to ensure the specificity of AR-dependent gene transcription.

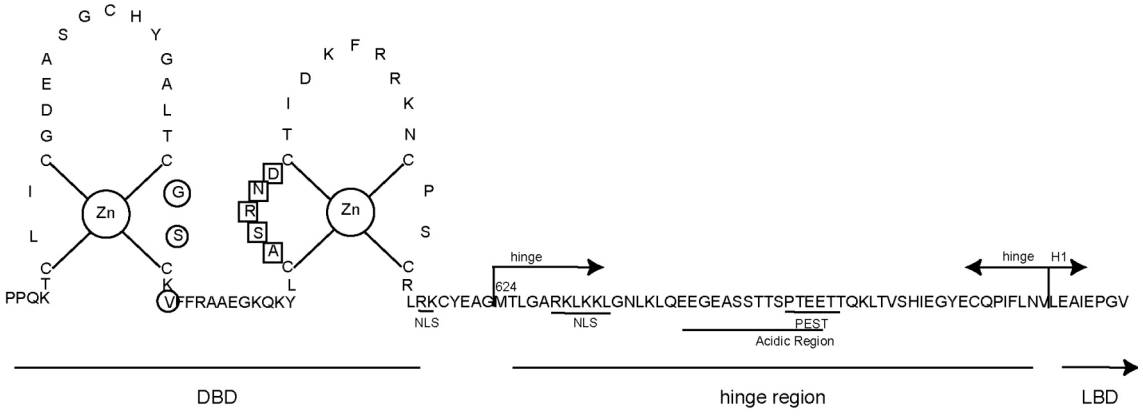

Fig. 8. Scheme of the AR DBD and adjacent hinge region. The DBD comprises two zinc-finger DNA-binding motifs. The residues of the P-box are shown in circles and those of the D-box are shown in squares. The numbering of the residues is according to Lubahan et al. (1988b). NLS, nuclear localization sequence; PEST, protein degradation sequence.

10.8 The androgen receptor hinge region

The hinge region spans amino acids 628-669 and was initially thought to serve as a relatively flexible connection region between the DBD and the LBD. It comprises the C-terminal end of the DBD and the first helix of the LBD. The hinge region of AR contains the bipartite nuclear localization signal that enables targeting of the activated AR to the nucleus (Poukka et al., 2000b). Mutation of the nuclear localization signal results in an AR that is exclusively cytoplasmic. The hinge region also plays some role in DNA binding (Haelens et al, 2003; Schoenmakers et al., 1999). Furthermore, the hinge region has a role in regulating transcriptional activity by modulating the NTD/LBD interaction (Zhou et al., 1994; Buchanan et al., 2001). In addition, the hinge region contains a PEST sequence that might be important for receptor degradation by the 26S proteasome and possibly an important phosphorylation site (Rechsteiner & Rogers, 1996; Sheflin et al., 2000; Tanner et al., 2004; Zhou et al., 1995; Gioeli et al., 2002). Several coregulatory proteins capable of interacting with the DBD and hinge region have been identified. Mutagenesis studies suggest that the hinge region might be the target for corepressor binding (Horlein et al., 1995). The hinge region has also been suggested to have a particular importance in the stabilization of the LBD in the presence of hormones or corepressors (Pissios et al., 2000).

10.9 The androgen receptor ligand-binding domain

In the unliganded form AR is a cytoplasmic protein. In the cytoplasm AR is bound by various molecular chaperones, such as the HSP 90, 70 and the cochaperone FKBP52-binding protein (Pratt & Toff, 2003; Cheung-Flynn et al., 2005). In the absence of androgens the HSPs hold the receptor in an 'inactive form'. Upon ligand binding, the receptor undergoes a conformational change, releases the HSP, translocates to the nucleus and regulates the transcription of AR responsive genes. *In vitro*, this translocation from cytoplasm to nucleus takes only 15-30 minutes (Poukka et al., 2000b; Karvonen et al., 2002). *In vivo*, there is probably always some active AR residing in the nucleus, even in the presence of low concentrations of androgens. Encoded by exons 4-8 the LBD resides at the C-terminal of AR. There has been substantial work on determining the tertiary structures of the NR LBDs

(Bourguet et al., 2000; Nagy & Schwabe, 2004). Despite differences between the AR primary sequences and the other SRs, the LBDs all adopt a similar three-dimensional structure (Matias et al., 2000; Sack et al., 2001). NR LBDs generally contain 12 α-helices. Following convention, the AR LBD has 12 α–helices and a short β-turn although the AR actually lacks what would be helix 2 (**Fig. 9**). The helices of the NR LBDs all take the 3 layer anti-parallel 'α-helical sandwich' fold (Bourguet et al., 2000; Nagy & Schwabe, 2004). The AR ligand-binding pocket (LBP) is formed by the helices 3, 4, 5, 7, 11 and 12, together with a β-turn preceding helix 6 (Matias et al., 2000; Williams & Sigler, 1998). There are a total of 18 amino acids that directly interact with the androgen bound in the LBP (Matias et al., 2000). Most of the amino acids are hydrophobic and directly interact with the androgen. Only a few amino acids are polar and may form hydrogen bonds to the polar atoms in the androgen, which is thought to determine stereo-specificity (Matias et al., 2000; Sack et al., 2001; Nagy & Schwabe; 2004).

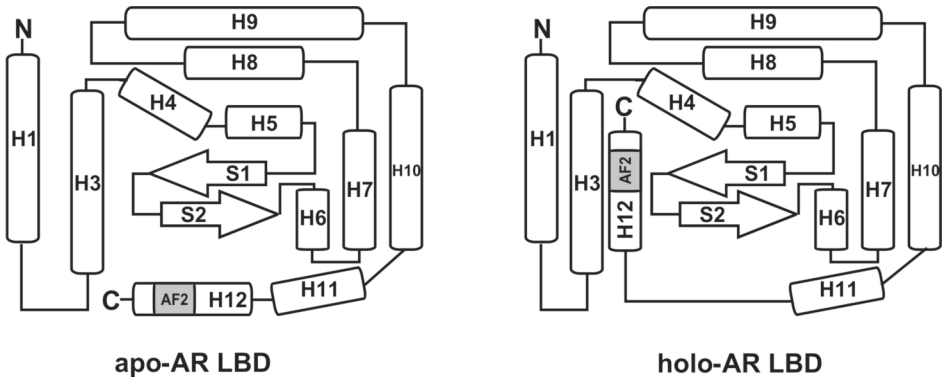

Fig. 9. Scheme of the AR LBD. In the absence of androgen (apo-AR LBD) and in the presence of androgen (holo-AR LBD). H1-H12 indicate the α-helices and S1 and S2 the β-strands. Shaded region represents the core androgen-dependent activation function 2 (AF2).

10.10 Activation function 2 of the LBD

The hormone-dependent AF2 of NR resides in the LBD. Unlike most other NRs, the AF2 of AR is transcriptionally weak (Moilanen et al., 1997; Ikonen et al., 1997). The receptor needs the AF2 region for the important ligand-dependent NTD/LBD interaction. The AF2 region also modulates the coregulator recruitment that is required for precise control of AR's transcriptional activity (Ikonen et al., 1997; He et al., 1999, 2000; Wärnmark et al., 2003;

Nagy & Schwabe, 2004). The AF2 comprises a core AD that is highly conserved throughout the NRs (amino acid sequence EMMAEIISV) and regions contributed by the helices 3, 5 and 12 (Danielian et al., 1992; Barettino et al., 1994; Slagsvold et al., 2000; Darimont et al., 1998; Shiau et al., 1998). Mutational analysis of the amino acid residues within the AF2 core AD demonstrate that each amino acid has a particular effect on receptor function that can disrupt the NTD/LBD interaction and/or responses to coregulators (Slagsvold et al., 2000). The most striking characteristic of the LBD is that upon ligand binding, it undergoes a dramatic and a well-documented conformational change. This change stabilizes the LBD structure and exposes the AF2 hydrophobic binding clefts for NTD and coregulator interactions (Bourguet et al., 2000). In the unliganded (apo-LBD) state, helix 12 points away from the main body of the LBD. Upon androgen binding (holo-LBD), helix 12 swings back into the LBD body and creates a 'lid,' trapping the ligand within the LBP (Parker & White, 1996; Bourguet et al., 2000). After helix 12 re-arrangement, a hydrophobic groove is formed by helices 3, 4, 5 and 12 (Darimont et al., 1998). The hydrophobic groove then interacts with the FXXLF motif of the NTD and/or the LXXLL of the coactivators and LXXXIXXX(I/L) motifs of corepressors (He et al., 2000; Heery et al., 1997; Hu & Lazar, 1999; Dubbink et al., 2004). The AR LBD can bind ligands that have an agonistic (activating) effect or ligands that have an antagonist (repressing) effect on receptor activity (Matias et al., 2000; Nagy & Schwabe, 2004). Both these types of ligand bind in the LBP. However only when the agonists are bound does it permit the closing of the H12 'lid' and the presentation of the AF2 interaction surfaces for a transcriptionally active receptor.

10.11 Antiandrogens-antagonists

AR antagontists can be divided into two groups. The first group of antagonists do not permit AR to engage in DNA binding but the receptor still undergoes nuclear translocation (Jenster et al., 1993; Karvonen et al., 2002). The second group of antagonists allows the receptor to bind DNA, but induces a conformation that disrupts associations with coregulators and inhibits the activation of the transcriptional machinery (Smith et al., 1997; Nagy & Schwabe, 2004). Androgen antagonists can either be non-steroidal or steroidal (**Fig. 10**). Non-steroidal antagonists include bicalutamide (casodex) and hydroxyflutamide. Steroidal antagonists include cyproterone acetate. Some of the steroidal antagonists have a bulky side-chain that

cannot be accommodated within the LBP (Duax et al., 1988; Bourguet et al., 2000; Giannoukos et al., 2001). These bulky side chains protrude out of LBP and cause distinct changes in LBD conformation (Nagy & Schwabe, 2004). Steroidal androgen antagonists have progesterone- and glucocortioid-like activities and are therefore considered non-selective androgen antagonists (Kuil & Brinkmann, 1996). The non-steroidal antagonists are considered selective antagonists, because they only block the actions of androgens. However, in doing so, they stimulate the hypothalamus-pituitary-gonadal axis to further produce T and DHT (Neumann & Topert, 1986; Raynaud & Ojasoo, 1986). The blocking of androgen action using antagonist is frequently used in treating advanced CaP. Unfortunately antagonists are not tissue-selective modulators and therefore can have quite severe side effects (Hirawat et al., 2003).

bicalutamide cyproterone acetate

Fig. 10. Chemical structures of two androgen antagonists. Bicalutamide, an example of a non-steriodal antiandrogen. Cyproterone acetate, an example of a steroidal antiandrogen.

10.12 Nongenomic androgen actions

According to the classical model of steroid hormone action, steroid hormones diffuse into the cell, bind to their cognate receptors and induce transcription of the target genes. The length of time between steroid hormone entry into the cell and the accumulation of significant amounts of protein to affect cell function can range from 15-30 min to several hours. Within the last decade, an increasing amount of information has been produced on a phenomenon termed the nongenomic actions of steroid hormones (Lösel & Wehling, 2003). The nongenomic actions of steroid hormones are defined as rapid cellular responses to steroid hormones that occur in the second to minute range of time, although they can occur over longer time periods. The effects of nongenomic signaling are not mediated by alterations in transcription and protein

synthesis, but rather the immediate activation/repression of cytoplasmic kinase-signaling cascades and intracellular calcium levels (Lösel & Wehling, 2003). The nongenomic effects of E have been studied in most detail on the production of nitric oxide in vascular endothelial cells (Levin, 2005; Kim & Bender, 2005; Manavathi & Kumar, 2006). Small pools of cytoplasmic ERα, localized to the plasma membrane are required for the rapid activation of endothelial nitric oxide synthase via the MAPK and PI3K/AKT signaling cascades. The activation of these cascades results in E-induced arterial vasodilation that does not immediately involve changes in E-regulated gene expression. The nongenomic effects of ERβ are still under investigation (Manavathi & Kumar, 2006). Recently, it has been shown that for progesterone a novel membrane-bound/associated PR exists, termed membrane associated PR (Zhu et al., 2003). Membrane associated PR seems to relay signals via the PI3K and extracellular-signal-regulated kinase/MAPK casades (Lösel & Wehling, 2003; Freeman et al., 2005). The existence/function of the nongenomic effects of androgens have not yet been satisfactorily proven and novel AR(s) have not yet been cloned (Heinlein & Chang, 2002b). Currently, it is believed that in the absence of a novel cytoplasmic/membrane AR, the androgen signal is relayed to the PI3K/AKT signaling pathway by the plasma membrane protein caveolin-1 (Li et al., 2003). The caveolin-1/PI3K/AKT pathway has been shown to promote cell survival and may be involved in the metastasis of CaP (Baron et al., 2004; Li et al., 2001; Freeman et al., 2005).

11 Androgen receptor and disease

Genetic defects of AR are implicated in several X-linked pathogenic states ranging from AIS to CaP (Quigley et al., 1995; Gelman et al., 2002; Shen & Coetzee, 2005). Four types of mutation can occur to the *AR* gene (Brinkmann, 2001):

- Single point mutations resulting in amino acid substitutions or premature stop codons
- Nucleotide insertions or deletions leading to frameshift and premature termination
- Complete or partial gene deletions (>10 nt)
- Intronic mutations in either splice donor or acceptor sites

Amino acid substitutions in different segments of the *AR* gene disturb AR function by distinct mechanisms (Lindzey et al., 1994). Amino acid substitutions can lead to both loss and gain of receptor function (www.mcgill.ca/androgendb, Gottlieb et al., 2004a). Due to the non-essential nature of AR function in embryonic development, the presence of only one copy of the *AR* gene on the X chromosome and a sometimes easily detectable phenotype, many of these amino acid substitutions have been detected and categorized. They have been listed in the AR mutations database www.mcgill.ca/androgendb (Gottlieb et al., 2004b). Substitutions in the DBD of the receptor appear to comprise a relativity homogenous group. These substitutions usually impair the capacity of the receptor to bind to HRE motifs and affect the function of AR modulated genes. Substitutions in the LBD have a more variable effect on receptor function. In some cases of LBD mutation the resulting effect is obvious as it disables the receptor to bind hormones. In other instances the effect is subtle and may result in the production of a receptor protein that displays qualitative abnormalities in hormone binding. But sometimes it is not possible to correlate a hormone-binding defect to an abnormal phenotype (McPhaul, 1999).

11.1 Androgen insensitivity syndromes

The syndromes of androgen resistance have attracted a great deal of interest for understanding the physiology of male sex differentiation and the mechanisms of androgen action. Androgen resistance is caused by loss-of-function mutations. Inhibition of androgen biosynthesis or AR mutations that lead to receptor dysfunction during fetal development will arrest androgen-dependent genital formation and result in defective or absent masculinization. AIS is estimated to be present in 1:20 000–64 000 male births. AIS is usually caused by missense mutations within the DBD or LBD. Due to the large size of the NTD and the homopolymeric amino acid tracts, mutations of this domain have not been as intensively investigated (Ahmed et al., 2000; Brinkmann, 2001; Avila et al., 2001; Yong et al., 2003). AR mutations that severely impair the amount, structure, or function of the receptor cause complete androgen insensitivity syndrome (CAIS). Due to the lack of androgen-dependent masculinization, CAIS individuals are born genetically male but with a normal external female appearance. These individuals have normal breast development and normal looking female genitals although they have vellous or scanty pubic hair. Internally,

CAIS individuals have testes located within the abdomen or in the labia majora (a condition earlier termed testicular feminization). They do not have a uterus or fallopian tubes (Avila et al., 2001; Ahmed et al., 2000). AR mutations that do not completely disrupt AR function cause partial AIS (PAIS). PAIS is presented as ambiguous external genitalia, including partial labial-scrotal fusion, hypospadias, bifid scrotum and micropenis. Detailed classifications, especially for PAIS phenotypes have been developed (Quigley et al., 1995; Sinnecker et al., 1997). As only 18 amino acids of the LBD are in close contact with the bound ligand it is unclear why certain mutations result in PAIS, whereas neighboring mutations result in CAIS (Matias et al., 2000; Yong et al., 1998; Gottleib et al., 2004a, b). Generally, mutations leading to PAIS tend to cluster in the regions located outside the structural helices 3, 4, 5 and 12 of the LBD. This clustering of mutations may not be coincidental and the regions between helices may have important roles in defining androgen binding and ligand specificity (Yong et al., 1998). Using modeling techniques it has been shown that LBD mutations that cause CAIS and are not involved in direct contact with the ligand, cause local structural distortions that affect the LBP conformation (Gottlieb et al., 2004a). Mild AIS (MAIS) is presented as impaired spermatogenesis and fertility that may or may not lead to total infertility (Yong et al 2003; Gottlieb et al., 2004a). MAIS may be caused by mutations in coregulators rather than of the receptor (Adachi et al., 2000; Yanase et al., 2004).

11.2 Prostate cancer

In contrast to AIS, CaP is usually associated with gain-of-function mutations. The growth of normal prostate is dependent on the presence of androgens and AR function. CaP is the most common malignancy among men in western societies (Dehm & Tindall, 2005; Parkin et al., 2005). CaP is associated with age, race, life style and family history. The steps that lead up to the initiation of CaP are not clear but men who are castrated during puberty do not develop CaP (Abate-Shen & Shen, 2000; Isaacs, 1994). In the initial stages when confined to the prostatic capsule, CaP is curable by surgical intervention and/or radiation therapy. However if not detected early, or in more aggressive forms of the disease, CaP can advance to stages characterized by local invasion of the seminal vesicles, followed by metastasis. There are about 85 AR mutations that have been found in CaP tissue. The mutations are nearly all

single-base somatic mutations found in both the LBD and NTD (Gottlieb et al., 2004a). The molecular events that lead to the progression of CaP from hormone-dependent to hormone-independent (hormone-refractory (HR)) are not understood. AR activity is important throughout all stages of the disease (Litvinov et al., 2003) and therefore the usual initial treatment of primary locally invasive CaP is androgen depletion therapy (ADT) by chemical or surgical castration. Initial response rates to ADT are high, however, in time, most CaPs become resistant to the ADT and generally patients get renewed tumor growth within 18-24 months (Edwards & Bartlett, 2005). This change in hormone dependency from hormone-dependent to HR CaP is termed 'androgen escape'. There are several proposed mechanisms behind androgen escape including somatic *AR* mutations during ADT (Haapala et al., 2001), *AR* gene amplification (Visakorpi et al., 1995; Linja et al., 2001), and altered coregulator interactions (Linja et al., 2004). Most HR CaPs overexpress AR (Linja et al., 2001), however this is not always due to AR gene amplification (Chen et al., 2004). Interestingly, Chen et al. demonstrated that overexpression of AR mRNA is required and sufficient to convert androgen-dependent CaP to HR CaP even without *AR* gene amplification. In addition, they demonstrated that the conversion from androgen-dependent CaP to HR CaP depended on the normal genomic actions of AR and that mutant receptors that could not bind androgens, could not induce the transition. They proposed that overexpression of AR dilutes the effects of androgen antagonists and promotes sensitivity to the available androgens (Chen et al., 2004). Another recent and fascinating finding is that two ETS transcription factors (Seth & Watson, 2005), ERG and ETV1, have been found to fuse at a very high frequency to the 5' end of the androgen-regulated *TMPRSS2* gene. This potentially generates an androgen-responsive fusion oncoprotein (Tomlins et al., 2005). TMPRSS2 codes for a prostate-specific serine protease that is overexpressed in many CaPs (Lin et al., 1999; Paoloni-Giacobino et al., 1997). ERG or ETV1 overexpression in high frequency is found in both primary and metastatic CaP, but not in benign prostatic hyperplasia (BPH). This suggests that translocation of either ERG or ETV1 to the TMPRSS2 locus could be one of the first steps of the invasive disease (Shand & Gelman, 2006).

BPH is a nonmalignant overgrowth that is common in aging men. The molecular mechanisms behind the BPH initiation/progression are not known. However, BPH is not associated with carcinoma (Abate-Shen & Shen, 2000). Although development of BPH is, in part, dependent

on androgens, to date no associated AR mutations have been identified (http://www.androgendb.mcgill.ca). Whatever the mechanism behind ADT relapse, all genetic anomalies result in an AR that is functional even in the presence of low circulating adrenal androgens. The alterations of AR LBD structure that have been detected in advanced forms of CaP may cause a relaxation of the exquisite specificity of normal androgen recognition, leading to activation of the receptor by steroid hormones that are not necessarily AR specific (Yong, 1998).

11.3 Male breast cancer

Male breast cancer is rare. Only 1% of all malignant tumors in men occur in the breast. Only two point mutations have been reported in the second zinc finger of the AR DBD and in both cases the patients had PAIS (Lobaccaro et al., 1993a). It is likely therefore that AR mutations are not a primary cause of male breast cancer.

11.4 Kennedy's disease

Of the nine known trinucleotide repeat expansions disorders, Kennedy's disease is an inherited neurodegenerative disorder caused by an expanded polyglutamine tract of the AR NTD (Evert et al., 2000). Kennedy's disease is a rare progressive disease that is characterized by proximal weakness, atrophy and fasciculation (Kennedy et al., 1968). Also known as SBMA, its onset usually occurs in adulthood. In normal individuals the polyglutamine tract varies between 11 and 34 CAG repeats whereas SBMA patients have 40–62 repeats (La Spada et al., 1991). Although the molecular steps leading to the neuropathology are unknown, it has been reported that a polyglutamine repeat length above 35 amino acids leads to a gradual decrease in the transcriptional activity of AR, which is presented as MAIS (Mhatre et al., 1993). Polyglutamine expansions result in a SBMA when the tract exceeds about 40 amino acids (Yong et al., 2000). The expanded AR may not have adequate functional interactions with the p160 coactivators (Irvine et al., 2000). At the cellular level, intracellular aggregates are characteristic of SBMA, but they do not necessarily correlate with motoneuronal cell death (Simeoni et al., 2000).

AIMS OF THE STUDY

Disturbances in AR functionality owing to receptor mutations (germline and somatic) and/or altered coregulator interactions appear to be linked to AIS and the pathogenesis of CaP. Advancing our understanding of how AR mutations influence intra-/inter-receptor interactions and the receptor's interactions with other coregulatory proteins would improve our success in treating male-specific syndromes. Furthermore, as the AR NTD is the most hyperviable region of the receptor, discovery of novel NTD interacting proteins, may result in exciting leads for CaP therapeutic drug targets.

The aims of this work were to study the transcriptional activation properties of human AR with the following specific objectives:

- To delineate the molecular consequences of clinically important AR mutations in patients with AIS and CaP with normal androgen binding properties.

- To investigate the occurrence of AR mutations in advanced CaP before the initiation of treatment.

- To investigate the occurrence of AR mutations in advanced CaP during ADT.

- To identify, characterize, and evaluate novel AR NTD interacting proteins.

MATERIALS AND METHODS

More detailed descriptions of the materials and methods used in this study are found in the original publications (I-IV) as indicated in **Table 5** below.

Table 5. Methods used in this study.

Method	**Original publication**
Bacterial two-hybrid screening and interaction assay	IV
Chromatin Immunoprecipition	IV
Coimmunoprecipitation	IV
Electrophoretic mobility shift assay	I, II, III
Fluorescence assisted cell sorting	IV
Fluorescence *in situ* hybridization	II, III
In vitro transcription and translation	I, III, IV
Lentivirus production and titration	IV
Mammalian cell culture	I, II, III, IV
Partial proteolytic digestion assay	I
Plasmid construction and recombinant DNA technology	I, II, III, IV
Production of recombinant protein in mammalian cells	I, II, III, IV
Quantitative Real-Time PCR	IV
RNA interference	IV
SDS-PAGE and immunoblotting	I, II, III, IV
Single strand conformation polymorphism analysis	II, III
Transduction of mammalian cells	IV
Transfection and reporter gene assay	I, II, III, IV
Whole-cell steroid binding assay	I

RESULTS AND DISCUSSION

1 Androgen insensitivity can be caused by AR LBD mutations that disrupt the NTD/LBD interaction (I)

Mutations of the AR LBD can lead to a wide spectrum of AIS and CaP. The mutations that lead to CAIS, PAIS, MAIS or CaP are not necessarily due to large deletions of amino acids from the LBD that abolish androgen binding or to gross changes in LBD structure. Clinical phenotypes, including PAIS, CAIS and HR CaP, can arise from single amino acid mutations within the LBD. These mutations can result in a broadening of the ligand binding properties, dramatically alter the LBD structure, and reduce or increase coregulator interactions. All these effects will either decrease or increase AR transcriptional activity (Quigley et al., 1995, Brinkmann, 2001; Gelmann, 2002; Gottlieb et al., 2004a).

In this study we investigated a selection of 7 single point AR LBD mutants (see **Table 6)** from the AR mutations database (Gottleib et al., 2004b). The selected AR mutants had been linked to patients with clinically identifible diseases but were found to harbor AR proteins with broadly normal androgen binding properties.

Table 6. AR LBD mutations investigated.

Mutation	Phenotype	Reference
V715M	CaP	Culig et al., 1993
F725L	PAIS	Quigley et al., 1995
R726L	CaP	Elo et al., 1995
M742V	PAIS	Batch et al., 1992
G743V	PAIS/CAIS	Nakao et al., 1993; Lobaccaro et al., 1993
F754L	PAIS/CaP	Weidemann et al., 1996; Takahashi et al., 1995
M886V	MAIS/oligospermia	Ghadessy et al., 1999

All the amino acid substitutions are hydrophobic conserved except R726L, which is a hydrophilic charged to hydrophobic change. The mutations were recreated into both full-

length and LBD- only AR mammalian expression vectors. The expressed mutant receptors were characterized with the following parameters:

- Androgen ([^{3}H]mibolerone (MB)) binding
- Transcriptional activation on minimal and complex reporter genes in the presence T, DHT, MB and methyltrienolone
- Interaction with the p160 coactivator GRIP1
- Receptor conformation analysis using partial trypsin digestion
- DNA binding ability
- NTD/LBD interaction using the fragments in isolation
- Repression of NF-κB

1.1 Conformation of the androgen bound LBD

This study revealed that the LBD of AR has an exquisitely balanced structure. It showed that there are no 'wasted' amino acid residues in the LBD. All residues contribute to the structure of the LBD, which in turn contributes to receptor function. Even subtle single amino acid substitutions can have profound effects on intra- and inter-receptor interactions and on DNA binding. The most dramatic effect on overall LBD structure was observed with LBD mutant M742V. The mutation has been reported to enlarge the LBP (Matias et al., 2001). In the presence of T, the M742 mutant did not demonstrate a trypsin resistant LBD fragment, unlike all the other LBD mutants, including neighboring residue G743V. Interestingly, although this mutant has a severely compromised LBD structure in the presence of T, the mutation was detected in a patient with PAIS rather than CAIS. This suggests that under certain conditions the M742V mutant receptor can display some activity. Remarkably, in the presence of the 'bulkier' androgens DHT and MB the conformation of M742V was rescued and demonstrated the trypsin resistant fragment. M742 is one of the 18 amino acid residues that contact the ligand and forms direct contacts with the sterol scaffold of DHT (Matias et al., 2000; Lill et al., 2005). The structural disruption caused by the M742V mutation clearly demonstrates the importance of different androgens in influencing receptor conformation. Therefore there must be flexibility within the LBP. However one may presume that the flexibility is limited to a number of 'inducible' active conformations. Somatic mutations of

amino acids that directly contact the ligand generally have quite devastating effects on CaP therapy. The amino acid preceding M742, residue W741, has been reported as being a hot-spot for mutation that may be important for androgen escape in CaP patients undergoing ADT by castration and androgen antagonist (Hara et al., 2003). Antagonists work by creating LBD conformations that prevent the induction of active receptor conformation. This can be done either by preventing DNA binding or by inhibiting NTD/LBD interactions and coregulator recruitment (Karvonen et al., 2002; Kang et al., 2002, 2004; Farla et al., 2005). The AR W741C and W741L mutations result in an AR that could be activated by the antiandrogen, bicalutamide (Hara et al., 2003). This suggests that the current treatment for primary CaP, ADT along with antiandrogens, may select for somatic receptor mutations that alter ligand specificity. Altering ligand specificity leads to androgen escape because the W741L mutation evokes an agonist bound conformation when bound with biculamide (Bohl et al., 2005). Therefore in the presence of biculamide the AR is activated, when it should be repressed. The broadening of ligand specificity is also seen with the AR T877A mutation (Veldscholte et al., 1990), which results in an AR that can be activated with progesterone. The structural abnormality caused by the T877A mutation is due to the space created by the alanine (A) residue that enable progesterone and other ligands to fit into the LBP, although in the presence of DHT the wild-type (WT) and T877A receptors take the same global structure (Sack et al., 2001).

1.2 LBD conformational changes and DNA binding

The single LBD point mutations examined occur throughout the LBD and are found in the helices and in the connecting loop regions. The study also reveals the importance of 'micro'-structural changes, caused by the amino acid substitutions. The LBD AF2 preferentially interacts with the NTD via the NTDs FXXLF motif to regulate transcription (He et al., 2001). This occurs via a hydrophobic groove formed by helices 3, 4, 5 and 12. This hydrophobic groove is also capable of binding the LXXLL motifs of the p160 coactivators, albeit with less affinity (Matias et al., 2000; He et al., 2004b). More recently it has also been shown that regions just before helix 3, between helices 5 and 6, and helix 10 are also important for the NTD/LBD interaction (Jääskeläinen et al., 2006). The LBD may have a certain degree of induced fit structural movements. Mutations that do not seriously alter the overall LBD

structure so as to render the receptor totally trypsinable, may still influence the receptors' local conformation. Local structural disruptions could influence the positioning of the LBD's interaction surfaces such as the AF2. The AF2 in WT AR binds with high affinity to the FXXLF interaction motif of the NTD. However slight structural changes could perturb the alignment of the FXXLF motif with the AF2. This would result in a 'faulty' receptor. On the same premise, the interaction surfaces of the LBD available for coregulator interactions, such as the p160 coactivators could also be disrupted by local structural changes. This speculation has been demonstrated, as AR bound with the nonsteriodal antiandrogen bicalutaimde fails to recruit p160 coactivators (Karvonen et al., 2002). In addition, different FXXLF and LXXLL peptides induce distinct conformations of the AR AF2 hydrophobic groove (Hur et al., 2004; He et al., 2004; Estebanez-Perpina et al., 2005).

Our research revealed that there are three classes of LBD mutations, those that severely impair the NTD/LBD interaction, those that moderately impair the NTD/LBD interaction, and those that slightly affected the NTD/LBD interaction. The LBD mutations V715M, R726L and M886V slightly impaired the NTD/LBD interaction when compared with WT AR. They had similar abilities to interact with GRIP1 and similar reporter activation ability. These mutations did have slightly altered properties in that the V715M mutant was slightly more active than the WT receptor in the presence of T and DHT. M886V was a little less active in T, but demonstrated activity levels similar to WT AR in the presence of DHT. R726L was a little less active than the WT receptor, but responded well to GRIP1. V715M and R726L were found to be activated better than WT AR by adrenal androgens and estradiol respectively (Culig et al., 1993; Elo et al., 1995). This may indicate that under ADT by surgical castration without androgen antagonists, the receptors are still active. A recent study showed that E709, a helix 3 amino acid, is critical in regulating the helix 3 and helix 12 interaction. The helix 3 and helix 12 interaction is required for optimal receptor activity. Furthermore this demonstrates the importance of the way in which amino acid substitution can affect the ligand binding properties of the LBD, even with residues that are not directly in contact with the ligand, but function in the stabilization of helix 12 positioning (Georget et al., 2006). The M886V was found in a patient with MAIS. The mutants V715M and M886V are located in helices 3 and 11, whilst R726L is located in a loop region between helices 3 and 4, reconfirming that location of the mutation *per se* is no indicator of how it will affect

receptor function. This is again highlighted by AR mutant F725L that is located in loop region next to F726L.

1.3 LBD mutations that severely impair the NTD/LBD interaction

F725L and M742V mutant ARs both have severely compromised NTD/LBD interactions in the presence of T. Like M742V, AR mutant F725L results in PAIS rather than CAIS. Both mutants have similar responses to DHT, MB, and methyltrienolone-dependent transcriptional activation. The F725L AR mutant has slightly more activity than M742V in the presence of T. It also demonstrates the trypsin resistance fragment, suggesting that the global conformation of the receptor may be intact. One possible mechanism that has been suggested for the loop regions is the positioning of helices. Mutating phenylalanine to leucine may reduce structural rigidity and compromise the positioning of helices 3, 4 and 5. It may also reduce/ compromise the interaction surface of GRIP1. A similar phenomenon was seen with residue N727K (Lim et al., 2000). When comparing mutants F725L, N727K and R726L, it is apparent how sensitive the LBD interaction surfaces are. Mutants F725L, M742V, G743V and F754L also have reduced DNA binding ability. M742, G743 and F754 reside in helix 5. It appears that helix 5 and also the positioning of helix 5 influences the function of the DBD and perhaps even the NTD. It therefore reflects that each domain does not work in isolation and reveals the intrinsic complexity of the working of the receptor. Interestingly, all mutants except M742V were able to repress RelA-induced gene activation to approximately the same extent. This indicates that other parts of the AR structure not involved in DNA binding influence the protein-protein interaction between RelA and AR.

1.4 LBD mutations that moderately impair the NTD/LBD interaction

G743V and F754L both reside in helix 5. The mutations cause the receptors to have approximately 30–50% of the transactivation activity of WT AR with all androgens tested. The interaction of the LBDs with GRIP1 was severely depressed, compared to WT receptor. As these two amino acids are not conserved between the NR3Cs, it suggests that they have an AR specific role, maybe with a role in the alignment of helices 3, 4 and 12. The binding of AR mutants G743V and F754L to DNA is weaker than that of WT receptor. As mentioned

above, residues in helix 5 may also influence the ability/specificity of the DBD to bind DNA. A recent study by Li and coworkers has also demonstrated that in the context of chromatin, the NTD/LBD interaction is important for the DNA binding process of the receptor to the *PSA* enhancer (Li et al., 2006). They showed this by using a mutant AR with the NTD FXXLF motif deleted (ARΔF), which reduced the NTD/LBD interaction. On nonchromtinized ARE constructs, the ARΔF had a reduced transactivation capability, but the receptor was still able to bind the DNA. However, in the presence of chromatin, the ARΔF mutant was not able to bind the same AREs, so the NTD/LBD interaction in a more 'natural' environment may also aid in the selection of the AREs used by the receptor.

1.5 Activation function 2 mutations

In addition to the 7 clinical mutations, we studied the effect of 5 other mutations located exclusively within the core AF2 region of helix 12. The four mutations were based on hypothetical substitutions that have been reported for VDR and were M894D, M894A, A896L and A896V (Slagsvold et al., 2000).

These AF2 mutants demonstrate that amino acid residue M984 is important for both the NTD interaction and the GRIP1 interaction. Mutating the residue to aspartate totally abolished the interaction of the LBD with both the NTD and GRIP1. Mutating the residue to alanine, the receptor retained between 30-50% of its ability to interact with the NTD but still did not interact at all with GRIP1. This phenomenon also highlights that the full-length receptor retained some 80% of its transactivation ability (Slagsvold et al., 2000). Both mutants A896L and A896V had highly reduced NTD/LBD interactions, but again, like M894A, were able to interact with GRIP1. Similar results to these have been achieved with other mutations within the core AF2 and throughout LBD. Examples include mutant I898T that had reduced NTD/LBD interactions, but interacted well with GRIP1. Mutant K720A lost the ability to bind GRIP1, but retained the inter-domain activity (He et al., 1999). Thus, the surfaces of AR LBD for GRIP1 and NTD interaction overlap, but are not identical. Under the outer surface of the AF2 coactivator binding site is an interface that is aligned against the LBP. The interface is thought to provide a communication link between the AF2 and bound androgen in regulating AR transcriptional activity (He et al., 2001). The binding of the FXXLF motif to

the AF2 stabilizes the LBP and reduces the androgen dissociation rate, activating transcription. LXXLL motifs have no stabilizing effect (He et al., 2004b; He et al., 2002). AF2 mutations that disrupt the FXXLF interaction, but do not change hormone-binding affinity, are therefore associated with AIS. This is because they increase the dissociation of the ligand, which reduces the transcriptional activation of the receptor (He et al., 2006). It should be pointed out, however, that defect(s) in coactivator protein(s) can also lead to CAIS. (Adachi et al., 2000; Holterhus et al., 2005).

The data of this work, together with the results obtained by numerous other groups since our study was undertaken (Quigley et al., 2004; Jääskeläinen et al., 2006), support the notion that disrupted NTB/LBD interaction is a potential molecular defect in many AIS patients. Therefore local structural changes in the LBD can influence global interactions, which may not always be predictable from direct modeling of the LBD. Therapeutic drugs that are designed to inhibit or enhance the NTD/LBD interaction may have unpredictable *in vivo* effects.

2 Androgen receptor mutations and prostate cancer (II and III)

Androgens are required for the growth and maintenance of normal prostate. CaP is caused by multiple genetic changes, which lead to uncontrolled cell proliferation and tumor formation. It not clear whether CaPs are caused directly by mutations of the *AR* gene (Linja & Visakorpi, 2004; Dehm & Tindall, 2005). Some predisposing genetic factors of CaP may be inherited and it has been suggested that genetic background can contribute a 25 to 40% increase in risk for developing CaP (Shand & Gelmann, 2006). The molecular changes behind the advancement of CaP are not understood, but AR activity is important throughout all stages of the disease, even upon transition to a HR state (Litvinov et al., 2003). The current therapy for CaP, ADT, was originally developed in the 1940s by Huggins and Hodges, who demonstrated that surgical castration reduced the size of prostate tumors (Huggins & Hodges, 1941). Testes derived androgens account for 90% of the circulating androgens, whilst 10% is produced by the adrenal glands (Shen & Coetzee, 2005). To achieve maximum androgen blockade, androgen antagonists are given to the patient to inhibit the action of the adrenal steroids (Linja & Visakorpi, 2004). ADT initially achieves good

response rates but patients generally relapse and get renewed androgen independent prostate tumor growth within 18-24 months (Edwards & Bartlett, 2005). Extensive evidence indicates that ADT relapse in advanced CaP is caused by *AR* gene amplification in about one third of CaP (Ford et al., 2003; Koivisto et al., 1997, 1996; Linja et al., 2001). It has been suggested that up to 50% of advanced-stage tumors can have somatic gain-of-function AR mutations (Taplin et al., 1999; Buchanan et al., 2001). It is thought that gene amplification and mutation arise to sensitize AR to the low circulating androgens placed on the tumor by the ADT. It has also been suggested that the pressures placed upon the tumor by ADT, selects for tumor cell colonies that are androgen-independent (Linja & Visakorpi, 2004; Dehm & Tindall, 2005). In these two studies we investigated the occurrence of somatic AR mutations in patients with advanced CaP before (II) and during (III) ADT by surgical castration, estrogen therapy or surgical castration with the cytotoxic drug estramustine phosphate (EMP) that also has androgen antagonist properties (Wang et al., 1998). The AR mutations functionally characterized in these two studies are presented in Table 7.

Table 7. AR mutants functionally characterized in studies II and III.

Mutation	Region	Transcriptional activity of mutant receptor compared to WT AR	Original publication
P514S	NTD/TAU5	Normal	III
G524A	NTD/TAU5	Normal	III
G524S	NTD/TAU5	Normal	III
P533S	NTD	Reduced	III
S646F	Hinge/LBD	Elevated	II
E653K	Hinge/LBD	Normal	II

2.1 Mutations of *AR* in advanced CaP before hormone therapy

In this study, 21 untreated, histologically poorly differentiated CaPs were investigated for AR mutations before the patients were surgically castrated. Fourteen samples were from primary tumors and 7 samples were from metastases. AR mutations were detected in 5 of the 14 primary tumors (36%). One mutation, (which was not detected in the corresponding primary

tumor) was detected in the 7 metastases (14%) samples. Of the 6 mutations detected, one was silent and all mutations were somatic. Age, Tumor, Node, Metastasis stage or histological differentiation did not differ between mutation positive and negative cases. All *AR* mutations identified were novel in CaP and were located in exon 1 and exon 4. We recreated the hinge region AR mutants S646F and E653K into AR expression vectors and characterized the mutants on their ability to transcactivate the complex natural probasin- (PB) or minimal TATA promoter-driven reporters. In addition, the mutant ARs were assessed for DNA-binding ability and protein stability. The E654D mutation was not studied, since it represented a conservative change that is likely to result in only minor structural alterations in AR. The S646F AR mutant displayed ~2-fold increase in transcriptional activity, compared to wild-type AR, on both the single ARE-containing PB reporter and on TATA-ARE_1 reporter constructs, at all concentrations of T and DHT tested. The S646F mutant was also slightly (~50%) more active than WT AR on the TATA-ARE_2 reporter. The activity of E653K AR mutant did not differ from that of WT AR. Neither mutant showed markedly altered activity on the double ARE-containing PB reporter. The mutations did not influence DNA binding or protein stability. The increased transcriptional activity of mutant S646F may be due to altered protein-protein interactions, possibly involving a coactivator. The ADT by surgical castration of the patient harboring the S646F mutation relapsed after six months. This suggests that the AR S646F mutation provided the tumor with a growth advantage in the presence of ADT levels of androgens. We can conclude that AR mutations are common in untreated, poorly differentiated CaPs. The S646F substitution found in the AR hinge region from a patient with a poor hormonal therapy response enhanced the activity of the receptor, and this may have contributed to the progression of the disease.

2.2 Mutations of *AR* in advanced CaP during hormone therapy

In this study 21 HR CaP from patients undergoing ADT by surgical castration, estrogen therapy or a combined therapy of surgical castration and EMP were analyzed for gene amplifications and mutations. *AR* gene amplifications were found in 4 of the 16 (25%) samples that were successfully analyzed by fluorescence *in situ* hybridization. Two of the 4 tumors with *AR* gene amplifications also harbored missense mutations. In total 7 somatic missense amino acid substitutions were found from the 21 (33%) tumors. Interestingly, 3 of

the 10 (30%) tumors from patients undergoing ADT by castration only had missense mutations localized in the LBD. However, 4 of the 5 (80%) tumors from patients undergoing ADT by castration and EMP had missense mutations. These 4 mutations were found in a region of the NTD that spans TAU5. Of the 6 patients undergoing estrogen treatment, 2 of the tumors harbored silent mutations and 1 of these two tumors also had *AR* gene amplification. No mutations were found in the DBD and only one constriction of the CAG repeat was observed. The two somatic LBD mutations detected were V866M and V757I. There are 13 reports on the AR mutations database of the V866M mutation. The V866M mutation is generally associated with reduced androgen binding and AIS (http://www.androgendb.mcgill.ca). V757I has never been reported, but an AR mutation of V757A has been reported (Marcelli et al., 2000). The specific functional studies are missing, but since codon 757 locates in the highly conserved helix 5 region of the LBD we predict that the ligand binding or DNA binding of the V757I mutant is altered when compared to WT AR.

We characterized functionally the 4 missense mutations from the patients undergoing ADT by castration and EMP. We recreated AR mutants P514S, G524A, G524S and P533S in AR expression vectors and their activity in the presence of T, DHT, androstenedione or estradiol on the full-length PB reporter containing two AREs was characterized. In PC-3 human CaP cells (AR negative) the transactivating ability of the mutants did not significantly differ from that of WT AR with any of the hormones tested. The mutants span TAU5, a region known to be involved in the recruitment p160 coactivators. We tested the mutant's response to GRIP1 and protein inhibitor of activated Stat1 an E3 ligase that promotes AR activity (Kotaja et al., 2000). The mutant receptors all responded to GRIP1 as WT receptor and displayed a 2- to 3-fold relative increase in transcriptional activity in the presence of either T or DHT in PC-3 cells. Similar results were also achieved with protein inhibitor of activated Stat1. The activities of the mutant receptors were also tested in COS-1 cells. The activity of the mutants was slightly lower than WT AR at 10 nM T. However, their activities were comparable to WT at all other T concentrations tested. Mutant P533S displayed a 20-30% loss in activity at 100 nM T, which was also seen with DHT. Similar results were seen with a minimal TATA-ARE_2 reporter in a COS-1 cell. The decrease of P533S activity was not due to reduced protein levels. All mutants were expressed at similar levels to WT receptor. Our results

showed that none of the mutations that arose caused broadening of ligand specificity or hypersensitivity to androgen. Under certain cellular conditions we even observed a decrease in mutant receptor activity.

It is not clear what mechanisms lead to ADT relapse. Our results indicate that mutations of the AR are frequent in both untreated and treated advanced CaP. Furthermore our results demonstrate a therapy-mediated mutation clustering. Our findings are in agreement with other studies that have demonstrated that castration plus antiandrogen selects for NTD mutations (Taplin et al., 1995, 1999; Scher et al., 2004). Surprisingly, the NTD mutations detected in our study did not increase receptor activity, even though they arose under maximum androgen blockade conditions. Other studies have suggested, however, that even under castration levels of androgens, intratumoral androgen concentrations may be sufficient to maintain tumor growth (Mohler et al., 2004). It has been shown that HR tumor cells increase the expression of enzymes involved in the steroid synthesis pathway (Holzbeierlein et al., 2004). Additionally, CaP tumors may be able to accumulate androgens by local synthesis of sex hormone binding globulin (Hryb et al., 2002). Therefore prostate tumors may never be completely free of androgens (Mohler et al., 2004). To reduce the detrimental effects of ADT mutation selection, future therapies may employ a rapid hormone cycling strategy (Scher et al., 2004). This strategy cycles between depleting androgens to cause a regression in tumor size and then replenishing androgens to prevent selection pressure and delaying the onset of HR CaP.

In addition to AR mutations, *AR* gene amplification has also been reported to be an adaptive mechanism leading to HR CaP. Previously, it has been reported that *AR* amplification and AR overexpression occurs in 20-30% of the CaPs relapsing during ADT (Culig et al., 2005; Chen et al., 2004; Edwards et al., 2003). In the present study, the prevalence of *AR* gene amplification was 25%. *AR* gene amplifications were equally distributed among the therapy groups. This equal distribution suggests that castration, estrogen therapy, or castration plus EMP cause a sufficient reduction in intra-prostatic androgen levels such that only cell clones with selective growth advantages, such as elevated AR copy number, are selected. Missense mutations were detected in 75% of the tumors with AR amplification, suggesting that AR mutations and amplification in the same tumor provide a synergistic growth advantage.

Others have shown that there is no difference in the time taken to relapse between patients who have *AR* amplifications and those without amplifications (Edwards et al., 2003; Edwards & Bartlett, 2005). Furthermore *AR* gene amplification is not the only mechanism to increase AR mRNA and protein expression. Increased AR protein expression may be as a result of dysregulated *AR* gene expression without amplification (Edwards et al., 2003). However gene amplification in untreated advanced CaP is as low as 1-2% (Edwards et al., 2003; Bubendorf et al, 1999). If AR mutations and *AR* gene amplication mechanisms were exclusive, independent events, 45-85% of ADT relapse could be explained (Dehm & Tindall, 2005). Therefore other mechanisms of ADT relapse are not all directed towards the *AR* gene. This suggests that growth factor signaling pathways and coregulatory proteins play a part in ADT relapse as well (Linja et al., 2004; Dehm & Tindall, 2005; Javidan et al., 2005; Shand & Gelmann, 2006).

Taken together, these studies clearly demonstrate that molecular aberrations of the *AR* gene are likely to underlie the ADT relapse of advanced CaP even before hormone therapy is started. Importantly, the selective pressure caused by various types of ADT may determine the nature of the somatic mutations that arise in AR. These findings therefore impact on the development of new hormonal therapy schemes aimed at reducing or delaying the onset of HR CaP.

3 Identification and characterization of small carboxyl-terminal phosphatase 2 as an androgen receptor coregulator (IV)

3.1 Bacterial two-hybrid screen recovered small carboxyl-terminal phosphatase 2 as an AR NTD interaction partner

Coregulators have a significant effect on AR function. Disturbances of AR functionality caused by up- or down-regulation of coregulator expression and receptor interaction have been linked to the advancement of CaP from clinically non-significant, low-grade CaP to metastatic, HR CaP (Gregory et al., 2001; Linja et al., 2004; Arnold & Isaacs, 2002; Abate-Shen & Shen, 2000; Rahman et al., 2004). Most of the currently known AR coregulators generally also regulate other NR3Cs as well (Xu & Li, 2003). Many of the AR coregulators

were identified using the AR DBD and LBD as bait in a yeast two-hybrid system (Jänne et al., 2000). Due to the sequence and structural conservation of these domains between the NRs, it is not surprising that most coregulators identified could be described as 'generic'. Identification of truly specific AR coregulators may lead to the discovery of potential therapeutic targets for syndromes concerning androgen action. The NTD of NRs is the most variable region between the receptors. Therefore coregulators identified using the AR NTD may not interact with the other NRs. The AR NTD harbors the hormone-independent AF1, which makes it unsuitable bait in the yeast system. Yeast is a eukaryotic organism and therefore the AR AF1 could recruit the yeast homologues of mammalian TF. The homologous yeast proteins/AF1 interaction would promote false positives. Bacteria are prokaryotes and, as such, do not have homologues of eukaryotic proteins. Therefore using a bacterial two-hybrid system we screened a human HeLa cell cDNA library with the AR NTD as bait. One of the potential AR coregulators recovered was small carboxyl-terminal domain, phosphatase 2 (SCP2) (Su et al., 1997).

3.2 Characteristics of SCP2

SCP2 mRNA is expressed in most tissues, including the prostate and encodes a protein of 283 amino acids. SCP2 is a 32-kDa protein composed of a novel protein phosphatase domain and short non-conserved amino- and carboxyl-termini. The novel phosphatase domain of SCP2 shares sequence homology with three other proteins, SCP1, SCP3/HYA22 and TFIIF-associating CTD phosphatase (FCP1) (Yeo et al, 2003; Kashuba et al., 2004; Lin et al., 2002). The family members are not splice variants as they are encoded by distinct genes. SCP1, SCP2 and SCP3 are present in all higher eukaryotes, but are not essential for cell survival. FCP1 is conserved from yeast to humans and is essential for cellular survival (Kobor et al., 1999). The SCP family members are nuclear proteins of a similar size. FCP1 is larger than the SCPs and has an additional breast cancer, protein-related carboxyl-terminal domain that is required for FCP1's interaction with the Pol II CTD (**Fig. 11**) (Yeo et al., 2003; Kamenski et al., 2004; Archambault et al, 1997). A putative substrate of SCP2 and the other family members is phosphorylated serine^{5} and/or phosphorylated serine^{2} of the Pol II CTD heptapeptide repeat (Su et al, 1997; Yeo et al, 2003). The phosphatase activity of the SCP family members is stimulated by TFIIF (Archambault et al., 1997; Yeo et al., 2003).

TFIIF is recruited by the AR NTD to the PIC, suggesting that SCP2 may play a role in AR mediated transcriptional regulation (McEwan & Gustafsson, 1997; Reid et al., 2002).

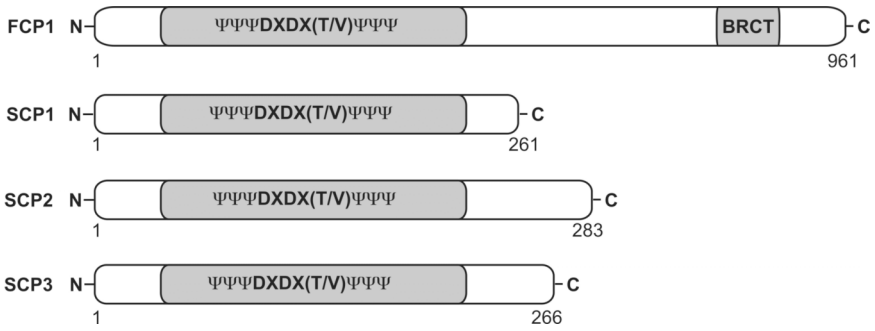

Fig. 11. Domain structures of FCP1 and SCP family proteins (adapted from Yeo et al., 2003). Shaded regions are functional domains. Numbers depict number of amino acids present in each protein. The shaded regions with the ΨΨΨDXDX(T/V)ΨΨΨ motifs, where Ψ represents a hydrophobic amino acid and X represents any amino acid, is the conserved Class C phosphatase domain. Also shown is the breast cancer protein-related carboxyl-terminal domain of FCP1.

3.3 SCP2 and AR interact *in vitro* and *in vivo*

We demonstrated that the bacterial two-hybrid interaction between SCP2 and AR also occurred in mammalian cells and that the interaction was hormone-independent. To examine whether the interaction of SCP2 with AR was direct, we performed glutathione S-transferase pull-down and coimmunoprecipitation assays. These results also confirmed that SCP2 could possibly function as a coregulator of AR function.

Next we coexpressed AR with increasing amounts of SCP2 and observed a marked reduction of AR-dependent transcription in a dose-dependent fashion from several androgen-regulated promoters. SCP2 also repressed ERα, GR and PR dependent-transcription. SCP2 is therefore not an AR specific corepressor and may regulate a transcriptional mechanism common to all SRs. In the development of CaP from a hormone-dependent to a HR state some coregulators are expressed at significantly lower amounts (Linja et al., 2004). Using lentiviral transduction of short hairpin RNAs against SCP2, we reduced the amount of endogenous SCP2 in LNCaP cells. LNCaP cells are a CaP cell line that expresses both AR and SCP2 proteins. The significant reduction in the amount of endogenous SCP2 protein was accompanied by a 50-

80% increase in androgen-dependent transcription of the *PSA* gene. The elevated PSA mRNA levels suggested that in the absence of SCP2, AR is more transcriptionally active. The loading of AR onto the *PSA* promoter and enhancer regions occurs in a cyclic manner (Kang et al., 2004). We performed chromatin immunoprecipitation assays and demonstrated that at times of maximal AR loading onto the *PSA* promoter and enhancer regions, there was 3–4 times more AR loaded under SCP2 knockdown conditions than in normal cells. Using the same short hairpin RNA constructs, endogenous SCP2 was reduced in LNCaP 1F5 cells. LNCaP 1F5 cells express AR and GR (Cleutjens et al., 1997). In the presence of glucocorticoids, GR can also bind to the AREs of the *PSA* gene and activate transcription (Cleutjens et al., 1997b). Once again, under SCP2 knockdown conditions we observed an increase in PSA mRNA levels and increased loading of GR onto the *PSA* gene.

The mRNA levels of the hormone-independent genes TBP and Sp1 were not affected by the knockdown of SCP2. Therefore SCP2 regulates a common transcriptional mechanism shared by the SRs. Similar results were seen with SCP1, suggesting the functions of SCP2 and SCP1 overlap.

3.4 Recruitment of Pol II to the *PSA* promoter

The loading of AR onto the *PSA* gene results in the recruitment of Pol II to the promoter (Shang et al., 2002). Pol II is recruited to the *PSA* promoter in a cyclic fashion, similar to that of AR. Our data revealed that at the peaks of Pol II recruitment, twice as much Pol II was present at the *PSA* promoter under SCP2 knockdown conditions than in normal cells. Furthermore, Pol II recruitment cycled more rapidly, suggesting that Pol II promoter clearance was occurring faster. We investigated the intragenic *PSA* intron1/exon 2 and intron 4/exon5 regions for the presence of Pol II. If more Pol II was present in these regions under the knockdown conditions, then Pol II leaves the promoter region faster than in normal cells. We observed significantly more Pol II within the *PSA* intragenic regions of the knockdown cells. This result suggested that at steady-state more Pol II is leaving the promoter, which accounts for the increases in PSA mRNA.

3.5 Hyperphosphorylation of Pol II CTD serine^5

In order to clear the promoter region of genes and enter the elongation phase of transcription, the CTD of Pol II has to be hyperphosphorylated on serine^5. The putative substrate for SCP2 phosphatase activity is phosphorylated serine^5 and/or phosphorylated serine^2 of the Pol II CTD heptapeptide repeat. Therefore under SCP2 knockdown conditions, there should be more serine^5 phosphorylated Pol II CTD at the promoter. There was no difference in the maximum levels of phosphorylated serine^5 between normal and knockdown LNCaP cells. However, under the knockdown conditions the cyclicity of serine^5 phosphorylation was approximately 2–3 times faster than in normal cells. The increase in phosphorylated serine^5 cyclicity indicated that hyperphosphorylation of serine^5 and Pol II promoter clearance was achieved faster in the absence of SCP2. We also demonstrated that in mammalian systems there is very little, if any serine^2 phosphorylation at the promoter region. In addition, the knockdown of SCP2 did not increase serine^2 phosphorylation, which occurred within the intragenic regions of the *PSA* gene. This suggests that the physiological substrate for SCP2 is promoter loaded serine^5 phosphorylated Pol II CTD.

3.6 Implications of SCP2 on steroid receptor-mediated transcription

When a patient presents a primary CaP, the illness is usually treated with surgical or chemical ADT. As mentioned above (II, III) the selective pressure of ADT promotes the growth of CaP cell colonies that have growth advantages in the presence of low androgens. It is possible that somewhere between 30-50% of HR tumors harbor a mutation of the *AR* or *AR* gene amplification that sensitize the cell to the decreased levels of androgens. *AR* mutation and gene amplification do not account for all the cases of ADT relapse. Recently, it has been shown that AR coregulators can also play a role in androgen escape (Gregory et 2001; Linja et al., 2004). We present data that suggests down-regulation of SCP2 expression could sensitize a CaP tumor to lower androgen concentrations. Down-regulating SCP2 would have two effects, it would sensitize cells to lower concentrations of androgens and it would also increase the elongation speed of Pol II.

The product of actively transcribing Pol II activity is heterogeneous nuclear RNA (hnRNA) that contains the intronic sequences of the gene. The intronic sequences of hnRNA must be efficiently and accurately spliced out and the remaining exons joined to produce mRNA. The resulting mRNA can then be translated into protein. Intron removal occurs cotranslationary on the nascent hnRNA transcript. The Pol II CTD acts as a platform for maturation of mRNA (Auboeuf et al., 2005; Kornblihtt, 2005). Alternative splicing affects about 60% of all human genes. In part, the elongation rate of Pol II transcript synthesis affects the intron splicing of an hnRNA species. Low elongation rates favor the inclusion of alternative exons, elevated rates favor exclusion of exons (Kornblihtt, 2005, 2006). Both abnormally high and low Pol II elongation rates can cause splicing errors (Kornblihtt, 2006; de la Mata et al., 2003). Mutations that add or remove single nucleotides to create or abolish splicing sites have been linked to a wide variety of diseases. The BRCA1 tumor suppressor gene that is involved in hereditary breast and/or ovarian cancer has a point mutation that disrupts splicing and results in a functionless protein (Liu et al., 2001; Yang et al., 2003). It has been speculated that splice variants of proteins found in cancer can be produced by the dysregulation of cotranslatory hnRNA splicing (Schozova et al., 2006). Increasing the overall steady state Pol II activity could therefore possibly be detrimental to cells. Increased Pol II activity could lead to aberrant mRNA processing and hence cause disruptions in cell function, which promote tumor development. Under SCP1 and SCP2 knockdown conditions Pol II activity is considerably elevated and therefore could possibly be linked to the progression of cancer. Our LNCaP data do not directly link SCP2 to CaP development and further examination of patient data is required. It is quite possible that SCP2 and SCP1 could be involved in the progression of other hormone-dependent cancers. The knockdown of SCP2 or SCP1 did not impair the LNCaP cells to grow in culture. However, further investigations are required to reveal whether the reduction of SCP2 or the other SCPs would give a growth advantage to cells *in vivo*. Reductions in SCP expression may be a molecular mechanism behind CaP and other cancers. Therefore preventing SCP2 dow-regulation or stimulating SCP2 activity in HR CaP patients may be a step towards controlling androgen escape.

CONCLUSIONS

AR consists of three major functional domains, the NTD, DBD and LBD. This work evaluated possible disruptions in androgen-dependent transcriptional regulation caused by aberrant intra- and inter-NTD and LBD interactions.

- Single point amino acid LBD mutations can lead to subtle differences in receptor conformation. These differences can have quite dramatic effects upon the receptor's NTD/LBD interaction. The NTD/LBD interaction is required for optimal receptor transcriptional regulation. Mutations can be receptor activating which lead to CaP, or receptor deactivating that can lead to various degrees of AIS. Additionally, the subtle structural differences caused by LBD mutations can also influence the DNA binding and coregulator interaction properties of the receptor.

- The molecular events that lead to the initiation of CaP are not clear, however androgen signaling is required throughout the entire progression of the disease. ADT relapse may be caused by spontaneous mutations of the AR that give tumors a growth advantage in low androgens. Furthermore, the types of ADT patients receive may provide a selective pressure for somatic mutations to arise and cluster to specific functional domains of the receptor.

- AR coregulator interactions are essential for controlled androgen-dependent gene expression. AR-dependent transcription is in part regulated by the novel protein phosphatase SCP2. SCP2 activity *in vivo* regulates the loading of AR onto the *PSA* promoter and enhancer regions and also the rate at which Pol II clears the promoter. Reduced expression of SCP2 results in more AR loading onto the *PSA* promoter and enhancer regions that, in turn, increases the rate at which Pol II clears the promoter.

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