PEPTIDES AND PROTEINS INTERACTING WITH THE ANDROGEN RECEPTOR

Dennis van de Wijngaart

Cover: 3-Dimensional representation of the androgen receptor ligand-binding domain (blue) in complex with a peptide containing the FxxLF motif as present in the androgen receptor N-terminal domain (yellow).

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Peptiden en eiwitten die interacteren met de androgeenreceptor

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CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
AF	activation function
AIS	androgen insensitivity syndrome
AR	androgen receptor
ARA	androgen receptor associated protein
ARE	androgen response element
BAF	BRG1-associated factor
BF-3	binding function-3
BRG1	brahma-related gene 1
BRM	brahma
CAIS	complete androgen insensitivity syndrome
CFP	cyan fluorescent protein
ChIP	chromatin immunoprecipitation
CPA	cyproterone acetate
CTE	carboxy-terminal extension
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
ER	estrogen receptor
ERR	estrogen-related receptor
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GR	glucocorticoid receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase
Hsp	heat-shock protein
LBD	ligand-binding domain
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
kDa	kiloDalton
MAIS	mild androgen insensitivity syndrome
MED	mediator
MMTV	mouse mammary tumour virus
MR	mineralocorticoid receptor
mRNA	messenger RNA
N/C interaction	interaction between NTD and LBD

NES	nuclear export signal
NLS	nuclear localization signal
NR	nuclear receptor
NTD	N-terminal domain
OH-F	hydroxyflutamide
PAIS	partial androgen insensitivity syndrome
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PSA	prostate-specific antigen
RAR	retinoic acid receptor
RNA	ribonucleic acid
RXR	retinoic X receptor
SBMA	spinal bulbar muscular atrophy
SRC	steroid receptor coactivator
TAU	transcription activation unit
TR	thyroid hormone receptor
TR-FRET	time-resolved fluorescence resonance energy transfer
VDR	vitamin D receptor
YFP	yellow fluorescent protein

CHAPTER 1

General introduction

1. GENERAL INTRODUCTION

Androgens are important sex steroid hormones. The androgens testosterone and dihydrotestosterone (DHT) are essential for normal male sexual differentiation and for the development and maintenance of male reproductive tissues, including the prostate. Androgens mediate their effects by binding to, and activation of, the androgen receptor (AR), which is a transcription factor belonging to the nuclear receptor (NR) family. Upon androgen binding, the AR is able to recognize specific DNA sequences from where it regulates the expression of its target genes. A disregulated androgen-AR pathway is involved in several diseases, such as prostate cancer, androgen insensitivity syndrome (AIS), and Kennedy's disease or spinal and bulbar muscular atrophy (SBMA).

1.1 THE NUCLEAR RECEPTOR FAMILY

NRs are members of a family of transcriptional regulators involved in many diverse cellular processes, including growth, differentiation, apoptosis, and metabolism (1-3). So far, forty-eight receptors have been identified in human, of which 24 require a ligand to be activated (3). NR ligands, such as steroids, retinoids, and fatty acids, are usually small hydrophobic molecules allowing easy crossing of the cell membrane. The other receptors are so-called orphan receptors. For these receptors, regulatory ligands have not yet been identified or they may function without a ligand.

NRs have a modular structure composed of an amino-terminal transcription activation domain (NTD), a central DNA-binding domain (DBD), and a carboxyl-terminal ligand-binding domain (LBD), which is connected to the DBD via flexible hinge region (as shown in Figure 1A for AR) (1). Phylogenetic tree reconstruction based on alignment of DBD- and LBD sequences classified the human NR family into six groups (4, 5). Group I contains the receptors for thyroid hormone (TRs), retinoic acids (RARs), and vitamin D (VDR). It also includes the peroxisome proliferator-activated receptors (PPARs) and some orphan receptors such as Rev-erb, RORs, and LXR. Members of Group II are the retinoic X receptors (RXRs) and the orphans COUP-TF, HNF4, TR2, and TR4. The steroid receptors, which include the AR, the progesterone receptor (PR), the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), and the estrogen receptors (ERs), as well as the closely related orphan estrogen-related receptors (ERRs) form Group III. Groups IV, V, and VI comprise various orphan receptors, such as NURR1 and steroidogenic factor 1 (SF1). A separate group, Group 0, consists of DAX1 and SHP, which lack a DNA-binding domain.

1.2 THE ANDROGEN RECEPTOR

The AR is expressed in a variety of tissues, with highest levels present in the male urogenital tract. Lower AR levels are found in many other tissues, including bone, muscle, hair follicles, liver, and brain (6, 7). The AR is encoded by a single copy gene located at chromosome band

Xq11.2-12 and consists of 8 exons (8, 9). Exon 1 encodes the AR NTD, exons 2 and 3 each encodes a zinc finger of the DBD, whereas exons 4 to 8 encode the hinge region (proximal part of exon 4) and the LBD. The size of the AR protein is 919 amino acid residues, but may vary between individuals because of variations in the lengths of poly-glutamine and poly-glycine stretches in the NTD. Based on a length of 919 amino acid residues, the AR NTD encompasses the first 557 residues. The DBD is formed by residues 558-623, the hinge region by residues 624-670, and the LBD by residues 671-919.

1.2.1 THE AMINO-TERMINAL DOMAIN

The NTD harbours the main transcription activation function in AR, termed activation function-1 (AF-1). So far, no crystallographic data are available for any of the NR NTDs, which is probably due to the high flexibility of this domain in solution (10, 11). Although the AR NTD structure is mainly disordered, several regions appear to be in an intermediate folded state (12, 13). This so-called molten globule conformation enables multiple protein-protein interactions without the need for high affinities and increases the contact surface for individual interactions (12).

Deletion mapping identified two different subdomains within AF-1, the so-called transcription activation units (TAUs; Figure 1A). TAU-1 (residues 100-370) is active in full-length ligand-activated AR, whereas there is a shift to TAU-5 (residues 360-485) in the absence of the AR LBD (14). The contribution of TAU-5 to transcriptional activity of full-length AR is dependent on the cellular- and promoter context, whereas TAU-1 is indispensable (14, 15). TAU-5 also serves as important protein interaction surface for p160 cofactors, which are the best described NR coregulators (15-17). Binding of p160 cofactors to TAU-5 is modulated by TAU-1, suggesting interplay between the two TAUs. Because there is no direct interaction between TAU-1 and TAU-5, it is thought that the effects of TAU-1 are indirect via induction of conformational changes or via recruitment of a secondary interaction partner (15, 18).

The NTD contains two stretches: a poly-glutamine tract, varying between 14-32 residues, and a poly-glycine tract, varying between 10-30 residues. AR transcriptional activity appears to be inversely correlated with the length of the glutamine stretch. A relatively short glutamine stretch has been weakly associated with an increased risk of developing prostate cancer. A strong correlation has been found between an expanded glutamine stretch and Kennedy's disease/SBMA (19-22). Whether abnormal lengths in the glycine tract also play a role in disease development is less clear. However, some studies indicate that a shorter stretch may be associated with the development of prostate cancer (21, 23).

The very N-terminus of the AR NTD contains a highly conserved FxxLF motif (residues 23-27), which resembles the LxxLL protein-protein interaction motif (24). The AR FxxLF motif was found to mediate the hormone-dependent interaction between AR NTD and AR LBD (25, 26). This interaction, which is also known as AR N/C interaction, will be discussed in more detail in section 1.3.3.

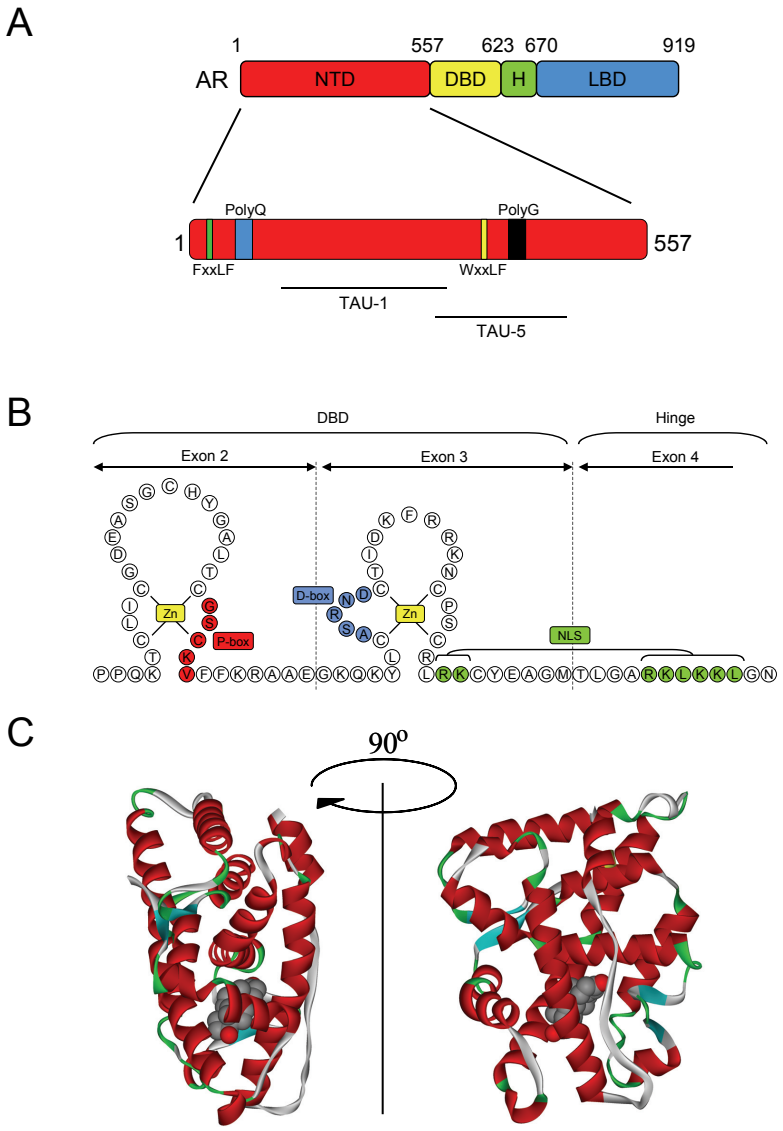

Figure 1. Functional domains of the AR. (A) Schematic representation of the AR functional domains with a focus on the subdomains present in the AR NTD. NTD, amino-terminal domain; DBD, DNA-binding domain; H, hinge region; LBD, ligand-binding domain; FxxLF, FxxLF motif; PolyQ, glutamine repeat; PolyG, glycine repeat; TAU, transcription activation unit; WxxLF, WxxLF motif. (B) The two zinc-finger coordinated modules of the AR DNA-binding domain. The single letter code for amino acids is used. Residues that are part of the P-box (red), the D-box (blue), or the nuclear localization signal (NLS; green) are highlighted. The fragments that are encoded by exon 2, exon 3 and part of exon 4 are indicated. Adapted from ref. 18. (C) Representation of the AR LBD crystal structure in complex with DHT (grey). The structure on the right is related to the structure on the left by a clockwise 90° rotation about the vertical axis.

1.2.2 THE DNA-BINDING DOMAIN

The DBD consists of three α-helices that are organized in two zinc finger modules and a relatively unstructured C-terminal extension (CTE; Figure 1B) (27-29). The α-helix in the first zinc finger contains the so-called proximal (P)-box sequence. This stretch of 5 amino acid residues (577-GSCKV-581) interacts directly with the major groove of the DNA and confers sequence recognition. Additional contacts with the DNA are made by the CTE. The second zinc finger, formed by the second and the third α-helix, contains the distal (D)-box (596-ASRND-600), which is involved in AR dimerization (18, 30). The DBD is further involved in nucleo-cytoplasmic shuttling, as it contains the first part of a bipartite nuclear localization signal (NLS; the second part is located in the hinge region) and a non-classical nuclear export signal (NES) (31, 32). A weaker NLS and an alternative NES have been identified in the LBD (33, 34).

1.2.3 THE HINGE REGION

The hinge region functions as a flexible linker to separate the DBD from the LBD. More recently, it has been demonstrated that the hinge region may play important roles in AR physiology as well. It regulates nucleo-cytoplasmic shuttling of the AR, stabilizes AR binding to DNA, and modulates AR N/C interaction and AR transcriptional activity (35, 36).

1.2.4 THE LIGAND-BINDING DOMAIN

The crystal structures of the AR LBD in complex with the natural androgens testosterone and DHT, and with the synthetic androgen R1881 (methyltrienolone or metribolone) have been solved (37-39). As can be deduced from these structures, the AR LBD is composed of 10-12 α-helices and 4 short β-strands (Figure 1C). The helices are arranged as a three-layered sandwich surrounding the central ligand-binding pocket. Although the overall structure of the AR LBD is similar to other agonist-bound NR LBDs, it lacks helix 2 (40). The other helices are numbered 1 to 12 and correspond with the general 12-helical numbering of other NR LBDs, which allows easy comparison.

Crystallographic studies demonstrate that NR activation involves major structural rearrangements in the LBD (reviewed in (41, 42)). Upon ligand binding, helix 12 is repositioned over the ligand-binding pocket to stabilize the bound ligand. This novel orientation also completes the formation of a hydrophobic groove on the surface of the LBD, also referred to as activation function-2 (AF-2). In most NRs this groove serves as a platform for high affinity interactions with LxxLL sequences present in several cofactors, including the members of the p160 family SRC1, TIF2, and SRC3 (24, 43, 44). Despite high sequence homology with LBDs of other NRs and a similar fold, the AR LBD has a relatively low affinity for these LxxLL motifs (45, 46). Instead, it prefers the binding of related FxxLF motifs mediating the recruitment of AR cofactors ARA54, ARA70, and Rad9 (46-48). High affinity binding was also observed to the FxxLF motif present in the AR NTD, which is essential for AR N/C interaction (see 1.3.3) (25, 26).

AR activation by agonistic ligands is counteracted by antagonists, such as hydroxyflutamide and bicalutamide (casodex). Antagonists compete with agonists for binding to the same ligand-binding pocket. So far, no crystal structures have been resolved of wild type AR LBD in complex with an antagonist. However, partial protease digestion assays demonstrated that antagonists induce a different AR LBD conformation than agonists (49, 50). In this alternative, inactive, conformation the AR is unable to recruit LxxLL and FxxLF peptides, and is unable to initiate transcription (51, 52).

Recently, a novel interaction site on the AR LBD surface, termed binding function (BF)-3, has been identified (53). Structural analysis demonstrated that BF-3 lies adjacent to the AR coactivator groove. It has been proposed that BF-3 functions as an allosteric regulatory surface that modulates cofactor binding to the coactivator groove.

1.3 AR TRANSCRIPTIONAL ACTIVATION

In its unliganded state, the AR is mainly located in the cytoplasm, where it is prevented from being active by interactions with heat shock protein complexes (Figure 2). Upon androgen binding, the AR undergoes conformational changes resulting in dissociation from these complexes. The AR then translocates to the nucleus, forms a homodimer, and subsequently binds to specific DNA sequences, or androgen response elements (AREs), which are located in enhancer and promoter regions of AR target genes. Cofactors, general transcription factors, and RNA polymerase II are recruited in order to allow expression of the target gene (reviewed in (54-56)). In the next paragraphs, the AR transcription activation process, starting with the synthesis and release of testosterone by the testis, will be discussed in more detail.

1.3.1 TESTOSTERONE SYNTHESIS AND CONVERSION TO DHT

The main circulating androgen, testosterone, is primarily synthesized from cholesterol by the Leydig cells in the testis and represents approximately 90% of the total circulating pool of androgens (57). Testosterone production is stimulated by luteinizing hormone (LH) from the pituitary, of which the release is regulated by LH-releasing hormone (LHRH) from the hypothalamus (Figure 2). Testosterone exerts a feedback mechanism. Other androgens, such as dehydroepiandrosterone (DHEA) and androstenedione, are mainly produced by the adrenal cortex and circulate at much lower concentrations (58). These adrenal androgens are converted to testosterone in target tissues.

Upon secretion into the blood stream, testosterone is transported to its target tissues bound to sex hormone-binding globulin (SHBG) or albumin (57). A small percentage circulates as free hormone. After entering the androgen target cell by diffusion, testosterone may be converted to the more potent metabolite DHT by 5α-reductase enzyme (Figure 2) (59). DHT appears to be the major androgen in male-specific tissues, such as the seminal vesicles and the prostate, whereas testosterone is most important in other tissues, such as skeletal muscle and bone (60).

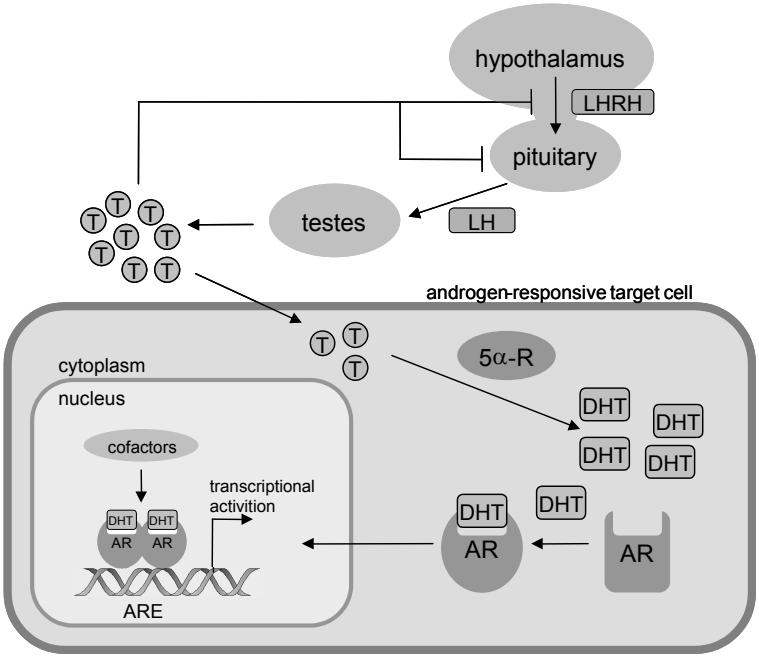

Figure 2. Schematic overview of the AR signalling axis. See section 1.3 for more details. Abbreviations used are: 5α-R, 5α-reductase; AR, androgen receptor; ARE, androgen response element; DHT, dihydrotestosterone; LH, luteinizing hormone; LHRH, luteinizing hormone releasing hormone; T, testosterone. Adapted from ref. 179.

1.3.2 AR NUCLEAR TRANSLOCATION

In the absence of hormone, cytoplasmic AR resides in large chaperone complexes containing heat-shock proteins (61). Chaperone complexes assist in maintaining the LBD in a relatively stable, partially folded, and inactive intermediate conformation with a high affinity for androgens. When bound to these complexes, the NLS is shielded, which prevents AR nuclear translocation (34, 62). Due to the AR conformational changes induced upon ligand binding, specific chaperone proteins are exchanged (63). As a result, the NLS is exposed, allowing transportation of the AR to the nucleus. Dynein or filamin may guide the AR to the nucleus along microtubules or actin filaments, respectively (63, 64).

1.3.3 AR DIMERIZATION AND N/C INTERACTION

Binding of androgens to the AR induces the formation of homodimers. So far, three forms of AR dimerization have been described (30). The first mechanism of ARs to dimerize is via LBD-LBD interactions (65, 66). However, the functional role of this type of dimerization is poorly

understood. The LBD-LBD interaction interface is unknown, but it does not seem to overlap with domains involved in N/C interaction (67). The second form of AR dimerization is mediated by the DBD and is dependent on the D-box (18, 30). This type of dimerization probably plays an important role in stabilizing AR binding to low-affinity AREs (68). N/C interaction is a third mechanism of AR dimerization, and will be discussed in more detail below.

Several studies demonstrated a hormone-dependent interaction between the NH_2- and COOH-termini of the AR, designated as N/C interaction (65, 69-72). Although communication between NTD and LBD has been proposed for other NRs as well, a direct interaction between these domains seems unique for the AR. Analysis of mutations in the coactivator groove of the AR LBD revealed that this groove is the primary interaction surface for the AR NTD (67, 73). Deletion mapping studies of the AR NTD indicated that the most important region for interaction with the LBD is constrained to residues 14-36 (67, 70). The FxxLF motif (AR residues 23-27) appeared to be crucially involved in the direct N/C interaction (25, 26). Searches for other LxxLL-like motifs in the AR NTD that might be involved in N/C interaction, revealed an LxxIL motif (residues 179-183) and a WxxLF motif (residues 433-437). However, the affinity for the LxxIL motif is probably too low to play a role in N/C interaction (16, 25). The role of the WxxLF motif is less clear. Although it was proposed that the WxxLF motif also contributes to N/C interaction, other studies failed to demonstrate an interaction between this motif and the AR LBD, excluding WxxLF as an autonomous LBD interaction motif (25, 26, 74). Another study showed that the WxxLF motif influences ligand-independent AR signalling by acting as an autonomous activation domain (74).

Until recently, it was not possible to determine whether AR N/C interaction takes place within one molecule (intramolecular) or that the NTD of one AR monomer interacts with the LBD of a second AR molecule (intermolecular). Experiments in living cells using fluorescence resonance energy transfer (FRET) started to decipher the molecular basis for AR N/C interaction. Using full length AR tagged with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) on either side of the protein, it was demonstrated that intramolecular N/C interaction is induced in the cytoplasm immediately upon addition of agonist, which is followed by nuclear translocation of the AR (75, 76). Intramolecular N/C interaction was also measured in the nucleus (75). Interaction between single tagged CFP-AR and AR-YFP, reflecting intermolecular N/C interaction, was detected exclusively in the nucleus (75). This suggests a nuclear transition from intramolecular N/C interaction to intermolecular N/C interaction, resulting in a dimerized AR prior to DNA binding.

Although the exact functional role of AR N/C interaction has so far not been fully established, the significance of N/C interaction in normal male physiology is supported by studies of naturally occurring AR mutations. Mutations in the coactivator groove that disrupt N/C interaction without affecting ligand binding affinity have been identified in patients with AIS (77-79). AR N/C interaction plays an important role in stabilizing AR by slowing down the androgen dissociation rate and by preventing the receptor from degradation (26, 71, 80, 81).

Some studies demonstrated that N/C interaction also selectively affects AR transcriptional activity from transiently transfected promoters (82, 83). Functional N/C interaction was essential for full AR transcriptional activity from reporters containing non-selective AREs, but not for transcription from reporters containing selective AREs. This contrasts with other data showing that AR N/C interaction is necessary for binding and activation of chromatinized templates, but not for activation of transiently transfected templates (84).

Using a combination of fluorescence recovery after photobleaching (FRAP) and FRET, it was demonstrated that AR N/C interaction in the nucleus preferentially takes place in the AR mobile fraction (76). Upon DNA binding N/C interaction is relieved, particularly in foci overlapping with sites of active transcription. In addition, a fragment of the AR cofactor ARA54 preferentially interacts with DNA-bound AR (76). This indicates that AR N/C interaction prevents unfavourable cofactor interactions and that the AR prefers cofactor recruitment after binding DNA. This is supported by another study, which showed that recruitment of p160 cofactors is hampered by N/C interaction (81). In contrast, in another study it was demonstrated that N/C interaction is still present in chromatin-bound AR (85).

1.3.4 AR-DNA BINDING

The high degree of conservation between the DBDs of AR, PR, GR, and MR is reflected by their ability to bind the same consensus DNA sequences, which are composed of an inverted (palindromic) repeat, separated by a 3-bp spacer sequence (5'-AGAACAnnnTGTTCT-3') (29, 86). The high affinity binding site of ER is different and consists of an inverted repeat of the 5'-AGGTCA-3' half site sequence. However, DNA binding sites in natural gene promoters and enhancers may deviate considerably from the consensus sequence. Crystal structures of GR DBD and ER DBD bound to inverted repeats demonstrated that the homodimers are orientated in a head-to-head conformation, reflecting the nature of the repeat (87, 88). Some DNA sequences have been described to be only recognized by AR, allowing AR specific gene transcription (89). These so-called selective AREs are more closely related to a direct repeat of the 5'-TGTTCT-3' sequence (90-93). It was expected that the AR monomers would bind parallel to the underlying DNA in a head-to-tail orientation. However, the resolved crystal structure of AR DBD bound to a selective ARE revealed that the AR DBDs dimerize also in the classic head-to-head arrangement to this type of ARE (Figure 3) (28). As a consequence, one AR DBD binds to a high-affinity ARE half site, whereas the second DBD binds to a low-affinity ARE half site. The increased affinity of AR DBD for direct repeats compared to PR and GR is most likely due to a stronger D-box dimerization interface, consisting of an extended vanderWaals surface and three additional hydrogen bonds (28). Stronger cooperative dimerization of AR allows more stable binding to the low-affinity second half site.

In vitro studies demonstrated that the AR is unable to bind to a single half site consensus sequence, which suggests that the AR preferentially binds DNA as a dimer (94). However, recent genome wide studies revealed that most AR binding sites differ considerably from

the consensus AREs, and include single ARE half sites and half sites separated by more than 3-bp (95-97). The mechanisms by which the AR binds to these types of AREs and how these interactions contribute to AR transcriptional activity requires further study.

AREs are usually located in enhancer or promoter regions of AR target genes. Recent studies showed that most AREs are located within intronic regions or in gene upstream regions relatively far (>10kb) from the transcription start site of the AR responsive gene (95, 97, 98). However, whether these AREs are also functional remains to be established.

ChIP-chip experiments estimate between 1,500 and 4,500 potential AR binding sites in the human genome (96, 97). Further sequence analysis reveals that consensus binding sequences for ETS transcription factors and AR single half sites co-occur in 70% of the AR-enriched promoters (96). In addition, ETS1 is recruited hormone-dependently to a subset of AR promoter targets and is required for expression of these AR target genes (96). Binding sequences for the transcription factors Forkhead, GATA, and Oct are also significantly enriched near AR half sites (97). GATA2 and Oct1, together with AR, form a regulatory hierarchy controlling androgen-dependent gene transcription. This demonstrates that other specific transcription factors may cooperate or cross-talk with AR to control the expression of AR target genes.

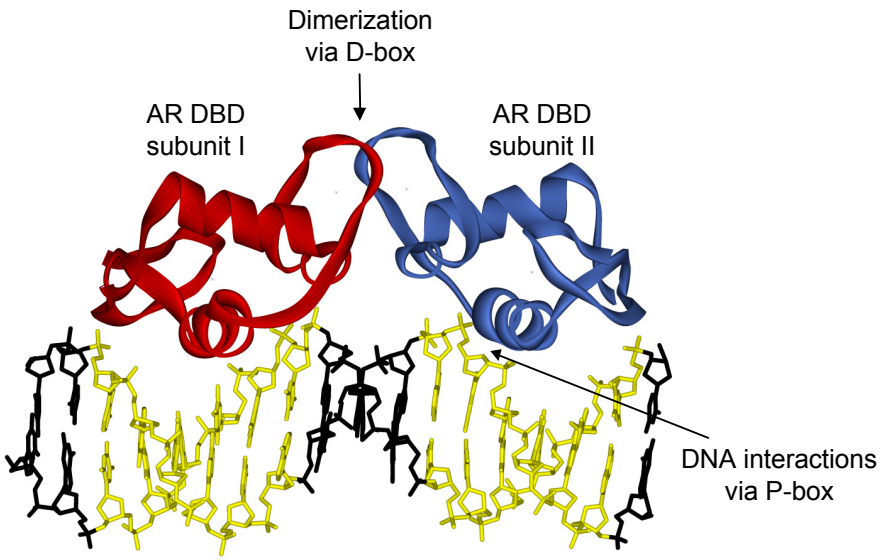

Figure 3. Overall architecture of the AR DBD bound to a selective ARE. The two AR DBD subunits are shown in red and blue. The two ARE half sites are indicated in yellow, and the spacer and flanking base pairs are shown in black. Adapted from ref. 28.

1.3.5 AR TARGET GENES

The majority of large-scale analyses to identify AR-regulated genes have been performed in the LNCaP prostate cancer cell line. Serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS) revealed that the total amount of genes expressed in LNCaP cells, the transcriptome, lies between 10,570 and 23,448 (reviewed in (99)). Various expression profiling studies found that 1.5% to 4.3% of the LNCaP transcriptome is either directly or indirectly induced or repressed by androgens (99).

The main function of the prostate is to produce secretions rich in proteins, organic solutes, lipids, and cholesterol. These secretions form a major component of the seminal fluid. It is not surprising that many AR-regulated genes encode proteins involved in the secretory pathway, polyamine synthesis, and lipogenesis (99, 100). Besides playing major roles in differentiation and maintaining prostate function, the AR controls a balance between cell proliferation, apoptosis, and survival.

The best-described AR-regulated gene is prostate-specific antigen (PSA), also termed kallikrein 3, which is secreted by the prostate. PSA, a serine protease, is a widely used clinical tumour marker for detection and monitoring progression of prostate cancer (101, 102). Increased PSA levels are an indication of prostate abnormalities. Another AR-regulated gene, TMPRSS2, has received much attention more recently. TMPRSS2 is a transmembrane serine protease highly enriched in prostate (103). Although TMPRSS2 itself is probably not involved in tumourigenesis, it has been found to be part of a gene fusion with genes encoding oncogenic ETS transcription factors in the majority of prostate cancers (104). Because of this fusion, expression of ETS factors are under control of the androgen-regulated and prostate-specific promoter of TMPRSS2 (discussed in more detail in paragraph 1.5). Other well-characterized AR-regulated genes include kallikrein 2, SARG, NDRG1, FKBP51, and NKX3.1 (89, 105, 106).

1.3.6 AR TRANSCRIPTIONAL REGULATION

Genomic DNA is tightly packaged in chromatin. The basic unit, the nucleosome, is composed of 146 base pairs of DNA wrapped around a histone octamer containing two copies of each of the four core histones H2A, H2B, H3, and H4 (107). The linker histone H1 occupies regions of DNA between nucleosomes. The condensed organization of chromatin generates a barrier for transcriptional activities. In order to allow transcription, DNA-bound AR requires the recruitment of cofactors and cofactor complexes that affect the local chromatin structure, or play a role in recruitment of general transcription factors and RNA polymerase II (Figure 4) (108-110). Cofactors may enhance (coactivators) or inhibit (corepressors) AR function. Complex interplay between cofactors and cofactor complexes ultimately determine AR transcriptional outcome.

At least two different mechanisms exist that are involved in altering the chromatin structure. The first is based on covalent modification of histone molecules by (de)acetylation, (de)methylation, (de)phosphorylation, ubiquitylation, and sumoylation (111). In most

cases, these modifications change the net charge of the nucleosome, resulting in loosening or tightening of the DNA-histone interactions (108). The best-described histone modifiers are the histone acetyltransferases (HATs), which catalyze the addition of an acetyl group to specific lysine residues. Histone acetylation loosens the nucleosomal structure and is therefore associated with transcription activation. Several AR cofactors are known to possess HAT activity, including CBP/p300 and P/CAF. Although members of the p160 cofactor family have been reported to possess (weak) HAT activity as well (112, 113), they probably function as adapter proteins for the recruitment of more potent HATs, like CBP/p300 and P/CAF (114, 115). HATs are antagonized by histone deacetylases (HDACs), which remove the acetyl group from the histone tails. This leads to reformation of the condensed nucleosomal structure and to silencing of transcriptional activity.

The second mechanism of regulating chromatin structure is by chromatin remodelling. Large complexes, such as SWI/SNF, WINAC, and NUMAC, use the energy derived from ATP hydrolysis to modulate the arrangement and stability of nucleosomes in a non-covalent manner in order to obtain a chromatin status that allows transcription (116, 117). All chromatin remodelling complexes consist of a catalytic subunit belonging to the SNF2 family of ATPases, usually BRG1 or BRM, and 10-12 BRG1-associated factors (BAFs), of which BAF47/INI/SNF5, BAF155, and BAF170 form the core (118). Other subunits commonly found include BAF53, BAF57, BAF60, BAF180, and BAF250 (119-122). Recruitment of SWI/SNF complexes is essential for transcriptional activity by AR (123). However, there appears to be a preference for complexes containing the BRM ATPase. Although both BRG1 and BRM may be recruited by chromatin-bound AR (85, 114), they do not appear to interact directly with AR (124). Recruitment seems to be dependent

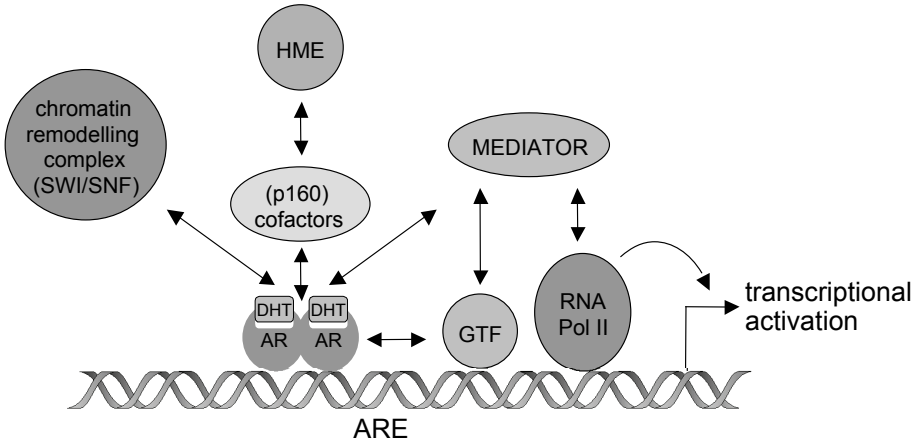

Figure 4. Schematic model of protein interactions regulating AR transcriptional activity. AR, androgen receptor; ARE, androgen response element; DHT, dihydrotestosterone; HME, histone modifying enzymes; GTF, general transcription factors; RNA pol II, RNA polymerase II.

on the BAF57 subunit, which binds to the AR DBD-hinge region (124, 125). In Chapter 4 we show that BAF60a is novel SWI/SNF subunit that directly interacts with the AR via the LBD.

Another multi-subunit complex that plays an important role in the AR transcriptional activation process is Mediator (MED), which is also known as TRAP/DRIP/ARC (126, 127). MED functions as a bridging factor between the receptor, and general transcription factors and RNA polymerase II. Recruitment of MED by the AR involves a direct interaction of the MED1 (TRAP220/DRIP205) subunit with the AR LBD via an extended LxxLL motif (128). However, direct interactions between AR and the general transcription factors TFIIF and TFIIH, as well as with the RPB2 subunit of RNA polymerase II, have also been described (129-132).

1.3.7 AR ENHANCER-PROMOTER DYNAMICS

Most of the work aimed at resolving how the AR transcriptional complex is orchestrated, has been done using the PSA gene as a model. The PSA gene contains two AREs in its proximal promoter region (ARE-I and ARE-II at –170 and –394, respectively) and one (ARE-III) in the enhancer region at approximately 4.2 kb upstream of the promoter (133, 134). Whereas the promoter region is weakly androgen inducible, the enhancer region responds much stronger. Combination of both regions synergistically result in maximum androgen regulation (133).

Several studies described the complex recruitment of AR and several coregulators to the PSA gene. However, the outcomes of the different studies are in some cases conflicting. Initially, ChIP experiments in LNCaP cells showed that in the presence of DHT the AR is recruited to both enhancer and promoter regions, but not to sequences in between (135). Several AR coactivators, including TIF2 and CBP, and RNA polymerase II are recruited to both regions as well. This contrasts with two other studies, which show more robust hormone-induced recruitment of the AR to the enhancer region than to the promoter (136, 137). However, whereas in the study of Louie *et al.* also the cofactors TIF2 and SRC3, and RNA polymerase II are mainly recruited to the enhancer region, Kang *et al.* found that RNA polymerase II is assembled on the promoter (136, 138). The reason for this discrepancy is not understood.

The occupancy of the PSA promoter and enhancer region by AR, and the recruitment of coactivators and RNA polymerase II are transient and cyclic events (136, 138). The AR is recruited to both regions within 15 minutes after hormone treatment, reaching a maximum after 45 minutes. The cycle is completed after 90 minutes. The second cycle, starting 105 minutes after hormone treatment, shows stronger promoter and enhancer loading by the AR. RNA polymerase II and the cofactors GRIP1 and CBP follow the same cyclical pattern, but start approximately 15 minutes after AR binding.

In the presence of the antiandrogen bicalutamide, AR is loaded on the PSA promoter but not on the enhancer (135, 136, 138, 139). In agreement with the pure antagonistic nature of bicalutamide, this antiandrogen fails to recruit TIF2, CBP, and RNA polymerase II to the enhancer or promoter of the PSA gene (135, 136, 138). Instead, the AR- bicalutamide complex promotes the recruitment of the corepressors N-CoR, SMRT, and HDACs 1 and 2 only to the

promoter (135, 136). Live cell imaging using FRAP demonstrated absence of stable DNA binding of the AR-bicalutamide complex, suggesting that the repressor complexes recruited by bicalutamide-bound AR are very short-lived (140).

Based on these studies, it was postulated that upon androgen-stimulated recruitment of the transcription initiation complex, the 4 kb region between the enhancer and promoter is "looped-out", thereby facilitating an interaction between these two regions (135). Another model involves a "facilitated tracking" mechanism, in which RNA polymerase II, after initial binding to the enhancer, trails along the entire 4.2 kb upstream sequence to the promoter (137). However, further studies are needed to elucidate which model is most likely and whether this can be generalized to other AR target genes.

1.4 AR AND DISEASE

The androgen-AR signalling pathway has been implicated in several diseases. These include Kennedy's disease or spinal and bulbar muscular atrophy (SBMA), androgen insensitivity syndrome (AIS), and prostate cancer.

1.4.1 KENNEDY'S DISEASE / SBMA

Kennedy's disease or SBMA is a progressive neurodegenerative disorder (141, 142). It is characterized by an adult-onset selective loss of motoneurons in the brain stem and in the anterior horn of the spinal cord, and of sensory neurons in the dorsal root ganglia. The underlying abnormality is an expanded glutamine stretch in the AR NTD (19, 143). In the presence of testosterone, this modified AR forms aggregates in the cytoplasm or nucleus of motoneurons leading to cell death. However, the exact molecular mechanism of SBMA is still unclear. Because an expanded glutamine stretch results in reduced AR transcriptional activity, SBMA is also associated with mild androgen insensitivity (see below).

1.4.2 ANDROGEN INSENSITIVITY SYNDROME

Androgen insensitivity syndrome or AIS is characterized by defective masculinization in 46,XY individuals (reviewed in (56, 144-146)). Phenotypic outcomes range from mild undervirilization or reduced fertility (mild AIS or MAIS) to several degrees of ambiguous genitalia (partial AIS or PAIS) or even completely female external genitalia (complete AIS or CAIS). Although AIS may be caused by defects in enzymes involved in the androgen biosynthesis pathway as well, this disorder is mainly caused by inactivating mutations in the AR. These include both frameshift mutations and/or deletions in the AR gene, usually leading to a truncated AR protein, and loss-of-function mutations in the AR protein. Loss-of-function mutations are primarily found in the AR DBD and LBD (147, 148). Such mutations partially or completely inactivate AR function by directly or indirectly affecting AR DNA binding, dimerization, ligand binding, N/C interaction, and/or cofactor recruitment.

1.4.3 PROSTATE CANCER

Prostate cancer is the most frequently diagnosed male cancer and the second leading cause of cancer deaths in men in Western countries (149). Like normal prostate development, initial prostate tumour growth is dependent on the androgen-AR axis (reviewed in (150-152)). Radical prostatectomy, i.e. surgical removal of the prostate, or local radiation are the main treatment options for men with organ-confined disease. When the tumour has metastasized, treatments are usually based on blocking AR function indirectly by blocking testicular androgen production using LHRH agonists (androgen withdrawal). Another option is to block AR function directly by applying antiandrogens, such as hydroxyflutamide and bicalutamide. Antiandrogens compete with residual androgens for binding to the AR ligand-binding pocket, but fail to induce the active conformation necessary to allow transcriptional activity. Despite an initial response, the tumour will recur in a form that is resistant to these hormonal manipulations. This stage of the disease is referred to as androgen-independent or hormone-refractory and is usually fatal within a few years (Figure 5). However, the majority of androgen-independent tumours retain high levels of AR expression (153-156). Moreover, the AR is predominantly nuclear and seems to be functionally active. The finding that knockdown of AR expression reduces AR-dependent target gene expression, cell proliferation, and survival in hormone-refractory prostate cell lines, further substantiates the importance of the AR in hormone-refractory prostate cancer (157-162). This suggests that androgen-independent prostate cancer cells continue to survive and proliferate through aberrant mechanisms of AR activation.

1.4.3.1 ANDROGEN-DEPENDENT PROSTATE CANCER

Because initial growth of prostate tumours is dependent on the androgen-AR axis, it was expected that one or more natural AR target genes would drive androgen-dependent tumour growth. Only recently, it was found that ERG and ETV1, which are members of the ETS family of oncogenes, are frequently overexpressed in clinical prostate cancers (104, 163). However, both genes are not direct targets of the AR. It was revealed that the overexpression of both ERG and ETV1 were due to a fusion to the androgen-regulated and prostate-specific TMPRSS2 gene (104). Because of interstitial deletion or intra- or inter-chromosomal translocations, the promoter and first exon(s) of TMPRSS2 are fused to coding sequences of the ETS factor, leading to androgen-regulated and prostate-specific expression of the ETS oncogene. TMPRSS2-ERG is the most common fusion identified and appears to be present in nearly 60% of the prostate cancers (104, 164). Fusions involving ETV1 are present in approximately 10% of the prostate cancers. Also fusions involving other members of the ETS family, including ETV4 and ETV5, and other 5' partners have been reported (165-170). These combinations appear to present at much lower frequencies (<2%). The majority of 5' partners are androgen-regulated and prostate-specific (166-168, 170).

The TMPRSS2-ERG fusion gene was expressed in a panel of androgen-dependent xenografts, but not in late-stage AR-negative xenografts, although this fusion gene was present

(171). This suggests that fusion genes play a role in early stages of prostate cancer. Controversial data exist on whether TMPRSS2-ERG fusions are associated with more aggressive prostate cancers and poorer prognosis (164, 172).

1.4.3.2 ANDROGEN-INDEPENDENT PROSTATE CANCER

Several mechanisms have been proposed to play a role in aberrant AR activation in prostate cancer. These include AR amplification and/or overexpression, AR mutations, ligand-independent AR activation, intratumoural androgen synthesis, and aberrant cofactor expression and function (Figure 5). In a subset of tumours the AR signalling pathway has been bypassed and is growth regulated by other signalling pathways. Each of the mechanisms will be described in more detail below.

AR amplification and/or overexpression

Amplification of the AR gene, resulting in AR overexpression, has been suggested as a mechanism that enables prostate cancer cells to become sensitive to the lower circulating androgen levels after androgen ablation. AR gene amplification is rare in untreated primary tumours, but has been detected in about 25-30% of hormone-refractory prostate tumours (173-178). In a study of matched paired androgen-dependent and androgen-independent tumours, 80% of the androgen-independent tumours with AR gene amplification also exhibited higher expression levels of AR protein (176). In addition, many tumours showed AR protein overexpression without having an amplified AR gene. This suggests that other mechanisms,

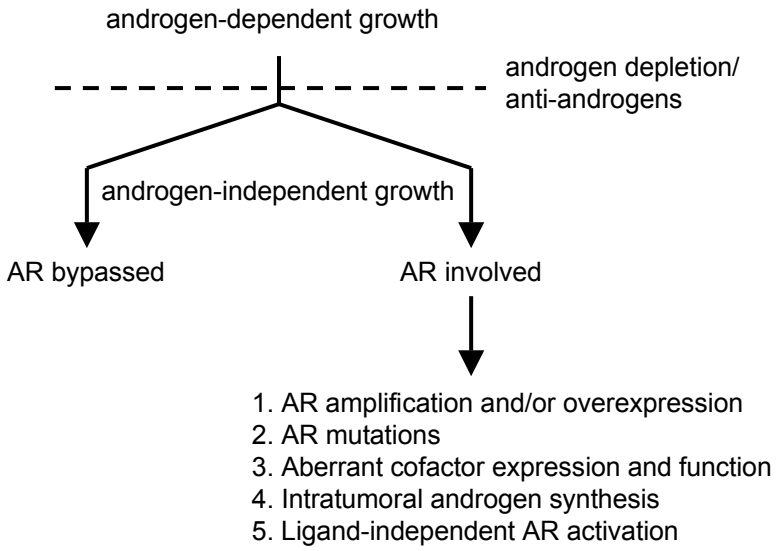

Figure 5. Schematic overview of the role of the AR in prostate cancer growth.

such as increased transcription rates, or stabilization of the AR mRNA or protein may account for the AR overexpression observed (179).

AR mutations

AR mutations are rare in localized primary lesions and locally progressive untreated prostate tumours (<5%). They appear to be more common in androgen-independent prostate cancer and in distant metastases of patients following endocrine therapy (10-30%), which is probably due to selective pressure by the antiandrogen used for treatment (180-185). A large study of bone marrow metastases has suggested that the rate of AR mutations in hormone-refractory prostate cancer is more around 10% (186).

The majority of AR mutations are located in the ligand-binding pocket in the LBD (148, 187, 188). Such mutations have been shown to alter the specificity of AR-ligand interactions, allowing inappropriate activation by non-androgenic ligands, such as estradiol, progesterone, glucocorticoids, and adrenal androgens, and even by antiandrogens, including hydroxyflutamide and bicalutamide (189-192). The most common AR mutations and their structural consequences allowing activation by non-androgenic ligands and antiandrogens will be discussed in more detail in section 2.

Aberrant cofactor expression and function

Some studies have implicated a direct role for AR cofactors in the development of androgen-independent prostate cancer (reviewed in (150, 179, 193, 194)). Aberrant expression of several AR cofactors has been described in prostate cancer tissues and cell lines, and include all members of the p160 family, CREB, CBP/p300, TIP60, PAK6, gelsolin, ARA54, and ARA70. This increased expression may enhance AR activity in a low androgen environment or may allow promiscuous activation of AR by low-affinity ligands. However, whether there is a causal relationship between cofactor expression levels and the initiation or progression of prostate cancer remains to be determined.

Ligand-independent AR activation

After androgen withdrawal, signal transduction pathways may be activated that can replace androgens for growth and survival of prostate cancer cells, either by bypassing the AR all together or by activating the AR so that it becomes active in the absence of androgens (reviewed in (150, 151, 179, 195, 196)). Pathways activated by epidermal growth factor (EGF), insulin-like growth factor (IGF)-I, keratinocyte growth factor (KGF), and interleukin (IL)-4 and –6, may be involved in these processes. Also the activation of oncogenes and the repression of copressors may play a role in tumour growth.

Intratumoural androgen synthesis

Because androgen ablation strongly reduces the levels of circulating testosterone, it was thought that tissue androgen levels were reduced correspondingly. However, it was recently demonstrated that tissue levels of testosterone in locally recurrent prostate cancer could be similar to those in patients who had not received androgen ablation therapy (197-199). Although DHT levels were lower than in normal tissues, the remaining concentrations were sufficient to activate the AR (200). Other studies demonstrated elevated levels of enzymes involved in steroid biosynthesis in recurrent metastases (201, 202) and that recurrent metastatic prostate tumours may even be capable of *de novo* androgen biosynthesis from cholesterol precursors (203, 204). Thus, residual androgen levels and elevated endogenous biosynthesis of androgens may be a driving mechanism for prostate cancer cells to proliferate after androgen withdrawal.

2. THE AR LIGAND-BINDING POCKET

The crystal structures of all steroid receptor LBDs in complex with their cognate ligand have been determined (39, 205-208). The first crystal structure of the AR LBD, in complex with the synthetic androgen R1881, was solved in 2000 (37). This report was soon followed by many other publications of AR LBD structures complexed with different ligands, but in none of them the LBD was bound to an antagonist. Although structures have been solved of antagonists bound to mutated AR LBDs, the antagonists functioned as agonists for these mutants. It is therefore not surprising, that these antagonist-bound mutant AR LBDs adopt similar conformations as agonist-bound wild-type AR LBD. Crystal structures of ER and GR LBDs have been solved in an alternate conformation bound to an antagonist in which helix 12 is displaced over the coactivator binding groove (208, 209). This antagonistic conformation, called 'active antagonism', prevents cofactor binding via LxxLL motifs. Another mechanism of antagonism, termed 'passive antagonism', is by stabilizing key residues in helix 11 and in the loop between helices 11 and 12 such that they fail to generate the proper anchoring points for helix 12 to obtain the active conformation (210). Whether antagonists induce similar conformations upon binding AR LBD is unknown. However, crystal structures of AR mutants in complex with an antagonist may explain not only how an antagonist functions as an agonist in these mutants, but may also provide clues about how such compounds serve as 'real' antagonist for wild-type AR LBD. The next paragraphs describe how agonists bind to the wild-type AR LBD and how AR mutants adopt an active conformation in the presence of an antagonist.

2.1 STRUCTURE OF THE AR LIGAND-BINDING POCKET

The ligand-binding pocket is composed of 18-20 amino acid residues belonging to helices 3, 4, 5, 7, 11, and 12 (37, 38, 211). It consists of a non-specific apolar cavity where mostly hydro-

phobic residues (L701, L704, L707, W741, M742, M745, M749, F764, M780, M787, L873, F876, L880, F891, and M895) are close enough to interact with the steroid backbone via vanderWaals contacts (Figure 6). The inherent non-specificity of the hydrophobic interactions combined with the fact that the side chains of these residues are flexible and can adopt variable positions may explain why the AR LBD can bind and stabilize several structurally different ligands (38). In addition to hydrophobic interactions, hydrogen bond (H-bond) networks between polar residues present at both extremities of the ligand-binding pocket (N705, Q711, R752, and T877) and polar atoms at either side of the steroid also play critical roles in stabilizing the bound ligand (Figure 6).

2.2 STRUCTURAL ANALYSIS OF AGONIST-BOUND WILD TYPE AR LBD

2.2.1 BINDING OF R1881, TESTOSTERONE, AND DHT

All potent androgens contain a ketone group at position 3 and a hydroxyl group at position 17β (Figure 7). The crystal structures of the wild-type AR LBD in complex with the synthetic androgen R1881 and the natural androgens testosterone and DHT show that these ligands bind the AR LBD in a similar way (37-39, 212). The 3-ketone group is hydrogen-bonded to Q711 (directly or indirectly via a water molecule) and R752 (Figure 8A). The 17β-OH group is stabilized via hydrogen bonds to N705 and T877. The steroid backbones are further stabilized via vanderWaals interactions with the hydrophobic residues scattered throughout the pocket.

2.2.2 ANDROGEN SPECIFICITY OF THE AR LIGAND-BINDING POCKET

Besides DHT and testosterone, most other steroids, including progesterone, cortisol, and aldosterone, also contain a ketone group at position 3. However, they differ at position 17β by the presence of larger substituents than the hydroxyl group in the androgens (Figure 7). The relatively large polar amino acids at AR positions 705 and 877 play a critical role in maintaining androgen specificity, as mutation to smaller residues enables the AR to bind the other steroids (37, 189, 211). Also the presence of a large hydrophobic leucine residue at AR position 880 (a smaller and polar threonine residue at position 894 in PR) probably prevents binding of progesterone to the AR LBD (37). Although estradiol, like DHT, contains a hydroxyl group at position 17β, it also has a hydroxyl group at position 3, instead of a ketone group. A weaker hydrogen bonding scheme around position 3 prevents estradiol from being an AR agonist (210).

2.3 BINDING OF ANTAGONISTS AND NON-ANDROGENIC LIGANDS TO MUTATED AR LBDs

So far, approximately forty unique amino acid mutations have been claimed to be identified in the AR LBD of prostate cancer patients. For only a few of these mutations, including T877A,

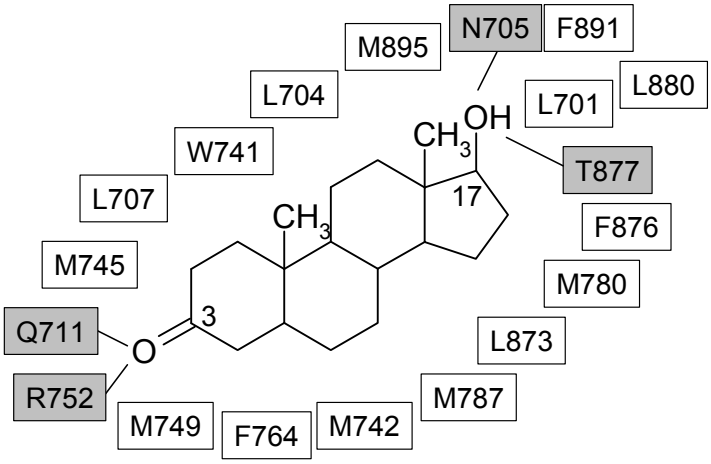

Figure 6. Interactions between AR LBD and DHT. Schematic representation of the relative position of residues in the AR LBD that contact DHT. Residues that contact DHT via vanderWaals interactions are indicated with white boxes, and residues stabilizing the ligand via hydrogen bonds (solid lines) are represented with grey boxes. Positions 3 and 17 of the steroid are indicated.

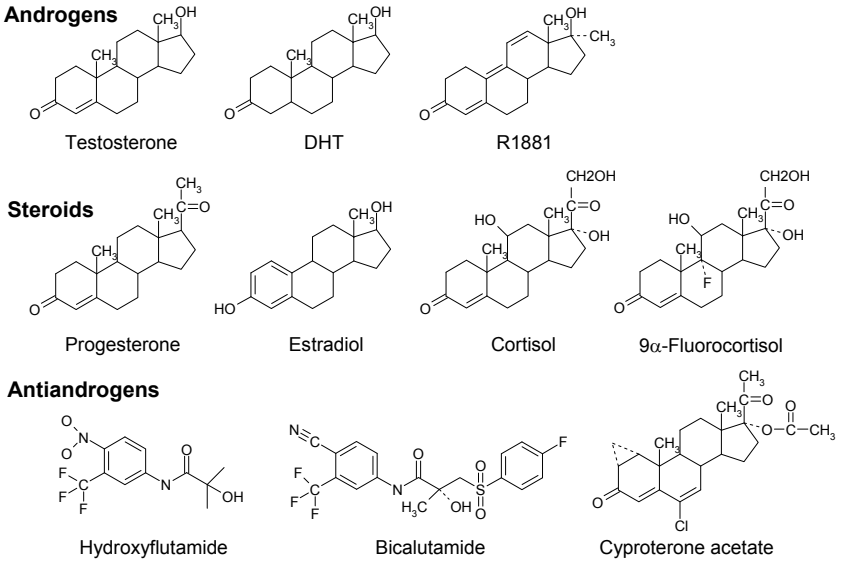

Figure 7. Chemical structures of androgens, non-androgenic steroids, and anti-androgens.

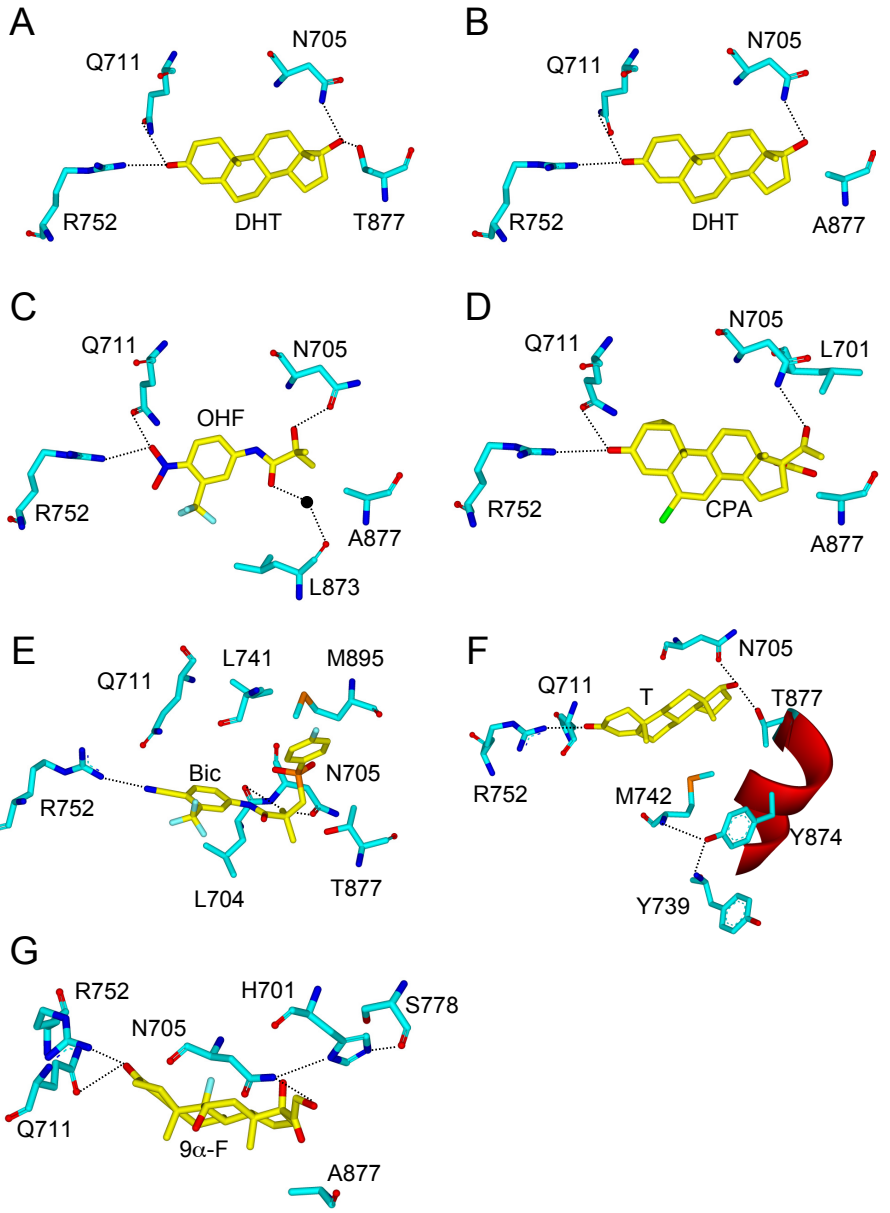

Figure 8. Structural representation of ligand binding to wild type AR and AR mutants. (A) Binding of DHT to wild type AR. (B-D) Binding of DHT (B), hydroxyflutamide (OHF; C), and cyproterone acetate (CPA; D) to the AR T877A mutant. (E) Binding of bicalutamide to the AR W741L mutant. (F) Binding of DHT to the AR H874Y mutant. (G) Binding of 9α-fluorocortisol to the AR L701H/T877A double mutant. Important LBD residues are indicated in blue; the ligand is indicated in yellow. Hydrogen bonds are indicated with dotted lines.

W741L, H874Y, and L701H/T877A, the effects on AR function have been studied in more detail. Functional and structural studies provided insight into how these mutations modify the ligand-binding pocket, allowing non-androgenic ligands and/or anti-androgens to bind and activate the AR. The functional and structural consequences of these four AR mutants are discussed in the next paragraphs

2.3.1 THE AR T877A MUTATION

The T877A mutation was the first AR mutation identified after sequencing the promiscuous AR in the LNCaP prostate cancer cell line, which was derived from a lymph node metastasis of an orchidectomized and estradiol treated patient (190, 213). Subsequently, this mutation was found to be an AR mutational hot-spot in endocrine therapy-resistant prostate cancer tissues (183, 214-216). Binding studies and transcription activation assays showed that AR T877A has a broadened ligand-specificity by responding to progesterone, estradiol, cortisol, aldosterone, and the antiandrogens hydroxyflutamide and cyproterone acetate (CPA) (189, 190, 217). Another AR prostate cancer mutant at the same position, T877S, shows similar responses to the non-androgenic ligands as the T877A mutant (184, 189, 218). Random mutagenesis of AR residue 877 identified two additional mutants, T877G and T877C, with a broadened ligand-specificity (189). However, these two mutations have never been identified in prostate cancer patients, probably because two base substitutions are necessary to substitute the threonine by a glycine or cysteine.

Structural analysis revealed that binding of DHT by wild-type AR and AR T877A is essentially identical except at the point of the mutation (Figure 8B) (39). Besides the loss of a polar interaction, mutation of threonine by the smaller alanine introduces more space around the D-ring allowing the accommodation of a larger substituent at position 17. This may explain the ability of the AR T877A mutant to bind a variety of other hormones, like progesterone. The loss of the polar interaction is partly compensated by N705 as there is only a slight loss in DHT activity of the AR T877A mutant (219, 220).

Wild-type AR LBD bound to an agonist resembling hydroxyflutamide shows that the side chain of T877 is rotated 180° from its position in the steroid-bound structures (219). Because of this orientation there is no hydrogen bond formed between T877 and the compound. The compound maintains the same binding conformation in the AR T877A mutant. However, the distance from the compound to the 877 residue side chain is increased in the T877A mutant leaving additional space to accommodate a water molecule. This water molecule bridges the compound's ketone group to the backbone oxygen of L873 through hydrogen bonds. The AR T877A-hydroxyflutamide structure shows the same hydrogen bond network between the ketone group and L873, suggesting that these water-mediated interactions stabilize hydroxyflutamide to function as an agonist (Figure 8C) (219).

The AR T877A-CPA structure shows that the 17α-acetate group of CPA induces movement of the L701 side chain resulting in partial unfolding of the C-terminal end of helix 11 and

displacement of the loop between helices 11 and 12 (Figure 8D) (220). This leads to an expansion of the ligand-binding pocket, which is supposed to accommodate the bulky group at position 17α of CPA. A threonine at position 877 would sterically hinder the C21 methyl group of CPA, and may repel helix 11 away from the binding pocket in wild-type AR in such a way that it prevents the AR from obtaining an active conformation.

2.3.2 THE AR W741C and AR W741L MUTATIONS

The AR W741C mutation has been reported in two individual prostate cancer patients treated with bicalutamide and in the KUCaP prostate cancer xenograft, which was obtained from an androgen-independent liver metastasis of a patient treated with LHRH agonists and bicalutamide (185, 186, 221). The same mutation and another one at the same position, W741L, occurred upon androgen deprivation and bicalutamide treatment of LNCaP cells (222). Transactivation assays demonstrated that both single W741 mutations were sufficient for the bicalutamide response of the AR (221, 222).

The structure of W741L in complex with bicalutamide has been solved (223). The structure shows that bicalutamide is in a bent conformation, because of intramolecular hydrogen bond formations (Figure 8E). The A-ring and the amide bond of bicalutamide overlap with the steroidal plane. The cyano group in the A-ring is hydrogen bonded in a similar way as the 3-ketone group in androgens. It forms a hydrogen bond with R752 and a water molecule, but the Q711 residue is slightly out of range. In contrast to steroidal bound AR structures, no hydrogen bond is formed between T877 and bicalutamide. However, the hydroxyl group in bicalutamide forms hydrogen bonds to N705 and the backbone of L704, mimicking the binding of the 17β-OH group of androgens. The bent conformation of bicalutamide causes the B-ring to fold away from the steroidal plane, pointing to the top of the ligand-binding pocket, where it makes direct contacts with residues in helix 12. Replacement of the bulky tryptophan by a smaller leucine at position 741 creates additional space to position the side chain of M895 near L741. This larger area allows the proper accommodation of the B-ring of bicalutamide, providing a platform for helix 12 to adopt an agonistic conformation. In the wild-type AR, the larger tryptophan residue at position 741 would displace M895 from the ligand-binding pocket upon binding of the B-ring. This promotes partial unfolding of the LBD and offers an explanation for bicalutamide antagonism in the AR.

2.3.3 THE AR H874Y MUTATION

The AR H874Y mutation has been identified in a few prostate cancer patients treated with hydroxyflutamide (184). The same mutation is also present in the androgen-independent CWR22 prostate cancer xenograft, which was isolated from a hormone-refractory bone marrow metastasis of a patient treated with hydroxyflutamide and LHRH agonists (224, 225). Random mutagenesis of AR residue H874 showed that only the H874Y mutation could broaden AR specificity (189). In addition to an enhanced response to testosterone, the AR

H874Y mutant was also activated by non-androgenic ligands, such as progesterone, estradiol, cortisol, aldosterone, and by the anti-androgen hydroxyflutamide (189, 212).

The crystal structure of the AR H874Y mutant has only been solved in the presence of the androgens testosterone and DHT (212). The 3-ketone and 17β-OH groups of both androgens were stabilized similarly as described for the wild-type AR (Figure 8F). Residue H874 does not line the ligand-binding pocket. Instead, it points the other way and therefore makes no direct contacts with the ligand. The substitution of the histidine by the larger and polar tyrosine residue replaces a water-mediated hydrogen bond network by more stable direct hydrogen bonds between the external helix 10 Y874 side chain and internal helix 4 Y739 and helix 5 M742 backbone atoms. This tethers M742, a key residue near the ligand-binding pocket, and Y739, near the coactivator binding groove, with exterior helix 10 via Y874. Direct hydrogen bonding likely improves binding of LxxLL and FxxLF motifs to the coactivator groove and decreases testosterone dissociation rate, which may explain the enhanced testosterone response of the AR H874Y mutant (212). The structure stabilizing effects of the H874Y substitution may also account for the responses to the non-androgenic ligands and hydroxyflutamide.

2.3.2 THE AR L701H/T877A DOUBLE MUTANT

The AR L701H mutation has been identified in two individual prostate cancer patients (216, 226). This mutant AR is strongly activated by cortisol, suggesting that tumour growth in these patients is driven by an endogenous hormone, and not, as described for other AR mutants, by the antiandrogen used for treatment. A crystal structure of the AR L701H mutant has so far not been elucidated. The mechanism by which cortisol functions as an AR agonist will be discussed in Chapter 2.

The L701H/T877A double mutation has been found in the AR in the prostate cancer cell lines MDA PCa 2a and 2b, which were originally derived from a bone metastasis of an orchiectomized prostate cancer patient (227, 228). Transactivation studies demonstrated that AR L701H/T877A is activated by the same steroids as the AR T877A single mutant, including progesterone, estradiol, and hydroxyflutamide (191). In addition, the AR double mutant is also strongly activated by cortisol, which is a property of the AR L701H mutant, and by other natural and synthetic glucocorticoids (191, 229). Thus, the AR L701H/T877A double mutant combines the characteristics of both AR single mutants.

AR L701H/T877A exhibits high affinities for cortisol and cortisone, but highest affinities are found for the synthetic glucocorticoid 9α-fluorocortisol (229). AR L701H/T877A in complex with 9α-fluorocortisol has also been crystallized (230). The 11β-OH group in the middle of the steroid is involved in a hydrogen bond formation with a water molecule, which is further hydrogen-bonded to N705 and M895. The T877A mutation creates the extra space to allow the binding of the bulky acetyl group at the 17β position (Figure 8G). This group is further stabilized via a hydrogen bond formed between the steroidal O21 and N705. The high affinity for 9α-fluorocortisol is probably obtained through the formation of a strong hydrogen bond

between the 17α-OH group and H701, which would not be possible with the hydrophobic wild-type leucine at this position (230). The histidine imidazole ring adopts a suitable orientation because of an additional hydrogen bond formed between H701 and the backbone oxygen of S778. This hydrogen bond between two different helices also enhances a more stable AR LBD fold.

3. PEPTIDE INTERACTIONS WITH THE AR COACTIVATOR GROOVE

3.1 THE NUCLEAR RECEPTOR COACTIVATOR GROOVE AND LXXLL MOTIF BINDING

Ligand binding induces a structural change in the LBD of NRs, which enables the association with a subset of cofactors. Functional studies revealed that a short motif, LxxLL (where L is leucine and x is any amino acid), is necessary and sufficient for binding of the cofactors to liganded NRs (24, 231). Cofactors containing one or more LxxLL motifs include TIF1α, CBP/p300, and the p160 cofactors SRC1, TIF2, and SRC3 (Figure 9).

Crystal structures of NR LBDs in complex with LxxLL motifs revealed that the motif binds to a groove on the LBD surface, which is formed by charged and hydrophobic residues in helices 3, 4, 5, and 12 (43, 232, 233). Because helix 12 completes the formation of this groove after ligand-dependent repositioning, this provides a molecular mechanism for the hormone-dependent association with cofactors. The bound LxxLL motifs adopt an amphipathic α-helical structure, in which the three leucine residues line one face of the helix and are oriented toward the LBD surface. The first and the last leucine of the motif, at positions +1 and +5, respectively, are embedded within the hydrophobic groove, whereas the leucine at position +4 rests on the periphery (see Figure 9 for residue numbering). The residues at positions +2 and +3 are solvent exposed. In addition to hydrophobic interactions with the leucine side chains, binding of the motif is also stabilized via hydrogen bonds between the peptide backbone and conserved charged residues at the opposite ends of the groove. A positively charged lysine (K) residue in helix 3 bonds with the backbone carbonyls within the C-terminal end of the motif, while a negatively charged glutamic acid (E) residue in helix 12 stabilizes the N-terminal end. Residues flanking the motif make additional contacts with the LBD surface (43, 234)

Several cofactors, such as SRC1, TIF2, and SRC3, contain multiple LxxLL motifs, also denoted as NR boxes. Particular NRs show overlapping but distinct preferences for individual NR boxes (44, 234-238). For example, NR box II of SRC1 preferentially interacts with ERα and ERβ, whereas NR box IV showed strongest interactions with PPARα, PPAR, GR, and AR (234). Using chimeric constructs, it was demonstrated that residues flanking the LxxLL motif, both N- and C-terminally, determine selectivity and affinity for a given receptor (234, 236). Conversely, the structure of the groove differs among the different NR LBDs, which may provide selectivity and affinity for LxxLL motifs (239, 240).

3.2 THE AR LBD PREFERENTIALLY BINDS FXXLF MOTIFS

Despite high sequence homology with LBDs of other NRs and a similar conformation, the AR LBD interacts relatively weakly with the LxxLL motifs present in p160 cofactors (44, 46, 73, 80, 234). Also the majority of non-natural NR-binding LxxLL sequences identified by peptide library screenings fails to interact with the LBD of AR (241-243). Instead, the AR LBD prefers the binding of related FxxLF sequences (F is phenylalanine). The FxxLF motif was initially identified in the AR NTD as a crucial mediator of AR N/C interaction (25, 26). Later it was shown that FxxLF motifs also play an important role in recruitment of AR cofactors ARA54, ARA55, ARA70, and hRAD9 to ligand-bound AR LBD (Figure 9) (47, 48).

The preference of the AR LBD for FxxLF sequences is further substantiated by F/L swapping experiments of FxxLF and LxxLL-based peptide motifs (46, 80). Substitution of the phenylalanine residues by leucines in the FxxLF motifs of AR, ARA54, and ARA70 reduce interaction with the AR LBD, showing that in these motifs the bulkier phenylalanines are essential for interaction. Substituting the leucine residues at positions +1 and +5 of LxxLL motifs by phenylalanines does not affect or even increases AR LBD affinity. However, the FxxLF motif is not sufficient for interaction with the AR LBD. FxxLF sequences present in TFIIEα and TAFII250 interact weakly with the AR LBD, whereas the FxxLF motifs in CBP, p300 and FHL2 do not interact at all (47). Thus, sequence determinants for binding to the AR LBD lie within and flanking the FxxLF motif.

LxxLL motifs

			-1	+1		+4+5	
SRC1	BOX I	627-S Q T S H K		**L**	V Q	**L L**	T T T A E-642
	BOX II	684-T A R H K I		**L**	H R	**L L**	Q E G S P-699
	BOX III	743-S K D H Q L		**L**	R Y	**L L**	D K D E K-758
	BOX IV	1429-A Q Q K S L		**L**	Q Q	**L L**	T E *-1441
TIF2	BOX I	636-S K G Q T K		**L**	L Q	**L L**	T T K S D-650
	BOX II	684-K E K H K I		**L**	H R	**L L**	Q D S S S-699
	BOX III	739-K K E N A L		**L**	R Y	**L L**	D K D D T-754
SRC3	BOX I	615-S K G H K K		**L**	L Q	**L L**	T C S S D-630
	BOX II	679-Q E K H R I		**L**	H K	**L L**	Q N G N S-694
	BOX III	732-K E N N A L		**L**	R Y	**L L**	D R D D P-747

FxxLF motifs

AR	17-K T Y R G A			**F**	Q N	**L F**	Q S V R E-32
ARA54	448-D P G S P C			**F**	N R	**L F**	Y A V D V-463
ARA70	322-R E T S E K			**F**	K L	**L F**	Q S Y N V-337
RAD9	355-T P P P K K			**F**	R S	**L F**	F G S I L-370

Figure 9. Alignment of NR boxes present in AR and in several cofactor proteins. The core motif residues are shown in bold. Amino acid numbering of motif residues is indicated.

FxxLF motifs have also been assessed for interaction with LBDs of other NRs (46, 47, 244). Most NR LBDs are unable to bind FxxLF sequences. Only the PR LBD appears to be able to interact with a subset of FxxLF motifs. However, F/L swapping experiments showed that although PR LBD is able to bind both LxxLL and FxxLF peptides, interactions with LxxLL are clearly preferred (46). Overall, phenylalanine residues at positions +1 and +5 strongly contribute to preferential and strong interaction with AR LBD and prevent binding to most other NR LBDs. This may provide specificity to protein-protein interactions with the AR LBD.

3.3 AMINO ACID REQUIREMENTS FOR PEPTIDE BINDING TO THE AR LBD

3.3.1 MUTATIONAL ANALYSIS OF THE AR FXXLF MOTIF

To study the role of individual amino acid residues for binding to the AR LBD, the AR FQNLF motif has been mutated extensively. Alanine-scanning of the AR FxxLF motif showed that the phenylalanine and leucine residues at positions +1, +4, and +5 are essential for binding to the AR LBD, whereas residues flanking this motif modulate the interaction capacity (25, 26).

Random mutagenesis of the phenylalanine residues at positions +1 and +5 of the AR FxxLF motif revealed that also FxxLM and FxxLW sequences are compatible with AR LBD binding (M is methionine; W is tryptophan (80)). However, both motifs interact weaker with AR LBD than the wild type FxxLF motif. Replacing F+1 by M or W resulted in non-interacting peptides, whereas the WxxLW variant interacted with an even lower affinity than the FxxLM and FxxLW peptides. This indicates that the AR coactivator groove is sufficiently flexible to allow other bulky hydrophobic residues at positions +1 and +5 as well. However, phenylalanines are indispensable for high affinity AR LBD interactions. The absence of leucine residues at both positions further substantiates the observations that the AR LBD does not favour binding of LxxLL sequences.

Substitution of L+4 in the AR FxxLF motif by any other amino acid revealed that this residue can be replaced by an F (FxxFF) or M (FxxMF) residue without losing AR LBD interaction capacity (see Chapter 3) (245). All other L+4 substituted motifs interact weakly or do not interact at all, demonstrating the preference for large hydrophobic residues at position +4 for binding AR LBD.

Also the importance of Q+2 and N+3 in the AR FxxLF motif has been investigated by random mutagenesis (46). Screening revealed that different combinations of a wide variety of amino acid residues are compatible with efficient AR LBD interactions. However, there was a preference for peptides with a negatively charged E at +2. The residue at +3 was less stringent, but consisted almost exclusively of polar and positively charged residues. The absence of hydrophobic residues at positions +2 and +3 is in agreement with the crystal structures of NR LBD-peptide complexes showing that these residues are solvent exposed and do not contact the AR LBD surface. Why there is a preference for certain types of residues is not understood, but it was proposed that they contribute to a more stable conformation of the peptide.

3.3.2 RANDOM SCREENINGS FOR AR-INTERACTING PEPTIDES

Phage display screenings have been carried out to select randomly for peptides that bind with high affinity to the AR LBD and full-length AR (244, 246, 247). These screenings yielded almost exclusively phenylalanine-rich motifs, including FxxLF, FxxFF, FxxVF, FxxYF, FxxLY, FxxFY, and FxxLW (V is valine; Y is tyrosine). Few peptides contained a W at +1 (WxxLF, WxxVW, and WxxLW), however, their interaction capacity with AR LBD are usually weaker than the phenylalanine-rich motifs, which corresponds with the mutagenesis studies of the AR FxxLF motif described above (80). This may also explain why the WxxLF motif in the AR NTD hardly contributes to AR N/C interaction (see section 1.3.3). The usually low affinity of the AR LBD for LxxLL sequences is reflected by the low number of LxxLL sequences retrieved from these screenings. In agreement with the mutagenesis studies, screening of random sequences demonstrated that a phenylalanine at +1 and bulky hydrophobic residues at positions +4 and +5 are indispensable for high-affinity AR LBD binding. These screenings did not reveal a clear-cut preference for specific residues outside the motif for AR LBD interaction.

3.4 STRUCTURAL ANALYSIS OF PEPTIDES BINDING TO THE AR LBD

Crystallographic studies of ligand-activated AR LBD complexed with peptide motifs provided detailed information about the structure of the coactivator-binding groove and the structural basis for the preference for FxxLF motifs (247-249). As reported for other NRs, the AR coactivator-groove is formed predominantly by hydrophobic residues in helices 3, 4, 5, and 12 (Figure 10A and B). Residues L712, F725, V716, I737, and I898 form the floor of the cleft, which is bounded on one side by residues V713, K717, and M894. The other ridge is formed by the helix 4 residues V730, Q733, M734, and Q738. The AR coactivator groove region further contains two oppositely charged amino acid clusters (250). A negatively charged cluster, formed by AR residues E709, E893, and E897, and a positively charged cluster, formed by residues K717, K720, and R726, are located at the opposite ends of the groove.

Superimposing the structures of AR LBD alone and AR LBD in complex with a peptide revealed no major rearrangements of the protein backbones upon peptide binding (247-249). Only side chains of residues that line the coactivator groove adopt a different orientation, which is consistent with an induced fit mechanism. Largest changes were observed for K720, M734, M894, and E897. These residues move away from each other to create a longer and wider groove, allowing the accommodation of the peptide side chains.

The structure of the AR LBD in complex with the AR FxxLF motif shows that the peptide forms, as predicted, an amphipathic α-helical structure in which the two phenylalanines at +1 and +5, and the leucine at +4 bind directly in the coactivator groove (Figures 10C and D) (249). Both phenylalanines bind face down in a deep and extended cleft on the LBD surface, rendering them almost completely solvent inaccessible. The coactivator groove of the AR LBD is deeper than of ER LBD, which allows more and better hydrophobic interactions with the bulky phenylalanines, resulting in a more optimal fit (60, 80, 249). L+4 binds a shallow hydrophobic depression and is

partly solvent exposed (249). The peptide is further stabilized with hydrogen bonds between the backbone amides of A-1 and F+1 and E897. The backbone carbonyl oxygens of F+5 and V+8 form hydrogen bonds with the side chain of K720. The residues at positions +2, +3, and +6 do not contact the AR LBD surface and are solvent exposed. Crystal structures of the AR LBD in complex with two other FxxLF peptides, of ARA70 and one derived by phage display, revealed that these peptides bound the coactivator groove similarly as the AR FxxLF peptide (247, 248). Both peptides were stabilized in the groove via interactions with K720 and E897.

Crystal structures of the AR LBD bound to LxxLL peptides, including TIF2 box III (Figure 10E and F) (248, 249), SRC3 box II (248), and a peptide derived from phage display (247), showed that the LxxLL peptides align along a similar helical axis as the FxxLF motifs. However, all LxxLL peptides are shifted in the groove toward K720, precluding interactions with E897. Besides distinct electrostatic interactions, the smaller leucine residues at positions +1 and +5 of LxxLL motifs result in fewer and less optimal hydrophobic interactions with the coactivator groove than phenylalanine residues.

The AR LBD has also been crystallized in complex with several peptides derived from the phage display screenings (247). These structures show that the tryptophan residues in WxxLF, FxxLW, and WxxVW motifs bind the coactivator groove in a similar orientation as phenylalanine residues. However, the larger indole ring of tryptophan causes the peptide backbones to shift toward K720 similarly as described for the LxxLL peptides. The main chain carbonyl groups are stabilized via hydrogen bonds with K720, but not with E897. Unlike LxxLL peptides, the tryptophan-containing peptides are stabilized to E897 via hydrogen bonds to a serine residue at position –2. Although the binding mode of an FxxFF peptide was nearly identical to that of the tryptophan peptides, binding of an FxxYF peptide resembled that of FxxLF. This conformation allowed the FxxYF peptide to be stabilized via direct backbone interactions to both K720 and E897. Because of the different helical geometry, the tyrosine residue of the FxxYF motif binds edgewise to the shallow +4 pocket, whereas the phenylalanine at +4 of the FxxFF motif binds face down.

Mutational analysis of the AR LBD largely confirmed the roles of the different charged residues in FxxLF and LxxLL peptide binding. As is shown in the crystal structures, binding of the AR and ARA70 FxxLF peptides involve both K720 and E897 (46, 80). However, E897 appeared to be crucial for binding the AR peptide, suggesting a dominant role of this residue in stabilizing the AR FxxLF motif. Binding of the ARA70 peptide was more dependent on the positively charged cluster in the AR LBD. The ARA54 FxxLF peptide, which has not been crystallized, was equally dependent on both K720 and E897 for AR LBD binding. This demonstrates that FxxLF peptides do not always depend on the same charged residues for binding to the AR LBD. This may be due to a slightly different conformation in the groove and/or to different interactions of sequences flanking the motif with the AR LBD surface. Consistent with the helical shifts observed in the crystal structures, binding of the majority of LxxLL peptides was dependent on K720, but not or weakly on E897 (46, 80). Remarkably, the dependency on the charged

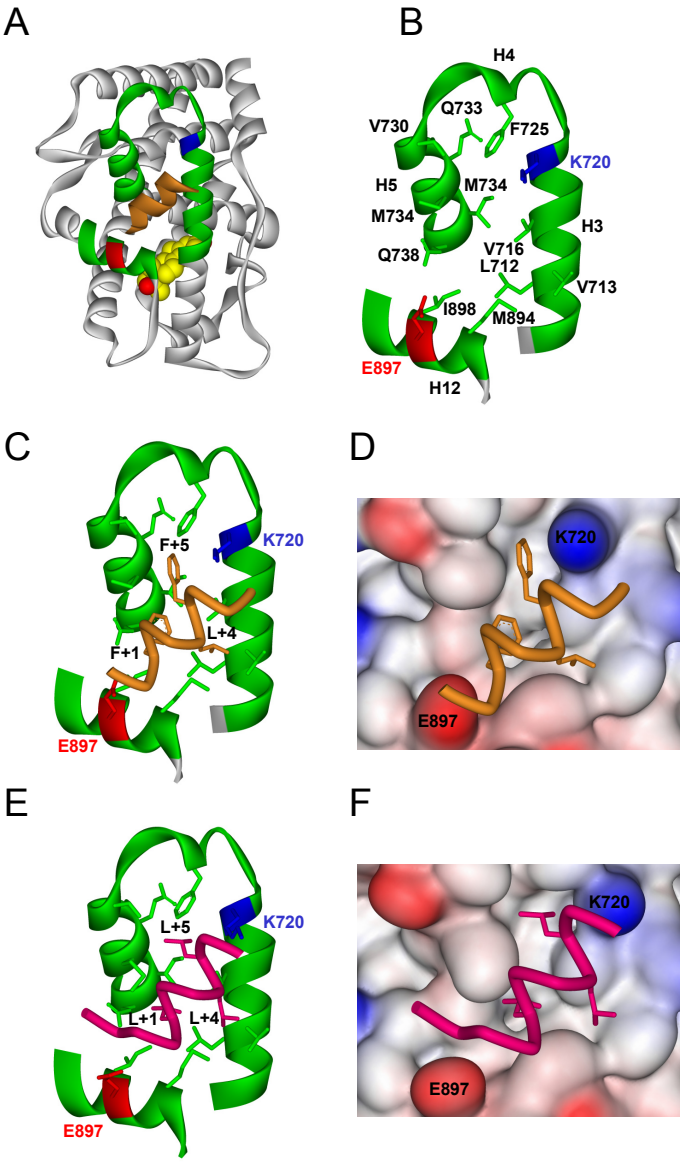

Figure 10. Structure of the AR FxxLF peptide bound to the AR LBD. (A) Global architecture of the AR FxxLF peptide (brown ribbon) and R1881 (space filled atoms yellow carbon and red oxygen) bound to AR LBD (white ribbon) with helices 3, 4, 5, and 12 (green ribbon) forming the coactivator binding site. Conserved charged residues (blue, positively charged lysine; red, negatively charged glutamic acid) at the opposite ends of the groove are indicated. (B) Close-up view of the AR coactivator groove. Helices 3, 4, 5, and 12 are shown (green ribbon). Side chains of residues composing the groove are shown as sticks. (C to F) Different representations of AR FxxLF (C and D) and TIF2 box III LxxLL (E and F) peptides bound to the AR LBD. The peptides are shown as orange and magenta Cα coils, respectively. For clarity only peptide side chains of the residues at positions +1, +4, and +5 are shown. The charged residues K720 and E897 are indicated for orientation.

residues in the groove is hardly affected by L to F substitutions of LxxLL peptides, indicating that in an identical amino acid context LxxLL peptides and their FxxLF variants have a similar position in the groove (46). This implies that flanking sequences are the main determinants for the observed differential dependency on charged residues.

Crystal structures and sequence alignments of AR with other NRs suggest that V713, V730, and M734 contribute to the preferential binding of FxxLF motifs versus LxxLL (247, 249). The unique combination of these residues in AR creates a deeper coactivator groove resulting in a flatter and smoother interaction surface, which has a higher complementarity to aromatic substituents than to branched aliphatic. The highly conserved tight binding mode of phenylalanine residues and tryptophan residues to the +1 pocket, suggests that other residues are not favoured at position +1 of the motif. The binding mode of the +5 residue is somewhat more variable, explaining why also other residues at this position, such as tyrosine, are allowed. The +4 binding site is shallow, surface exposed, and is characterized by non-specific interactions, explaining the assortment of residues found at the +4 position.

Overall, the shape of the groove explains why the interaction of a peptide with the AR LBD is preferentially driven by bulky aromatic residues (F, W, and Y) at positions +1 and +5, instead of smaller branched aliphatic leucine residues. This may also explain why the AR LBD has a relatively low affinity for p160 cofactors. The residue at position +4 appears to be less critical, whereas residues flanking the motif largely determine the specific mode of interaction of a peptide with the groove.

3.5 AR MUTATIONS AND LIGANDS AFFECT PEPTIDE RECRUITMENT TO THE AR LBD

3.5.1 AR GROOVE MUTATIONS

Several mutations in residues lining the AR coactivator groove have been identified in patients with MAIS (I737T), PAIS (L712F, F725L, Q733H, and I737T), and CAIS (I898T). Functional analysis demonstrated that these mutations severely impaired AR transcriptional activity. Binding studies showed that the affinities for R1881 were not affected by the mutations and were similar to wild type AR (73, 77, 79). Although the F725L, I737T, and Q733H mutations resulted in increased R1881 dissociation rates from full length AR, this was not observed if only the AR DBD-LBD fragment was used. This suggests that the hormone is insufficiently stabilized in the AR LBD because of defective N/C interaction. Indeed, mammalian interaction studies demonstrated that these mutations impaired N/C interaction, p160 recruitment, and binding of LxxLL and FxxLF peptides. Similar observations were made for the L712F and I898T mutants.

AR groove mutations have also been described in prostate cancer patients, and include V715M, K720E, V730M, and R726L. The V730M mutation did not affect recruitment of the AR FxxLF motif, but increased LBD affinity for SRC1 and TIF2 LxxLL motifs (249). Thus, aberrant cofactor recruitment may be a mechanism for increased activity of the AR V730M mutant.

AR V715M showed stronger FxxLF and LxxLL motif binding, suggesting that this mutation stabilizes N/C interaction and cofactor recruitment.

3.5.2 EFFECTS OF THE LIGAND-BINDING POCKET ON PEPTIDE RECRUITMENT

Peptide recruitment to the AR LBD may also be affected by the bound ligand. A study by Kazmin *et al.* showed that in the presence of androgens other FxxLF-like peptides were recruited to the AR than in the presence of agonistic selective androgen receptor modulators (SARMs) (251). Computer modelling showed that these ligands had distinct effects on the size and shape of the coactivator groove and on the distance between the charged clusters at the opposite ends of the groove.

Besides an effect of the bound ligand, peptide recruitment may also be influenced by mutations in the ligand-binding pocket. It has been reported that the AR T877A mutant recruits peptides more efficiently than wild type AR (52). This may be due to increased flexibility of amino acid residues in the groove and a larger solvent accessible surface than wild type AR, as was determined by molecular modelling (252). Another study showed that the AR mutants T877A, T877S, and H874Y preferentially bind LxxLL motifs in the presence of CPA, but preferred binding of FxxLF-like motifs in the presence of hydroxyflutamide (51). In agreement, ARA70, containing an FxxLF motif, was a more effective coactivator of AR T877A in the presence of hydroxyflutamide than SRC1, which contains LxxLL motifs (51). The opposite was found with CPA. Overall, this suggests that ligand- and mutation-dependent differences in motif preferences are a result of distinct receptor conformations.

3.5.3 BINDING FUNCTION-3

Functional and x-ray screens identified several compounds that bind the AR surface and block AR transcriptional activity (53). Crystal structures revealed that these compounds (the nonsteroidal anti-inflammatory drugs flufenamic acid and tolfenamic acid, and the thyroid hormones TRIAC and T3) preferentially bind to a surface adjacent to the coactivator groove, designated as binding function (BF)-3. Binding of these compounds to BF-3 result in reduced interactions of FxxLF and LxxLL peptides, suggesting that BF-3 functions as an allosteric regulatory site for protein interactions with the coactivator groove. However, the natural role of BF-3 in AR function remains to be uncovered.

Summarizing, motif interactions with the AR LBD are not only dependent on the integrity of the coactivator groove itself, but can be modulated by other AR LBD regions as well.

4. SCOPE OF THIS THESIS

The AR is essential for development and maintenance of the male phenotype, and plays an important role in several diseases, including prostate cancer. AR transcriptional activity is

triggered by hormone binding and is regulated by protein interactions. The best-studied protein interaction site in the AR is the coactivator groove in the LBD. This groove is induced upon hormone binding and serves as high-affinity docking surface for FxxLF motifs present in a subset of AR cofactors and in the AR NTD. The aim of this thesis is to investigate the interaction of the coactivator groove in the AR LBD with peptide motifs and with proteins. This knowledge is applied to investigate whether blockade of protein interactions with the AR coactivator groove may serve as an alternative target to inhibit AR transcriptional activity.

Chapter 2 describes a systematic structure-function analysis of AR amino acid residue 701, to obtain further insight in the cortisol response of the AR L701H mutant in prostate cancer. Transcription activation studies using a panel of structurally related hormones and molecular modelling provide detailed insight in the properties of AR L701H and related mutants.

As part of ongoing studies of peptide interactions with the AR LBD, **Chapter 3** describes a systematic mutational analysis of L+4 in the AR FxxLF motif. This screening reveals that also other residues are allowed at position +4 without losing AR LBD interaction capacity. We show that these novel motifs drive the interaction of two previously indentified AR cofactors.

Chapter 4 describes a random and a more focused functional screening of FxxLF-like motifs present in potentially AR-interacting proteins. Using this approach, BAF60a is identified as a novel AR cofactor. Further studies show that BAF60a, a subunit of the SWI/SNF chromatin remodelling complex, interacts with the AR via a novel type of motif and that it plays a critical role in the expression of a subset of AR-regulated genes.

In **Chapter 5** it is investigated whether the AR LBD can function as a target for blocking AR activity. The AR LBD is targeted with peptides containing FxxLF-like motifs, which were delivered into cells either by transfection of vectors expressing the peptide or by a cell-penetrating peptide. The effects of these peptides on AR-protein interactions and on AR transcriptional activity are studied.

In **Chapter 6**, the results obtained in the previous chapters are discussed in more detail and are put together in a broader context. Furthermore, directions for further research are suggested.

5. REFERENCES

1. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839
2. Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, Hochberg RB, McKay L, Renoir JM, Weigel NL, Wilson EM, McDonnell DP, Cidlowski JA 2006 International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev 58:782-797
3. Germain P, Staels B, Dacquet C, Spedding M, Laudet V 2006 Overview of nomenclature of nuclear receptors. Pharmacol Rev 58:685-704
4. 1999 Nuclear Receptors Nomenclature Committee: A unified nomenclature system for the nuclear receptor superfamily. Cell 97:161-163
5. Noy N 2007 Ligand specificity of nuclear hormone receptors: sifting through promiscuity. Biochemistry 46:13461-13467
6. Kimura N, Mizokami A, Oonuma T, Sasano H, Nagura H 1993 Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. J Histochem Cytochem 41:671-678
7. Ruizeveld de Winter JA, Trapman J, Vermey M, Mulder E, Zegers ND, van der Kwast TH 1991 Androgen receptor expression in human tissues: an immunohistochemical study. J Histochem Cytochem 39:927-936
8. Kuiper GG, Faber PW, van Rooij HC, van der Korput JA, Ris-Stalpers C, Klaassen P, Trapman J, Brinkmann AO 1989 Structural organization of the human androgen receptor gene. J Mol Endocrinol 2:R1-4
9. Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS 1989 Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc Natl Acad Sci U S A 86:9534-9538
10. Lavery DN, McEwan IJ 2005 Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem J 391:449-464
11. Reid J, Kelly SM, Watt K, Price NC, McEwan IJ 2002 Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions. J Biol Chem 277:20079-20086
12. Lavery DN, McEwan IJ 2006 The human androgen receptor AF1 transactivation domain: interactions with transcription factor IIF and molten-globule-like structural characteristics. Biochem Soc Trans 34:1054-1057
13. Lavery DN, McEwan IJ 2008 Structural characterization of the native NH2-terminal transactivation domain of the human androgen receptor: a collapsed disordered conformation underlies structural plasticity and protein-induced folding. Biochemistry 47:3360-3369
14. Jenster G, van der Korput HA, Trapman J, Brinkmann AO 1995 Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 270:7341-7346
15. Callewaert L, Van Tilborgh N, Claessens F 2006 Interplay between two hormone-independent activation domains in the androgen receptor. Cancer Res 66:543-553
16. Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B 1999 The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 19:6085-6097
17. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG 1999 The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 19:8383-8392

18. Claessens F, Denayer S, Van Tilborgh N, Kerkhofs S, Helsen C, Haelens A 2008 Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Recept Signal 6:e008
19. Palazzolo I, Gliozzi A, Rusmini P, Sau D, Crippa V, Simonini F, Onesto E, Bolzoni E, Poletti A 2008 The role of the polyglutamine tract in androgen receptor. J Steroid Biochem Mol Biol 108:245-253
20. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH 1991 Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77-79
21. Linja MJ, Visakorpi T 2004 Alterations of androgen receptor in prostate cancer. J Steroid Biochem Mol Biol 92:255-264
22. Casella R, Maduro MR, Lipshultz LI, Lamb DJ 2001 Significance of the polyglutamine tract polymorphism in the androgen receptor. Urology 58:651-656
23. Ding D, Xu L, Menon M, Reddy GP, Barrack ER 2005 Effect of GGC (glycine) repeat length polymorphism in the human androgen receptor on androgen action. Prostate 62:133-139
24. Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733-736
25. Steketee K, Berrevoets CA, Dubbink HJ, Doesburg P, Hersmus R, Brinkmann AO, Trapman J 2002 Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor. Eur J Biochem 269:5780-5791
26. He B, Kemppainen JA, Wilson EM 2000 FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986-22994
27. Freedman LP 1992 Anatomy of the steroid receptor zinc finger region. Endocr Rev 13:129-145
28. Shaffer PL, Jivan A, Dollins DE, Claessens F, Gewirth DT 2004 Structural basis of androgen receptor binding to selective androgen response elements. Proc Natl Acad Sci U S A 101:4758-4763
29. Zilliacus J, Wright AP, Carlstedt-Duke J, Gustafsson JA 1995 Structural determinants of DNA-binding specificity by steroid receptors. Mol Endocrinol 9:389-400
30. Centenera MM, Harris JM, Tilley WD, Butler LM 2008 The Contribution of Different Androgen Receptor Domains to Receptor Dimerization and Signaling. Mol Endocrinol
31. Black BE, Holaska JM, Rastinejad F, Paschal BM 2001 DNA binding domains in diverse nuclear receptors function as nuclear export signals. Curr Biol 11:1749-1758
32. Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM 1994 A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. J Biol Chem 269:13115-13123
33. Jenster G, van der Korput JA, Trapman J, Brinkmann AO 1992 Functional domains of the human androgen receptor. J Steroid Biochem Mol Biol 41:671-675
34. Saporita AJ, Zhang Q, Navai N, Dincer Z, Hahn J, Cai X, Wang Z 2003 Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor. J Biol Chem 278:41998-42005
35. Haelens A, Tanner T, Denayer S, Callewaert L, Claessens F 2007 The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor. Cancer Res 67:4514-4523
36. Wang Q, Lu J, Yong EL 2001 Ligand- and coactivator-mediated transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J Biol Chem 276:7493-7499
37. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Basler S, Schafer M, Egner U, Carrondo MA 2000 Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 275:26164-26171
38. Pereira de Jesus-Tran K, Cote PL, Cantin L, Blanchet J, Labrie F, Breton R 2006 Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity. Protein Sci 15:987-999

39. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR, Jr., Weinmann R, Einspahr HM 2001 Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci U S A 98:4904-4909
40. Bourguet W, Germain P, Gronemeyer H 2000 Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol Sci 21:381-388
41. Greschik H, Moras D 2003 Structure-activity relationship of nuclear receptor-ligand interactions. Curr Top Med Chem 3:1573-1599
42. Moras D, Gronemeyer H 1998 The nuclear receptor ligand-binding domain: structure and function. Curr Opin Cell Biol 10:384-391
43. Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ, Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 12:3343-3356
44. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, Stallcup MR 1998 Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 12:302-313
45. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19:8226-8239
46. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, Trapman J 2006 Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping. Mol Endocrinol 20:1742-1755
47. He B, Minges JT, Lee LW, Wilson EM 2002 The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226-10235
48. Wang L, Hsu CL, Ni J, Wang PH, Yeh S, Keng P, Chang C 2004 Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol 24:2202-2213
49. Kuil CW, Berrevoets CA, Mulder E 1995 Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. Studies on the mechanism of antiandrogen action. J Biol Chem 270:27569-27576
50. Kuil CW, Mulder E 1994 Mechanism of antiandrogen action: conformational changes of the receptor. Mol Cell Endocrinol 102:R1-5
51. Brooke GN, Parker MG, Bevan CL 2008 Mechanisms of androgen receptor activation in advanced prostate cancer: differential co-activator recruitment and gene expression. Oncogene 27:2941-2950
52. Ozers MS, Marks BD, Gowda K, Kupcho KR, Ervin KM, De Rosier T, Qadir N, Eliason HC, Riddle SM, Shekhani MS 2007 The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay. Biochemistry 46:683-695
53. Estebanez-Perpina E, Arnold AA, Nguyen P, Rodrigues ED, Mar E, Bateman R, Pallai P, Shokat KM, Baxter JD, Guy RK, Webb P, Fletterick RJ 2007 A surface on the androgen receptor that allosterically regulates coactivator binding. Proc Natl Acad Sci U S A 104:16074-16079
54. Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, Wang C, Mizokami A 1995 Androgen receptor: an overview. Crit Rev Eukaryot Gene Expr 5:97-125
55. Lee HJ, Chang C 2003 Recent advances in androgen receptor action. Cell Mol Life Sci 60:1613-1622

56. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16:271-321
57. Imamoto T, Suzuki H, Yano M, Kawamura K, Kamiya N, Araki K, Komiya A, Nihei N, Naya Y, Ichikawa T 2008 The role of testosterone in the pathogenesis of prostate cancer. Int J Urol 15:472-480
58. Miller WL 1988 Molecular biology of steroid hormone synthesis. Endocr Rev 9:295-318
59. Russell DW, Wilson JD 1994 Steroid 5 alpha-reductase: two genes/two enzymes. Annu Rev Biochem 63:25-61
60. Gao W, Bohl CE, Dalton JT 2005 Chemistry and structural biology of androgen receptor. Chem Rev 105:3352-3370
61. Prescott J, Coetzee GA 2006 Molecular chaperones throughout the life cycle of the androgen receptor. Cancer Lett 231:12-19
62. Black BE, Vitto MJ, Gioeli D, Spencer A, Afshar N, Conaway MR, Weber MJ, Paschal BM 2004 Transient, ligand-dependent arrest of the androgen receptor in subnuclear foci alters phosphorylation and coactivator interactions. Mol Endocrinol 18:834-850
63. Davies TH, Ning YM, Sanchez ER 2002 A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem 277:4597-4600
64. Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN 2000 Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol Endocrinol 14:1618-1626
65. Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J 1997 Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Biochemistry 36:1052-1064
66. Nemoto T, Ohara-Nemoto Y, Shimazaki S, Ota M 1994 Dimerization characteristics of the DNA- and steroid-binding domains of the androgen receptor. J Steroid Biochem Mol Biol 50:225-233
67. Berrevoets CA, Doesburg P, Steketee K, Trapman J, Brinkmann AO 1998 Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2). Mol Endocrinol 12:1172-1183
68. van Royen ME, Houtsmuller AB, Trapman J 2009 A two-step model for androgen receptor dimerization in living cells. Submitted
69. Langley E, Kemppainen JA, Wilson EM 1998 Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. J Biol Chem 273:92-101
70. Langley E, Zhou ZX, Wilson EM 1995 Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. J Biol Chem 270:29983-29990
71. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM 1995 Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 9:208-218
72. Ikonen T, Palvimo JJ, Janne OA 1997 Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem 272:29821-29828
73. He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM 1999 Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J Biol Chem 274:37219-37225
74. Dehm SM, Regan KM, Schmidt LJ, Tindall DJ 2007 Selective role of an NH2-terminal WxxLF motif for aberrant androgen receptor activation in androgen depletion independent prostate cancer cells. Cancer Res 67:10067-10077
75. Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, Miner JN, Diamond MI 2005 The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. Proc Natl Acad Sci U S A 102:9802-9807

76. van Royen ME, Cunha SM, Brink MC, Mattern KA, Nigg AL, Dubbink HJ, Verschure PJ, Trapman J, Houtsmuller AB 2007 Compartmentalization of androgen receptor protein-protein interactions in living cells. J Cell Biol 177:63-72
77. Quigley CA, Tan JA, He B, Zhou ZX, Mebarki F, Morel Y, Forest MG, Chatelain P, Ritzen EM, French FS, Wilson EM 2004 Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH(2)- and carboxyl-terminal interaction. Mech Ageing Dev 125:683-695
78. Jaaskelainen J, Deeb A, Schwabe JW, Mongan NP, Martin H, Hughes IA 2006 Human androgen receptor gene ligand-binding-domain mutations leading to disrupted interaction between the N- and C-terminal domains. J Mol Endocrinol 36:361-368
79. He B, Gampe RT, Jr., Hnat AT, Faggart JL, Minges JT, French FS, Wilson EM 2006 Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity. J Biol Chem 281:6648-6663
80. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J 2004 Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132-2150
81. He B, Bowen NT, Minges JT, Wilson EM 2001 Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 276:42293-42301
82. Callewaert L, Verrijdt G, Christiaens V, Haelens A, Claessens F 2003 Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements. J Biol Chem 278:8212-8218
83. He B, Lee LW, Minges JT, Wilson EM 2002 Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. J Biol Chem 277:25631-25639
84. Li J, Fu J, Toumazou C, Yoon HG, Wong J 2006 A role of the amino-terminal (N) and carboxyl-terminal (C) interaction in binding of androgen receptor to chromatin. Mol Endocrinol 20:776-785
85. Klokk TI, Kurys P, Elbi C, Nagaich AK, Hendarwanto A, Slagsvold T, Chang CY, Hager GL, Saatcioglu F 2007 Ligand-specific dynamics of the androgen receptor at its response element in living cells. Mol Cell Biol 27:1823-1843
86. Glass CK 1994 Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr Rev 15:391-407
87. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 352:497-505
88. Schwabe JW, Chapman L, Finch JT, Rhodes D 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell 75:567-578
89. Steketee K, Ziel-van der Made AC, van der Korput HA, Houtsmuller AB, Trapman J 2004 A bioinformatics-based functional analysis shows that the specifically androgen-regulated gene SARG contains an active direct repeat androgen response element in the first intron. J Mol Endocrinol 33:477-491
90. Barbulescu K, Geserick C, Schuttke I, Schleuning WD, Haendler B 2001 New androgen response elements in the murine pem promoter mediate selective transactivation. Mol Endocrinol 15:1803-1816
91. Schoenmakers E, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, Claessens F 2000 Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses. J Biol Chem 275:12290-12297
92. Verrijdt G, Haelens A, Claessens F 2003 Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. Mol Genet Metab 78:175-185
93. Verrijdt G, Schoenmakers E, Alen P, Haelens A, Peeters B, Rombauts W, Claessens F 1999 Androgen specificity of a response unit upstream of the human secretory component gene is mediated by

differential receptor binding to an essential androgen response element. Mol Endocrinol 13:1558-1570
94. Wong CI, Zhou ZX, Sar M, Wilson EM 1993 Steroid requirement for androgen receptor dimerization and DNA binding. Modulation by intramolecular interactions between the NH2-terminal and steroid-binding domains. J Biol Chem 268:19004-19012
95. Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR 2007 Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev 21:2005-2017
96. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, Mills IG 2007 New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. EMBO Rep 8:871-878
97. Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M 2007 A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 27:380-392
98. Takayama K, Kaneshiro K, Tsutsumi S, Horie-Inoue K, Ikeda K, Urano T, Ijichi N, Ouchi Y, Shirahige K, Aburatani H, Inoue S 2007 Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis. Oncogene 26:4453-4463
99. Dehm SM, Tindall DJ 2006 Molecular regulation of androgen action in prostate cancer. J Cell Biochem 99:333-344
100. Kaarbo M, Klokk TI, Saatcioglu F 2007 Androgen signaling and its interactions with other signaling pathways in prostate cancer. Bioessays 29:1227-1238
101. Lilja H 2003 Biology of prostate-specific antigen. Urology 62:27-33
102. Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW 1998 Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 35:275-368
103. Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L, Nelson PS 1999 Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res 59:4180-4184
104. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM 2005 Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644-648
105. Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B 2002 The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 99:11890-11895
106. Velasco AM, Gillis KA, Li Y, Brown EL, Sadler TM, Achilleos M, Greenberger LM, Frost P, Bai W, Zhang Y 2004 Identification and validation of novel androgen-regulated genes in prostate cancer. Endocrinology 145:3913-3924
107. Zlatanova J, Seebart C, Tomschik M 2008 The linker-protein network: control of nucleosomal DNA accessibility. Trends Biochem Sci 33:247-253
108. Heemers HV, Tindall DJ 2007 Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 28:778-808
109. Kinyamu HK, Archer TK 2004 Modifying chromatin to permit steroid hormone receptor-dependent transcription. Biochim Biophys Acta 1677:30-45
110. Trotter KW, Archer TK 2007 Nuclear receptors and chromatin remodeling machinery. Mol Cell Endocrinol 265-266:162-167
111. Rosenfeld MG, Lunyak VV, Glass CK 2006 Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 20:1405-1428

112. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM 1997 Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90:569-580
113. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194-198
114. Huang ZQ, Li J, Sachs LM, Cole PA, Wong J 2003 A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. Embo J 22:2146-2155
115. Sheppard HM, Harries JC, Hussain S, Bevan C, Heery DM 2001 Analysis of the steroid receptor coactivator 1 (SRC1)-CREB binding protein interaction interface and its importance for the function of SRC1. Mol Cell Biol 21:39-50
116. Trotter KW, Archer TK 2008 The BRG1 transcriptional coregulator. Nucl Recept Signal 6:e004
117. Fan HY, He X, Kingston RE, Narlikar GJ 2003 Distinct strategies to make nucleosomal DNA accessible. Mol Cell 11:1311-1322
118. Phelan ML, Sif S, Narlikar GJ, Kingston RE 1999 Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. Mol Cell 3:247-253
119. Lemon B, Inouye C, King DS, Tjian R 2001 Selectivity of chromatin-remodelling cofactors for ligand-activated transcription. Nature 414:924-928
120. Nie Z, Xue Y, Yang D, Zhou S, Deroo BJ, Archer TK, Wang W 2000 A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol Cell Biol 20:8879-8888
121. Wang W, Cote J, Xue Y, Zhou S, Khavari PA, Biggar SR, Muchardt C, Kalpana GV, Goff SP, Yaniv M, Workman JL, Crabtree GR 1996 Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. Embo J 15:5370-5382
122. Wang W, Xue Y, Zhou S, Kuo A, Cairns BR, Crabtree GR 1996 Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev 10:2117-2130
123. Marshall TW, Link KA, Petre-Draviam CE, Knudsen KE 2003 Differential requirement of SWI/SNF for androgen receptor activity. J Biol Chem 278:30605-30613
124. Link KA, Burd CJ, Williams E, Marshall T, Rosson G, Henry E, Weissman B, Knudsen KE 2005 BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. Mol Cell Biol 25:2200-2215
125. Link KA, Balasubramaniam S, Sharma A, Comstock CE, Godoy-Tundidor S, Powers N, Cao KH, Haelens A, Claessens F, Revelo MP, Knudsen KE 2008 Targeting the BAF57 SWI/SNF subunit in prostate cancer: a novel platform to control androgen receptor activity. Cancer Res 68:4551-4558
126. Vijayvargia R, May MS, Fondell JD 2007 A coregulatory role for the mediator complex in prostate cancer cell proliferation and gene expression. Cancer Res 67:4034-4041
127. Wang Q, Sharma D, Ren Y, Fondell JD 2002 A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression. J Biol Chem 277:42852-42858
128. Coulthard VH, Matsuda S, Heery DM 2003 An extended LXXLL motif sequence determines the nuclear receptor binding specificity of TRAP220. J Biol Chem 278:10942-10951
129. McEwan IJ, Gustafsson J 1997 Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc Natl Acad Sci U S A 94:8485-8490
130. Reid J, Murray I, Watt K, Betney R, McEwan IJ 2002 The androgen receptor interacts with multiple regions of the large subunit of general transcription factor TFIIF. J Biol Chem 277:41247-41253
131. Lee DK, Duan HO, Chang C 2000 From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. J Biol Chem 275:9308-9313
132. Lee DK, Li M, Chang C 2003 The second largest subunit of RNA polymerase II interacts with and enhances transactivation of androgen receptor. Biochem Biophys Res Commun 302:162-169

133. Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J 1997 An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol Endocrinol 11:148-161
134. Riegman PH, Vlietstra RJ, van der Korput JA, Brinkmann AO, Trapman J 1991 The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol 5:1921-1930
135. Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. Mol Cell 9:601-610
136. Kang Z, Janne OA, Palvimo JJ 2004 Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. Mol Endocrinol 18:2633-2648
137. Louie MC, Yang HQ, Ma AH, Xu W, Zou JX, Kung HJ, Chen HW 2003 Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc Natl Acad Sci U S A 100:2226-2230
138. Kang Z, Pirskanen A, Janne OA, Palvimo JJ 2002 Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. J Biol Chem 277:48366-48371
139. Masiello D, Cheng S, Bubley GJ, Lu ML, Balk SP 2002 Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. J Biol Chem 277:26321-26326
140. Farla P, Hersmus R, Trapman J, Houtsmuller AB 2005 Antiandrogens prevent stable DNA-binding of the androgen receptor. J Cell Sci 118:4187-4198
141. Adachi H, Waza M, Katsuno M, Tanaka F, Doyu M, Sobue G 2007 Pathogenesis and molecular targeted therapy of spinal and bulbar muscular atrophy. Neuropathol Appl Neurobiol 33:135-151
142. Poletti A, Negri-Cesi P, Martini L 2005 Reflections on the diseases linked to mutations of the androgen receptor. Endocrine 28:243-262
143. Monks DA, Rao P, Mo K, Johansen JA, Lewis G, Kemp MQ 2008 Androgen receptor and Kennedy disease/spinal bulbar muscular atrophy. Horm Behav 53:729-740
144. Brinkmann AO 2001 Molecular basis of androgen insensitivity. Mol Cell Endocrinol 179:105-109
145. Hughes IA, Deeb A 2006 Androgen resistance. Best Pract Res Clin Endocrinol Metab 20:577-598
146. Trapman J, Dubbink HJ 2007 The role of cofactors in sex steroid action. Best Pract Res Clin Endocrinol Metab 21:403-414
147. Mooney SD, Klein TE, Altman RB, Trifiro MA, Gottlieb B 2003 A functional analysis of disease-associated mutations in the androgen receptor gene. Nucleic Acids Res 31:e42
148. Gottlieb B, Beitel LK, Wu JH, Trifiro M 2004 The androgen receptor gene mutations database (ARDB): 2004 update. Hum Mutat 23:527-533
149. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ 2008 Cancer statistics, 2008. CA Cancer J Clin 58:71-96
150. Agoulnik IU, Weigel NL 2006 Androgen receptor action in hormone-dependent and recurrent prostate cancer. J Cell Biochem 99:362-372
151. Feldman BJ, Feldman D 2001 The development of androgen-independent prostate cancer. Nat Rev Cancer 1:34-45
152. McPhaul MJ 2008 Mechanisms of prostate cancer progression to androgen independence. Best Pract Res Clin Endocrinol Metab 22:373-388
153. Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A 1995 Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 55:3068-3072
154. Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, Verleun-Mooijman MC, Trapman J, Brinkmann AO, Santerse AB, Schroder FH, van der Kwast TH 1994 Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. Am J Pathol 144:735-746
155. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, Trapman J 1991 Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 48:189-193

156. Mohler JL 2008 A role for the androgen-receptor in clinically localized and advanced prostate cancer. Best Pract Res Clin Endocrinol Metab 22:357-372
157. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ 2002 Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 62:1008-1013
158. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE 2005 Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. J Steroid Biochem Mol Biol 96:251-258
159. Eder IE, Haag P, Basik M, Mousses S, Bektic J, Bartsch G, Klocker H 2003 Gene expression changes following androgen receptor elimination in LNCaP prostate cancer cells. Mol Carcinog 37:181-191
160. Liao X, Tang S, Thrasher JB, Griebling TL, Li B 2005 Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. Mol Cancer Ther 4:505-515
161. Compagno D, Merle C, Morin A, Gilbert C, Mathieu JR, Bozec A, Mauduit C, Benahmed M, Cabon F 2007 SIRNA-directed in vivo silencing of androgen receptor inhibits the growth of castration-resistant prostate carcinomas. PLoS ONE 2:e1006
162. Li TH, Zhao H, Peng Y, Beliakoff J, Brooks JD, Sun Z 2007 A promoting role of androgen receptor in androgen-sensitive and -insensitive prostate cancer cells. Nucleic Acids Res 35:2767-2776
163. Petrovics G, Liu A, Shaheduzzaman S, Furusato B, Sun C, Chen Y, Nau M, Ravindranath L, Chen Y, Dobi A, Srikantan V, Sesterhenn IA, McLeod DG, Vahey M, Moul JW, Srivastava S 2005 Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. Oncogene 24:3847-3852
164. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM 2008 Recurrent gene fusions in prostate cancer. Nat Rev Cancer 8:497-511
165. Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, Mehra R, Chinnaiyan AM 2008 Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. Cancer Res 68:73-80
166. Hermans KG, Bressers AA, van der Korput HA, Dits NF, Jenster G, Trapman J 2008 Two unique novel prostate-specific and androgen-regulated fusion partners of ETV4 in prostate cancer. Cancer Res 68:3094-3098
167. Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, Chinnaiyan AM 2006 TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res 66:3396-3400
168. Hermans KG, van der Korput HA, van Marion R, van de Wijngaart DJ, Ziel-van der Made A, Dits NF, Boormans JL, van der Kwast TH, van Dekken H, Bangma CH, Korsten H, Kraaij R, Jenster G, Trapman J 2008 Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer. Cancer Res 68:7541-7549
169. Mehra R, Tomlins SA, Yu J, Cao X, Wang L, Menon A, Rubin MA, Pienta KJ, Shah RB, Chinnaiyan AM 2008 Characterization of TMPRSS2-ETS gene aberrations in androgen-independent metastatic prostate cancer. Cancer Res 68:3584-3590
170. Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, Yu J, Wang L, Montie JE, Rubin MA, Pienta KJ, Roulston D, Shah RB, Varambally S, Mehra R, Chinnaiyan AM 2007 Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature 448:595-599
171. Hermans KG, van Marion R, van Dekken H, Jenster G, van Weerden WM, Trapman J 2006 TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. Cancer Res 66:10658-10663
172. Narod SA, Seth A, Nam R 2008 Fusion in the ETS gene family and prostate cancer. Br J Cancer 99:847-851

173. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T 2001 Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 61:3550-3555
174. Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, Willi N, Mihatsch MJ, Sauter G, Kallioniemi OP 1999 Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. Cancer Res 59:803-806
175. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP 1995 In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 9:401-406
176. Edwards J, Krishna NS, Grigor KM, Bartlett JM 2003 Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer 89:552-556
177. Ford OH, 3rd, Gregory CW, Kim D, Smitherman AB, Mohler JL 2003 Androgen receptor gene amplification and protein expression in recurrent prostate cancer. J Urol 170:1817-1821
178. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP 1997 Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 57:314-319
179. Dehm SM, Tindall DJ 2005 Regulation of androgen receptor signaling in prostate cancer. Expert Rev Anticancer Ther 5:63-74
180. Newmark JR, Hardy DO, Tonb DC, Carter BS, Epstein JI, Isaacs WB, Brown TR, Barrack ER 1992 Androgen receptor gene mutations in human prostate cancer. Proc Natl Acad Sci U S A 89:6319-6323
181. Tilley WD, Buchanan G, Hickey TE, Bentel JM 1996 Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res 2:277-285
182. Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ 2000 Androgen receptor mutations in prostate cancer. Cancer Res 60:944-949
183. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP 1999 Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 59:2511-2515
184. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP 1995 Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393-1398
185. Haapala K, Hyytinen ER, Roiha M, Laurila M, Rantala I, Helin HJ, Koivisto PA 2001 Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide. Lab Invest 81:1647-1651
186. Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EJ, Cancer and Leukemia Group BS 2003 Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol 21:2673-2678
187. Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD 2001 Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res 7:1273-1281
188. Bergerat JP, Ceraline J 2008 Pleiotropic functional properties of androgen receptor mutants in prostate cancer. Hum Mutat
189. Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO, Trapman J 2002 Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer 100:309-317
190. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E 1990 A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 173:534-540

191. Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM, Feldman D 2000 Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. Nat Med 6:703-706
192. Shi XB, Ma AH, Xia L, Kung HJ, de Vere White RW 2002 Functional analysis of 44 mutant androgen receptors from human prostate cancer. Cancer Res 62:1496-1502
193. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM 2007 Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 120:719-733
194. Rahman M, Miyamoto H, Chang C 2004 Androgen receptor coregulators in prostate cancer: mechanisms and clinical implications. Clin Cancer Res 10:2208-2219
195. So A, Gleave M, Hurtado-Col A, Nelson C 2005 Mechanisms of the development of androgen independence in prostate cancer. World J Urol 23:1-9
196. Jenster G 1999 The role of the androgen receptor in the development and progression of prostate cancer. Semin Oncol 26:407-421
197. Mohler JL, Gregory CW, Ford OH, 3rd, Kim D, Weaver CM, Petrusz P, Wilson EM, French FS 2004 The androgen axis in recurrent prostate cancer. Clin Cancer Res 10:440-448
198. Nishiyama T, Hashimoto Y, Takahashi K 2004 The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. Clin Cancer Res 10:7121-7126
199. Mostaghel EA, Nelson PS 2008 Intracrine androgen metabolism in prostate cancer progression: mechanisms of castration resistance and therapeutic implications. Best Pract Res Clin Endocrinol Metab 22:243-258
200. Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS 2007 Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res 67:5033-5041
201. Holzbeierlein J, Lal P, LaTulippe E, Smith A, Satagopan J, Zhang L, Ryan C, Smith S, Scher H, Scardino P, Reuter V, Gerald WL 2004 Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. Am J Pathol 164:217-227
202. Stanbrough M, Bubley GJ, Ross K, Golub TR, Rubin MA, Penning TM, Febbo PG, Balk SP 2006 Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer. Cancer Res 66:2815-2825
203. Locke JA, Guns ES, Lubik AA, Adomat HH, Hendy SC, Wood CA, Ettinger SL, Gleave ME, Nelson CC 2008 Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 68:6407-6415
204. Montgomery RB, Mostaghel EA, Vessella R, Hess DL, Kalhorn TF, Higano CS, True LD, Nelson PS 2008 Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth. Cancer Res 68:4447-4454
205. Bledsoe RK, Madauss KP, Holt JA, Apolito CJ, Lambert MH, Pearce KH, Stanley TB, Stewart EL, Trump RP, Willson TM, Williams SP 2005 A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. J Biol Chem 280:31283-31293
206. Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE 2002 Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell 110:93-105
207. Williams SP, Sigler PB 1998 Atomic structure of progesterone complexed with its receptor. Nature 393:392-396

208. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753-758
209. Kauppi B, Jakob C, Farnegardh M, Yang J, Ahola H, Alarcon M, Calles K, Engstrom O, Harlan J, Muchmore S, Ramqvist AK, Thorell S, Ohman L, Greer J, Gustafsson JA, Carlstedt-Duke J, Carlquist M 2003 The three-dimensional structures of antagonistic and agonistic forms of the glucocorticoid receptor ligand-binding domain: RU-486 induces a transconformation that leads to active antagonism. J Biol Chem 278:22748-22754
210. Nahoum V, Bourguet W 2007 Androgen and estrogen receptors: potential of crystallography in the fight against cancer. Int J Biochem Cell Biol 39:1280-1287
211. Poujol N, Wurtz JM, Tahiri B, Lumbroso S, Nicolas JC, Moras D, Sultan C 2000 Specific recognition of androgens by their nuclear receptor. A structure-function study. J Biol Chem 275:24022-24031
212. Askew EB, Gampe RT, Jr., Stanley TB, Faggart JL, Wilson EM 2007 Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. J Biol Chem 282:25801-25816
213. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA 1980 The LNCaP cell line--a new model for studies on human prostatic carcinoma. Prog Clin Biol Res 37:115-132
214. Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, Srivastava S 1994 Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res 54:2861-2864
215. Suzuki H, Akakura K, Komiya A, Aida S, Akimoto S, Shimazaki J 1996 Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome. Prostate 29:153-158
216. Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J 1993 Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol 46:759-765
217. Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E 1992 The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol 41:665-669
218. Fenton MA, Shuster TD, Fertig AM, Taplin ME, Kolvenbag G, Bubley GJ, Balk SP 1997 Functional characterization of mutant androgen receptors from androgen-independent prostate cancer. Clin Cancer Res 3:1383-1388
219. Bohl CE, Miller DD, Chen J, Bell CE, Dalton JT 2005 Structural basis for accommodation of nonsteroidal ligands in the androgen receptor. J Biol Chem 280:37747-37754
220. Bohl CE, Wu Z, Miller DD, Bell CE, Dalton JT 2007 Crystal structure of the T877A human androgen receptor ligand-binding domain complexed to cyproterone acetate provides insight for ligand-induced conformational changes and structure-based drug design. J Biol Chem 282:13648-13655
221. Yoshida T, Kinoshita H, Segawa T, Nakamura E, Inoue T, Shimizu Y, Kamoto T, Ogawa O 2005 Antiandrogen bicalutamide promotes tumor growth in a novel androgen-dependent prostate cancer xenograft model derived from a bicalutamide-treated patient. Cancer Res 65:9611-9616
222. Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, Miyamoto M 2003 Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. Cancer Res 63:149-153
223. Bohl CE, Gao W, Miller DD, Bell CE, Dalton JT 2005 Structural basis for antagonism and resistance of bicalutamide in prostate cancer. Proc Natl Acad Sci U S A 102:6201-6206
224. Wainstein MA, He F, Robinson D, Kung HJ, Schwartz S, Giaconia JM, Edgehouse NL, Pretlow TP, Bodner DR, Kursh ED, et al. 1994 CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. Cancer Res 54:6049-6052
225. Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL, French FS 1997 Dehydroepiandrosterone activates mutant an-

drogen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. Mol Endocrinol 11:450-459
226. Watanabe M, Ushijima T, Shiraishi T, Yatani R, Shimazaki J, Kotake T, Sugimura T, Nagao M 1997 Genetic alterations of androgen receptor gene in Japanese human prostate cancer. Jpn J Clin Oncol 27:389-393
227. Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM, Johnston D, Pollack A, Pathak S, von Eschenbach AC, Logothetis CJ 1997 Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin Cancer Res 3:2493-2500
228. Zhao XY, Boyle B, Krishnan AV, Navone NM, Peehl DM, Feldman D 1999 Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a. J Urol 162:2192-2199
229. Krishnan AV, Zhao XY, Swami S, Brive L, Peehl DM, Ely KR, Feldman D 2002 A glucocorticoid-responsive mutant androgen receptor exhibits unique ligand specificity: therapeutic implications for androgen-independent prostate cancer. Endocrinology 143:1889-1900
230. Matias PM, Carrondo MA, Coelho R, Thomaz M, Zhao XY, Wegg A, Crusius K, Egner U, Donner P 2002 Structural basis for the glucocorticoid response in a mutant human androgen receptor (AR(ccr)) derived from an androgen-independent prostate cancer. J Med Chem 45:1439-1446
231. Le Douarin B, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R, Chambon P 1996 A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. Embo J 15:6701-6715
232. Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, Milburn MV 1998 Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature 395:137-143
233. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927-937
234. Needham M, Raines S, McPheat J, Stacey C, Ellston J, Hoare S, Parker M 2000 Differential interaction of steroid hormone receptors with LXXLL motifs in SRC-1a depends on residues flanking the motif. J Steroid Biochem Mol Biol 72:35-46
235. Leers J, Treuter E, Gustafsson JA 1998 Mechanistic principles in NR box-dependent interaction between nuclear hormone receptors and the coactivator TIF2. Mol Cell Biol 18:6001-6013
236. McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Inostroza J, Torchia J, Nolte RT, Assa-Munt N, Milburn MV, Glass CK, Rosenfeld MG 1998 Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev 12:3357-3368
237. Warnmark A, Treuter E, Gustafsson JA, Hubbard RE, Brzozowski AM, Pike AC 2002 Interaction of transcriptional intermediary factor 2 nuclear receptor box peptides with the coactivator binding site of estrogen receptor alpha. J Biol Chem 277:21862-21868
238. Ko L, Cardona GR, Iwasaki T, Bramlett KS, Burris TP, Chin WW 2002 Ser-884 adjacent to the LXXLL motif of coactivator TRBP defines selectivity for ERs and TRs. Mol Endocrinol 16:128-140
239. Darimont BD 2003 Finding specificity within a conserved interaction site. Chem Biol 10:675-676
240. Li Y, Suino K, Daugherty J, Xu HE 2005 Structural and biochemical mechanisms for the specificity of hormone binding and coactivator assembly by mineralocorticoid receptor. Mol Cell 19:367-380
241. Chang CY, McDonnell DP 2002 Evaluation of ligand-dependent changes in AR structure using peptide probes. Mol Endocrinol 16:647-660
242. Hall JM, Chang CY, McDonnell DP 2000 Development of peptide antagonists that target estrogen receptor beta-coactivator interactions. Mol Endocrinol 14:2010-2023
243. Kurebayashi S, Nakajima T, Kim SC, Chang CY, McDonnell DP, Renaud JP, Jetten AM 2004 Selective LXXLL peptides antagonize transcriptional activation by the retinoid-related orphan receptor RORgamma. Biochem Biophys Res Commun 315:919-927

244. Chang CY, Abdo J, Hartney T, McDonnell DP 2005 Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol Endocrinol 19:2478-2490
245. van de Wijngaart DJ, van Royen ME, Hersmus R, Pike AC, Houtsmuller AB, Jenster G, Trapman J, Dubbink HJ 2006 Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain. J Biol Chem 281:19407-19416
246. Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C 2003 The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691-23698
247. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ 2004 Recognition and Accommodation at the Androgen Receptor Coactivator Binding Interface. PLoS Biol 2:E274
248. Estebanez-Perpina E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, Buehrer BM, Webb P, Fletterick RJ, Guy RK 2005 The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280:8060-8068
249. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM 2004 Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425-438
250. He B, Wilson EM 2003 Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs. Mol Cell Biol 23:2135-2150
251. Kazmin D, Prytkova T, Cook CE, Wolfinger R, Chu TM, Beratan D, Norris JD, Chang CY, McDonnell DP 2006 Linking ligand-induced alterations in androgen receptor structure to differential gene expression: a first step in the rational design of selective androgen receptor modulators. Mol Endocrinol 20:1201-1217
252. Southwell J, Chowdhury SF, Gottlieb B, Beitel LK, Lumbroso R, Purisima EO, Trifiro M 2008 An investigation into CAG repeat length variation and N/C terminal interactions in the T877A mutant androgen receptor found in prostate cancer. J Steroid Biochem Mol Biol 111:138-146

CHAPTER 2

Differential ligand-responsiveness of androgen receptor L701 mutants

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ABSTRACT

One of the mechanisms of prostate tumours to escape from androgen ablation therapies is by mutation of the androgen receptor (AR). The L701H mutation results in an AR that is strongly stimulated by cortisol. We performed a systematic structure-function analysis of AR residue 701 to increase our knowledge of AR function. Our results demonstrate that most L701 substitutions are allowed for activation by dihydrotestosterone (DHT). Further analysis of the AR 701 variants showed that AR L701M and AR L701Q, like AR L701H, had a changed ligand responsiveness. AR L701M was strongly activated by progesterone, but not by cortisol, whereas the opposite was observed for AR L701Q and AR L701H. By analysing a panel of structurally related steroids, we studied which of the OH-groups at positions 11β, 17α, and 21, which discriminate cortisol from progesterone, underlied the differential responses to both hormones. It turned out that the presence of the 17α-OH group was essential for activation of AR L701H and AR L701Q, whereas its absence was important for activation AR L701M. Modelling indicated a conserved H-bonding network between the steroidal 17α-OH group, and H701 or Q701, and the backbone of S778. This network is absent in L701 or in other mutants. A hydrophobic leucine or methionine at position 701 is unfavourable for the 17α-OH group. In conclusion, two novel AR L701 variants that broaden ligand-responsiveness were identified (L701M and L701Q). The data indicate that the properties of the 701 residue, the interaction with the backbone of S778, and the steroidal 17α-hydroxyl group play crucial roles in the distinct transcriptional responses to progesterone and cortisol.

INTRODUCTION

The androgen receptor (AR) is a ligand-dependent transcription factor, which is activated by the androgens testosterone and dihydrotestosterone (DHT). The androgen-AR axis is essential for normal male development and plays a pivotal role in maintaining the functions of male-specific organs, including the prostate (1). A disturbed androgen-AR axis has been implicated in a number of malignancies, including androgen insensitivity syndrome (AIS) and prostate cancer (2, 3). AIS is caused by AR inactivation and is characterized by defective masculinization of 46,XY individuals. It ranges from mild undervirilization (MAIS) to partial (PAIS) or even complete (CAIS) female phenotypic outcomes. An active AR pathway is involved in prostate cancer. Initially, prostate cancer growth is dependent on androgens. Treatment of metastasized tumors, therefore, aims at inhibiting the AR pathway by suppressing testicular androgen production by LHRH-analogues or by blocking AR activity using anti-androgens. Despite an initial response, tumors eventually regain the ability to grow leading to an endocrine-therapy resistant stage of the disease. Although at this stage tumor growth is androgen-independent, the AR pathway still appears to be active (4, 5). Several mechanisms have been proposed that may underlie therapy failure, including *AR* amplification and *AR* mutations (6-9).

Like other nuclear receptors, the AR contains separate functional domains: an N-terminal transactivation domain, a central DNA-binding domain and a C-terminal ligand-binding domain (LBD) (10). The LBD is composed of 12 α-helices of which amino acid residues in helices 3, 5, 7, and 11 form the ligand-binding pocket (11, 12). Upon binding of an agonistic ligand, the LBD undergoes major structural rearrangements to obtain an active conformation. Helix 12 closes the ligand-binding pocket and becomes part of the coactivator-binding groove. This groove then serves as high affinity docking site for short amphipathic α-helical FxxLF-like sequences present in cofactors (13-15). In response to binding of an antagonist, helix 12 adopts a different orientation preventing the AR LBD to obtain the active conformation necessary to induce transcription (16).

The incidence of AR mutations is low in primary prostate tumors, but increases in advanced disease during endocrine therapy (7, 8, 17). The majority of AR mutations collocate at several regions in the LBD mapping to amino acid residues 670-678, 701-730, and 874-919 (18). The first AR mutation reported was a threonine to alanine substitution at position 877 that was identified in the LNCaP prostate cancer cell line (19). Later, it was found that residue T877 serves as an AR mutational hot spot in recurrent prostate tumors (20). Crystallographic analysis and functional studies of the AR T877A mutant demonstrated that this amino acid substitution alters the size and shape of the ligand-binding pocket, allowing several non-natural ligands and even anti-androgens to bind and activate the receptor (12, 21-23). It seems that AR T877A drives tumor growth through aberrant activation by the anti-androgen used for treatment.

A second AR mutational hot spot identified in prostate cancers is residue 701. The AR mutation substituting Leu for His at position 701 (L701H) has been reported in hormone-refractory prostate cancer patients (24, 25). The same mutation, in combination with T877A, was also present in the AR of the MDA PCa cell lines, which were originally derived from a bone metastasis of an orchiectomized prostate cancer patient (26, 27). AR L701H and AR L701H/T877A are somewhat less sensitive to androgens, but highly responsive to the glucocorticoids cortisol and cortisone, which circulate at concentrations high enough to activate both mutant receptors (28, 29). So, androgen-independent growth of prostate tumors containing the AR L701H mutation differs from those containing the T877A mutation by being driven by endogenously circulating ligands.

Previously, the T877A substitution has been studied in detail (23). In the present study we performed a systematic structure-function analysis of AR residue 701 to obtain further insight in the ligand-responsiveness of AR L701H. Screening revealed that in addition to AR L701H, also AR L701M and AR L701Q had a changed, but differential, ligand-specificity. Functional studies with a panel of structurally related steroids showed that the presence of a hydroxyl group at position 17α was critical for activation of AR L701H and AR L701Q, but not of AR L701M. Modelling of the various mutations in the AR LBD structure revealed that a unique hydrogen-bond network involving H701 or Q701, the steroidal 17α-OH group and the backbone oxygen of S778 plays an important role in the cortisol response.

EXPERIMENTAL PROCEDURES

Hormones

5α-dihydrotestosterone (DHT), progesterone, cortisol, and 11-desoxycorticosterone were purchased from Steraloids (Wilton, NH), 17α-hydroxyprogesterone, 11β-hydroxyprogesterone, 11-desoxycortisol, and 21-desoxycortisol were from Sigma (St Louis, MO), and hydroxyflutamide from Schering (Bloomfield, NJ). Corticosterone was kindly provided by Dr. Albert Brinkmann (Erasmus MC, Rotterdam, the Netherlands) and bicalutamide (casodex) was a gift from AstraZeneca (Macclesfield, UK).

Plasmids

All AR L701X mutants were generated by Quikchange Site-Directed Mutagenesis on mammalian AR cDNA expression vector pSVAR0 (30), according to the manufacturer's protocol (Stratagene, La Jolla, CA). Primers used were 5'-CTTTGCAGCCTTG**NNN**TCTAGCCTCAATG-3' (bases encoding AR residue 701 are indicated in bold) and its complementary sequence. AR L701H/T877A was generated by Quikchange of AR residue Leu701 in the pSVARL vector, which expresses the T877A mutant AR (19), using primer 5'-CTTTGCAGCCTTG**CAC**TCTAGCCTCAATG-3' and its complementary sequence (bases encoding mutated AR residue 701 are

indicated in bold; base substitution is underlined). All mutations were verified by sequence analysis and Western blots were performed to analyze size and expression of the mutant ARs.

Construction of the mammalian expression plasmid encoding Gal_4DBD-AR FxxLF peptide and the $(ARE)_2$TATA-LUC reporter ($(PRE)_2$-E1b-LUC) have been described previously (31, 32). The $(UAS)_4$TATA-LUC reporter construct was kindly provided by Magda Meester.

Mammalian cell culture, transient transfections, and luciferase assay

Hep3B cells were maintained in αMEM (Bio-Whittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) and antibiotics. For our transient transfection assays, Hep3B cells were plated at a density of $5x10^4$ cells per well of a 24-well plate and were allowed to grow for 24 hours. Four hours prior to transfection, the medium was replaced by αMEM supplemented with 5% charcoal-stripped FCS, antibiotics, and hormone or vehicle. Twenty-four hours after addition of transfection mixtures (described below), cells were lysed and luciferase activities measured as described previously (31).

Transcription activation assays were performed using Fugene 6 (Roche Diagnostics, Mannheim, Germany), 50 ng AR expression construct and 100 ng $(ARE)_2$TATA-LUC reporter construct per well. In case of mammalian one-hybrid assays, Fugene 6 mixtures contained 50 ng Gal_4DBD-peptide, 50 ng AR expression constructs, and 150 ng $(UAS)_4$TATA-LUC reporter per well.

Western blot analysis

For Western blot analysis, Hep3B cells were transfected with 50 ng AR expression construct as described above. Twenty-four hours after transfection, cells were lysed in 100 µl Laemmli buffer (50 mM Tris, 10 mM DTT, 10% glycerol, 2% SDS and 0.001% Bromophenol Blue). Lysates were boiled and subjected to electrophoresis on a 10% SDS polyacrylamide gel, after which proteins were transferred to a nitrocellulose membrane. Blots were incubated with monoclonal antibodies directed against the AR N-terminal domain (F39.4.1), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark). Proteins were visualized using SuperSignal West Pico Chemiluminescent blotting substrate from Pierce (Rockford, IL), followed by exposure to X-ray film.

Modelling

Residue 701 was mutated systematically in a proprietary wild type AR structure bound to DHT and the publicly available AR L701H structure (33). Each mutation has been minimized in Yasara using the Yamber2 force field (34) and visually inspected.

RESULTS AND DISCUSSION

Most AR L701 mutants are responsive to DHT

We performed a systematic structure-function analysis of AR residue 701 to obtain further insight in the effect of the L701H mutation on AR ligand-responsiveness. AR L701 was substituted by any other amino acid residue and the resultant mutant AR was tested for DHT (100 nM) response in transiently cotransfected Hep3B cells. Using the $(ARE)_2$TATA-Luc as reporter, most AR mutants, including AR L701H, were strongly activated by DHT (Fig. 1A). Responses were somewhat less if a polar residue (Asn, Gln, Ser, Thr, Tyr) was present at AR position 701. Substitution of the Leu by a Gly or a Trp strongly reduced activation by DHT, whereas substitution by a charged residue (Asp, Glu, Arg, Lys) completely abrogated AR transcriptional activity. Similar results were obtained if MMTV-Luc was used as reporter construct (data not shown). Western blot analysis demonstrated that all AR L701 mutants were expressed at levels comparable to wild type AR (Fig. 1B).

AR L701I and AR L701F have previously been identified in patients with PAIS and CAIS, respectively (35, 36). Despite normal responses at high DHT concentrations (100 nM) used for initial analysis of the substituted ARs (Fig. 1A), the transcriptional activities of AR L701I and AR L701F were less than wild type AR at lower DHT concentrations (data not shown). These reduced responses may account for the AIS phenotypes observed in individuals carrying these mutations.

Analysis of the X-ray structure of wild type AR in complex with DHT showed that the 3-keto group of DHT is hydrogen-bonded to Q711 and R752 (Fig. 1C) (12). The 17β-OH group is stabilized via hydrogen bonds to N705 and T877. L701 is located near the steroidal D-ring and has hydrophobic contacts with C17. The side chain of L701 is buried in a predominantly hydrophobic pocket consisting of residues F697, L700, L704, S778, M780, F876, L880, and V889 (Fig. 1D). We modelled the replacement of L701 by each amino acid and evaluated the structure. These analyses showed that in addition to the wild type leucine, also other aliphatic residues (Val, Ile, Met, and Ala) were tolerated sterically and formed varying degrees of favourable vanderWaals interactions with the surrounding hydrophobic pocket (data not shown). A701 and G701 naturally formed less hydrophobic contacts in the receptor than the other aliphatic residues, potentially resulting in a less stable protein. This may be the cause of the strongly reduced DHT response of AR L701G. Although polar residues were sterically tolerated at position 701, their lower DHT responses are most likely due to their presence in an unfavourable hydrophobic pocket. Large residues such as Trp, Phe and Tyr could not be tolerated in the L701 hydrophobic pocket without causing significant clashes with the protein structure. The presence of these large residues required structural modification of the receptor, which likely explains the strongly reduced DHT responses and the CAIS phenotype observed in the individual carrying the AR L701F mutation. The lack of a charged residue

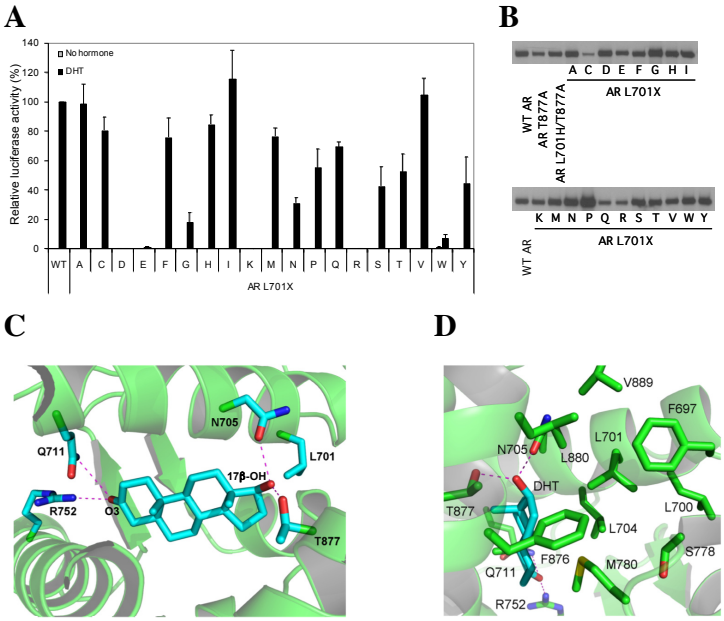

Figure 1. Activation of AR L701 mutants by DHT. (A) Transcriptional responses of wild type (WT) AR and AR L701 mutants to DHT (100 nM). The amino acid single letter code of the L701 substitution is indicated on the x-axis. Hep3B cells were transiently cotransfected with AR expression constructs and $(ARE)_2$TATA-Luc reporter. Transcriptional activation of wild type AR by DHT was set to 100%. Bars represent mean relative luciferase activities of three independent experiments performed in duplicate (+/- SEM). (B) Western blot analysis showing protein expression of the AR L701 mutants. (C and D) Structural representations of wild type AR LBD in complex with DHT showing the relative position of residue L701 in the ligand-binding pocket. The steroidal 3-keto and 17β-OH group are indicated. Red dotted lines represent hydrogen bonds.

in the environment surrounding L701 prohibited mutation to a charged residue. Without a charged partner this would result in the destabilization of the receptor.

AR L701H, AR L701M, and AR L701Q display modified ligand-responsiveness

We next studied the transcriptional activities of the AR L701 mutants induced by progesterone and cortisol. In control experiments, we first determined transcriptional responses of wild type AR, the AR single mutants L701H and T877A, and the AR L701H/T877A double mutant to both steroids using the $(ARE)_2$TATA-Luc reporter. In Hep3B cells, all receptor mutants responded similarly as described previously for CV-1 cells and an MMTV-Luc reporter (Fig. 2A; (28)). Whereas the wild type AR was activated only by DHT, AR L701H was additionally activated by cortisol but not by progesterone. The opposite was observed for AR T877A.

AR L701H/T877A displayed combined characteristics of both single mutants by responding strongly to both progesterone and cortisol.

Although the majority of AR L701 mutants was activated by DHT (Fig. 1A), they showed weak or no responses to progesterone and cortisol (Fig. 2B). However, apart from AR L701H, two mutants had a modified, but distinct, ligand-responsiveness. Transcriptional activity of AR L701M was highly induced upon incubation with progesterone but not with cortisol, whereas AR L701Q, like AR L701H, was induced by cortisol and not by progesterone (Fig. 2B). Similar results were obtained using an MMTV-Luc reporter construct (data not shown).

Differential responsiveness of AR L701M, AR L701Q, and AR L701H is determined by the 17α-OH group

Progesterone and cortisol are structurally highly similar (Fig. 3A). Cortisol differs from progesterone by the presence of hydroxyl groups at positions 11β, 17α, and 21. To study which of these hydroxyl groups differentially affects transcriptional activation of AR L701M, AR L701H, and AR L701Q, we tested a panel of steroids intermediary between progesterone and cortisol with varying combinations of hydroxyl groups at positions 11β, 17α, and 21 (Fig. 3A).

Our results demonstrated that wild type AR and AR L701M on one hand and AR L701H and AR L701Q on the other hand display two different transcription activation profiles (Figs. 3B-G), which are characterized by differential responses to steroids containing a hydroxyl group at

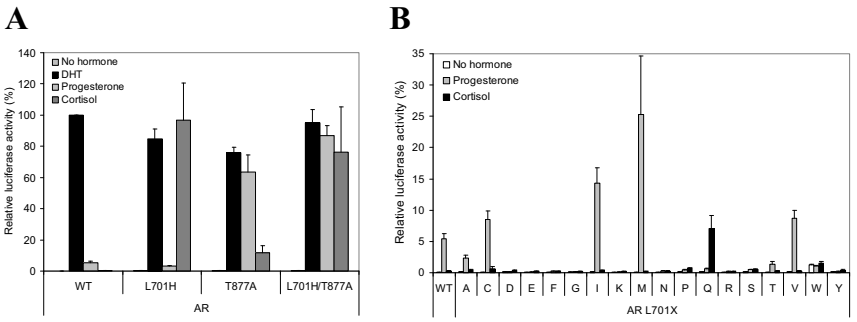

Figure 2. AR L701H, L701M, and L701Q display modified ligand-specificity. (A) Transcriptional activation of wild type (WT) AR, the AR single mutants L701H and T877A, and the AR L701H/T877A double mutant by DHT (100 nM), progesterone (100 nM), and cortisol (1 μM). Transcriptional activation of wild type AR by DHT was set to 100%. Bars represent mean relative luciferase activities of three independent experiments performed in duplicate (+/- SEM). Protein expression of these AR mutants is shown in Figure 1B. (B) Screening of AR L701 mutants for responses to progesterone (100 nM) and cortisol (1 μM). Bars represent mean relative luciferase activities of three independent experiments performed in duplicate (+/- SEM). Transcriptional activation of wild type AR by DHT was set to 100%.

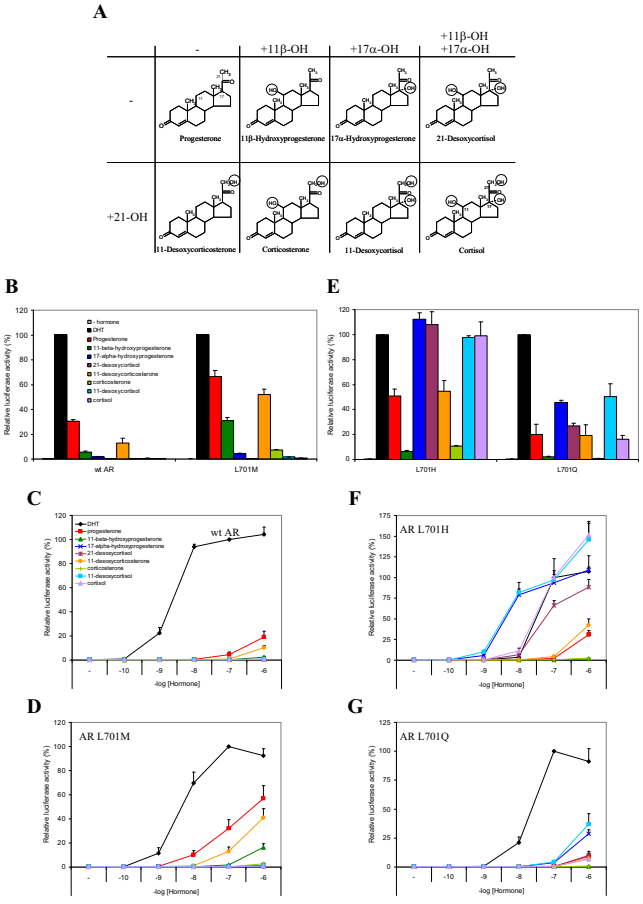

Figure 3. Differential responsiveness of AR L701M, AR L701Q, and AR L701H is determined by the 17α-OH group. (A) Chemical structures of steroids used in our panel. Cortisol (lower right) differs from progesterone (upper left) by the presence of hydroxyl groups at positions 11β, 17α, and 21 (indicated in the structures). All other steroids are structurally intermediary between progesterone and cortisol, and differ by the positions of the hydroxyl groups. Steroids presented in the lower row differ from the steroids in the upper row by the presence of a hydroxyl group at position 21. (B and E) Transcriptional responses of wild type AR and AR L701M (B), and AR L701H and AR L701Q (E) to the panel of structurally related steroids. Hep3B cells were transiently transfected with expression vectors encoding the different ARs together with the $(ARE)_2$TATA-Luc reporter plasmid. Cells were incubated for 24 h with 100 nM DHT or 1 μM of the other steroids. For each receptor the transcriptional activity in response to DHT was set to 100%. The responses to the other steroids are relative to their respective DHT response. Bars represent the mean relative luciferase activities of three independent experiments performed in duplicate (+/- SEM). (C, D, F, G) Dose-response curves of wild type AR (C), AR L701M (D), AR L701H (F), and AR L701Q (G) to the panel of steroids. Hep3B cells were incubated for 24 h with different concentrations of ligands ranging from 0.1 nM to 1 μM. Other procedures are similar as described in the legend to Figure 1A. For each receptor the transcriptional activity in response to 100 nM DHT was set to 100%. Data represent the mean of three independent experiments performed in duplicate (+/- SEM).

position 17α (17α-hydroxyprogesterone, 21-desoxycortisol, 11-desoxycortisol, and cortisol). AR L701M and wild type AR, which contain a hydrophobic residue at position 701, did not respond at all to these steroids (Figs. 3B, C, and D). In contrast, AR L701H and AR L701Q, which harbour a polar residue at the 701 position, were strongly stimulated by steroids containing the 17-OH group (Figs. 3E, F, and G).

All steroids containing a hydroxyl group at position 11β (11β-hydroxyprogesterone, corticosterone, 21-desoxycortisol, and cortisol) were less capable of activating the AR mutants than steroids without this hydroxyl group (progesterone, 11-desoxycorticosterone, 17α-hydroxyprogesterone, and 11-desoxycortisol, respectively) (Figs. 3B-G). This demonstrated that the 11β-OH group is unfavourable for AR activation. Steroids with the 21-OH group (progesterone, 11β-hydroxyprogesterone, 17α-hydroxyprogesterone, and 21-desoxycortisol) or without the 21-OH group (11-desoxycorticosterone, corticosterone, 11-desoxycortisol, and cortisol) were equally capable of activating AR L701H and AR L701Q, suggesting that the 21-OH group does not play a role in activating these mutant receptors (Figs. 3E-G). Transcriptional activities of AR L701M and wild type AR were slightly affected if the 21-OH group was present (Figs. 3B-D).

We also determined transcriptional responses of AR T877A and AR L701H/T877A to the same panel of steroids (Suppl. Figs. 1 and 2). Although both mutants were stimulated by all steroids tested, activities of AR L701H/T877A were much stronger and combined the characteristics of the AR L701H (Fig. 3F) and AR T877A (Suppl. Fig. 2A) single mutants. The strong responses to progesterone, 11-desoxycorticosterone, 11β-hydroxyprogesterone, and corticosterone were shared with AR T877A. The strong responses to 17α-hydroxyprogesterone, 11-desoxycortisol, 21-desoxycortisol, and cortisol were shared with AR L701H (Suppl. Fig. 2B).

Structural analysis and modelling reveals that a conserved hydrogen-bond network can explain the cortisol response of AR L701H and AR L701Q

The crystal structure of wild type AR bound to DHT showed that L701 has hydrophobic contacts with C17 of the steroid. The structure of the AR L701H/T877A double mutant has been elucidated in complex with 9α-fluorocortisol (33). This structure revealed an H-bond between the steroidal 17α-OH group and the polar H701. The histidine residue was also well positioned to make an H-bond to the backbone of S778 (Fig. 4A). Based on this structure, we modelled a Gln and a Met at position 701. Modelling reveals that Q701 is able to make an H-bond to a 17α-OH group of the ligand in a manner similar to H701 (Fig. 4B). In addition, Q701 also replicates the H-bond with the backbone of S778 that is seen in the L701H structure (Fig. 4B). None of the other amino acid substitutions at 701 were able to reproduce this H-bonding network. The other amide-containing side chain amino acid, Asn (Fig. 4C), as well as the small polar residues Thr, Ser, and Cys are sterically tolerated and were able to make the H-bond with the steroid. However, unlike H701 or Q701 these residues are unable to interact with S778. The polar interaction of Asn, Thr, and Ser with the steroids 17α-OH group should prevent the

burying of this hydrophilic group into a hydrophobic part of the pocket. Despite this, L701N, L701T, and L701S mutations do not show strong activation by cortisol as seen for L701H and L701Q (Fig. 2B). We therefore conclude that the additional interaction to S778 that is unique for L701H and L701Q must have an important role in activating the receptor, perhaps by further stabilizing the protein.

Contrary to this, modelling showed that the 17α-OH group in the wild type structures and L701M mutation is unfavourably buried in a hydrophobic pocket, explaining the lack of activity upon incubation with steroids containing the 17α-OH group (Fig. 3). M701 actually fills the pocket marginally better than L701 and results in an electrostatically favourable sulphur-sulphur contact between M701 and M780 (Figs. 4D and E). This improved packing and sulphur-sulphur contacts have a positive effect on protein stability, which may explain the increased activation of the L701M mutation compared to the wild type receptor.

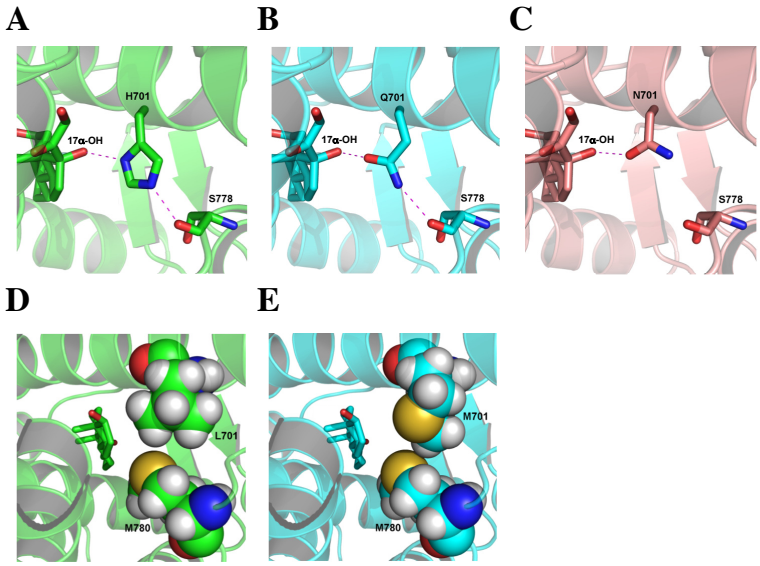

Figure 4. A conserved hydrogen-bonding network around position 701 defines the cortisol response. (A) X-Ray structure of L701H mutant structure (33) shows an H-bond network between the 17α-OH group of 9α-Fluorocortisol to the ND1 nitrogen of H701 and from the NE2 nitrogen of H701 to the backbone carbonyl of S778. The same H-bond network is maintained in the L701Q structure with the 17α-OH group interacting with the OE1 oxygen of Q701 and the NE2 nitrogen of Q701 forming an H-bond to the backbone of S778 (B). Contrary to this, in the L701N structure, an interaction between the mutated residue and the steroids 17α-OH group is possible, but the interaction with S778 is missing (C). (D and E) Modelling shows that M701 moderately improved packing with M780, including an electrostatically favourable sulphur-sulphur contact (E), compared to the interaction between wild type L701 and M780 (D).

As shown in Figure 3, the presence of the 11β-OH group is unfavourable for activating all AR L701 mutants. Modelling suggests that the most likely explanation is a clash of the steroidal 11β-OH group with M895, which is also a Met residue in PR but a smaller Leu in GR and MR (data not shown). This may explain the general tolerance of 11β-OH steroids in MR and GR, but not in AR and PR.

Transcriptional activation of AR L701 mutants corresponds with AR FxxLF peptide interaction

Different coactivator groove-interacting peptides display distinct binding modes (31). For example, LxxLL peptides are shifted in the AR groove towards K720 compared to FxxLF peptides (37-40). In addition, AR T877A bound to cyproterone acetate (CPA) strongly interacts with LxxLL motifs but less with FxxLF motifs, whereas the opposite is observed if bound to hydroxyflutamide (41, 42). We therefore determined interaction capacities of distinct peptides containing FxxLF, FxxFF, FxxMF, and LxxLL sequences with the different AR L701 mutants in the presence of steroids from our panel. Peptide interactions were studied in mammalian one-hybrid assays as described previously (31).

As shown in Figure 5, for all AR L701 mutants the relative interaction of the AR FxxLF peptide correlated with the relative transcription activation capacity. Similar results were obtained for AR T877A and AR L701H/T877A (Suppl. Fig. 3). Although we also found that AR T877A prefers binding of LxxLL motifs in the presence of CPA and FxxLF motifs in the presence of hydroxyflutamide, we did not observe a similar effect for the AR L701 mutants to bind the distinct motifs in the presence of the panel of ligands (data not shown). This suggests that, unlike T877A, amino acid residue 701 in the AR does not directly or indirectly influence the conformation of the coactivator groove.

AR L701 mutants hardly respond to anti-androgens

Many AR mutations found in prostate cancer, including T877A and W741C, result in anti-androgen responsive receptors, leading to failure of anti-androgen treatment of metastasised prostate cancer (23, 43). As shown previously, AR T877A is strongly activated by hydroxyflutamide and CPA, but not by bicalutamide (Figure 6) (23). Vice versa, AR W741C is strongly activated by bicalutamide, but not by hydroxyflutamide (43) and CPA (Figure 6). It was previously shown that AR L701H is not responsive to hydroxyflutamide (28).

Here, we extended these observations by investigating the effects of the anti-androgens hydroxyflutamide, bicalutamide and CPA on transcriptional activities of AR L701H and the other AR L701 mutants. Figure 6 shows that, similar to the responses to the panel of steroids (Figure 3), AR L701H and AR L701Q on one hand and wild type AR and AR L701M on the other hand displayed different responses to the anti-androgens. AR L701H and AR L701Q were neither activated by hydroxyflutamide, nor by bicalutamide or CPA. AR L701M showed weak agonistic responses to hydroxyflutamide and CPA, which were comparable to wild type

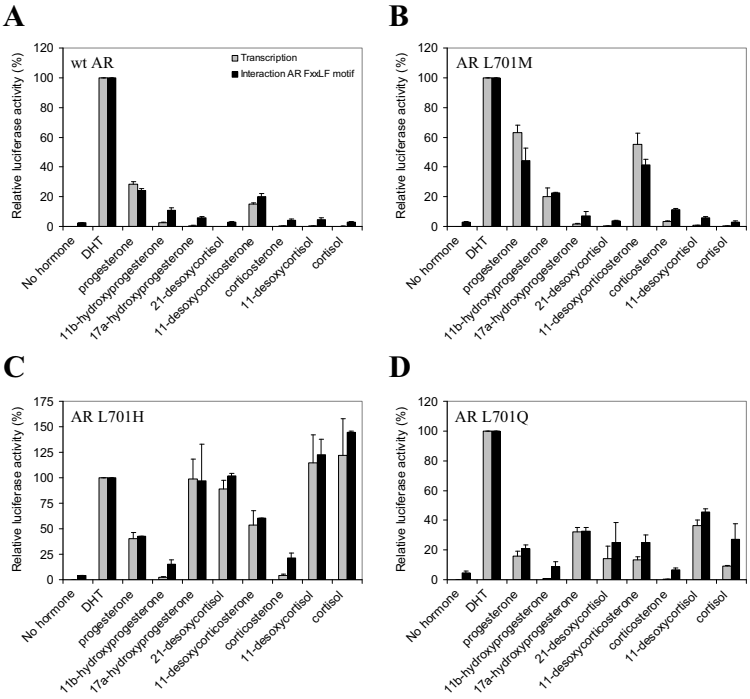

Figure 5. Transcriptional activaties of wild type AR and selected AR mutants correspond with AR FxxLF peptide interaction capacities. Using the panel of structurally related natural steroids, transcription activation (grey bars) of wild type AR (A), AR L701M (B), AR L701H (C), and AR L701Q (D) was compared with the capacity to bind the AR FxxLF peptide (black bars). Transcription activation was determined as described in the legends to Figure 1. Mammalian one-hybrid assays were carried out to determine peptide interactions. Hep3B cells were transiently transfected with expression constructs encoding the peptide fused to Gal_4DBD, which served as bait for the different AR constructs. In case of interaction between the peptide and the AR, the AR NTD transactivates the luciferase reporter. Cells were incubated for 24 h with 100 nM DHT or with 1 μM of the other steroids. For each receptor both the transcriptional activity and the AR FxxLF peptide interaction in response to DHT were set to 100%. The responses and interactions in the presence of the other steroids are relative to their respective DHT response. Bars represent the mean relative luciferase activities of two independent experiments performed in duplicate (+/- SD).

AR. Both receptors could not be activated by bicalutamide. These results indicate that the AR L701H mutant does not drive tumour growth because of activation by anti-androgens, suggesting a different mechanism of AR activation as compared to AR T877A and AR W741C.

Conclusions

In addition to the L701H mutation we found two other mutations, L701M and L701Q, that result in an AR with modified ligand-specificity. We showed that these mutants could be

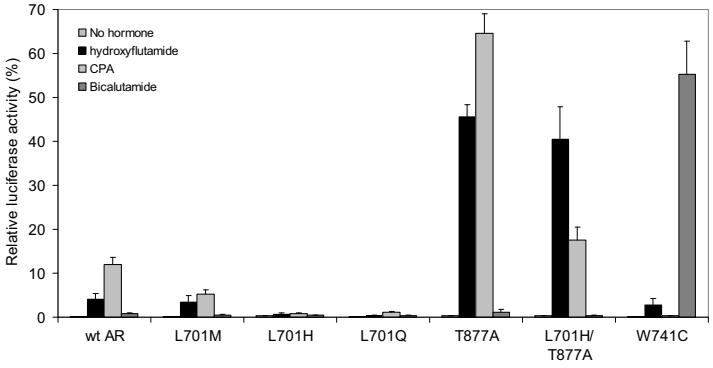

Figure 6. Transcriptional activities of wild type AR and selected AR mutants by anti-androgens. Hep3B cells were transiently transfected with expression constructs encoding wild type AR or the indicated mutant receptors. Cells were treated for 24 h with hydroxyflutamide (10 μM), cyproterone acetate (CPA; 1 μM), or bicalutamide (10 μM). Responses to the different anti-androgens are relative to the DHT (100 nM) response, which was set to 100% for each receptor (not shown). AR T877A and AR L701H/T877A served as controls for hydroxyflutamide and CPA, whereas AR W741C served as control for activation by bicalutamide (43). Bars represent the mean relative luciferase activities of three independent experiments performed in duplicate (+/- SEM).

subdivided on basis of their transcription activation profiles and structural conformation, in which the interaction between the AR 701 residue, the backbone of S778, and the steroidal 17α-hydroxyl group play a crucial role.

The His to Leu substitution is the only mutation found at position 701 in prostate cancer patients (24, 25, 27). AR L701M and AR L701Q mutations have never been found, possibly because two base substitutions are needed to mutate the Leu codon into a codon for Met or Gln. The lack of activation of the AR L701H mutant by anti-androgens strongly suggests that this mutant AR does not drive prostate tumor growth upon binding of the anti-androgen used for treatment. This provides an additional clue that in these cases tumor growth is dependent on endogenously circulating ligands, such as cortisol. This finding indicates a different mechanism of tumor growth than observed for the AR T877A and W741C mutants, which are dependent on anti-androgens for their transcriptional activity.

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REFERENCES

1. Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, Trapman J 1999 Mechanisms of androgen receptor activation and function. J Steroid Biochem Mol Biol 69:307-313
2. Brinkmann AO 2001 Molecular basis of androgen insensitivity. Mol Cell Endocrinol 179:105-109
3. Taplin ME 2007 Drug insight: role of the androgen receptor in the development and progression of prostate cancer. Nat Clin Pract Oncol 4:236-244
4. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, Trapman J 1991 Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 48:189-193
5. Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS 2007 Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res 67:5033-5041
6. Trapman J 2001 Molecular mechanisms of prostate cancer. Eur J Cancer 37 Suppl 7:S119-125
7. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP 1999 Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 59:2511-2515
8. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP 1995 Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332:1393-1398
9. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP 1995 In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 9:401-406
10. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839
11. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Basler S, Schafer M, Egner U, Carrondo MA 2000 Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 275:26164-26171
12. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR, Jr., Weinmann R, Einspahr HM 2001 Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci U S A 98:4904-4909
13. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, Trapman J 2006 Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping. Mol Endocrinol 20:1742-1755
14. He B, Minges JT, Lee LW, Wilson EM 2002 The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226-10235
15. van de Wijngaart DJ, van Royen ME, Hersmus R, Pike AC, Houtsmuller AB, Jenster G, Trapman J, Dubbink HJ 2006 Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain. J Biol Chem 281:19407-19416
16. Masiello D, Cheng S, Bubley GJ, Lu ML, Balk SP 2002 Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. J Biol Chem 277:26321-26326
17. Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ 2000 Androgen receptor mutations in prostate cancer. Cancer Res 60:944-949
18. Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD 2001 Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res 7:1273-1281

19. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E 1990 A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 173:534-540
20. Gottlieb B, Beitel LK, Wu JH, Trifiro M 2004 The androgen receptor gene mutations database (ARDB): 2004 update. Hum Mutat 23:527-533
21. Bohl CE, Gao W, Miller DD, Bell CE, Dalton JT 2005 Structural basis for antagonism and resistance of bicalutamide in prostate cancer. Proc Natl Acad Sci U S A 102:6201-6206
22. Bohl CE, Wu Z, Miller DD, Bell CE, Dalton JT 2007 Crystal structure of the T877A human androgen receptor ligand-binding domain complexed to cyproterone acetate provides insight for ligand-induced conformational changes and structure-based drug design. J Biol Chem 282:13648-13655
23. Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO, Trapman J 2002 Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer 100:309-317
24. Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J 1993 Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol 46:759-765
25. Watanabe M, Ushijima T, Shiraishi T, Yatani R, Shimazaki J, Kotake T, Sugimura T, Nagao M 1997 Genetic alterations of androgen receptor gene in Japanese human prostate cancer. Jpn J Clin Oncol 27:389-393
26. Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM, Johnston D, Pollack A, Pathak S, von Eschenbach AC, Logothetis CJ 1997 Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin Cancer Res 3:2493-2500
27. Zhao XY, Boyle B, Krishnan AV, Navone NM, Peehl DM, Feldman D 1999 Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a. J Urol 162:2192-2199
28. Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM, Feldman D 2000 Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. Nat Med 6:703-706
29. Krishnan AV, Zhao XY, Swami S, Brive L, Peehl DM, Ely KR, Feldman D 2002 A glucocorticoid-responsive mutant androgen receptor exhibits unique ligand specificity: therapeutic implications for androgen-independent prostate cancer. Endocrinology 143:1889-1900
30. Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris C, Klaassen P, van der Korput JA, Voorhorst MM, van Laar JH, Mulder E, Trapman J 1989 The human androgen receptor: domain structure, genomic organization and regulation of expression. J Steroid Biochem 34:307-310
31. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J 2004 Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132-2150
32. Jenster G, Spencer TE, Burcin MM, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor induction of gene transcription: a two-step model. Proc Natl Acad Sci U S A 94:7879-7884
33. Matias PM, Carrondo MA, Coelho R, Thomaz M, Zhao XY, Wegg A, Crusius K, Egner U, Donner P 2002 Structural basis for the glucocorticoid response in a mutant human androgen receptor (AR(ccr)) derived from an androgen-independent prostate cancer. J Med Chem 45:1439-1446
34. Krieger E, Darden T, Nabuurs SB, Finkelstein A, Vriend G 2004 Making optimal use of empirical energy functions: force-field parameterization in crystal space. Proteins 57:678-683
35. Ahmed SF, Cheng A, Dovey L, Hawkins JR, Martin H, Rowland J, Shimura N, Tait AD, Hughes IA 2000 Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. J Clin Endocrinol Metab 85:658-665
36. Chavez B, Vilchis F, Zenteno JC, Larrea F, Kofman-Alfaro S 2001 Novel molecular defects in the androgen receptor gene of Mexican patients with androgen insensitivity. Clin Genet 59:185-188

37. Askew EB, Gampe RT, Jr., Stanley TB, Faggart JL, Wilson EM 2007 Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. J Biol Chem 282:25801-25816
38. Estebanez-Perpina E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, Buehrer BM, Webb P, Fletterick RJ, Guy RK 2005 The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280:8060-8068
39. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM 2004 Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425-438
40. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ 2004 Recognition and Accommodation at the Androgen Receptor Coactivator Binding Interface. PLoS Biol 2:E274
41. Brooke GN, Parker MG, Bevan CL 2008 Mechanisms of androgen receptor activation in advanced prostate cancer: differential co-activator recruitment and gene expression. Oncogene 27:2941-2950
42. Ozers MS, Marks BD, Gowda K, Kupcho KR, Ervin KM, De Rosier T, Qadir N, Eliason HC, Riddle SM, Shekhani MS 2007 The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay. Biochemistry 46:683-695
43. Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, Miyamoto M 2003 Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. Cancer Res 63:149-153

SUPPLEMENTARY INFORMATION

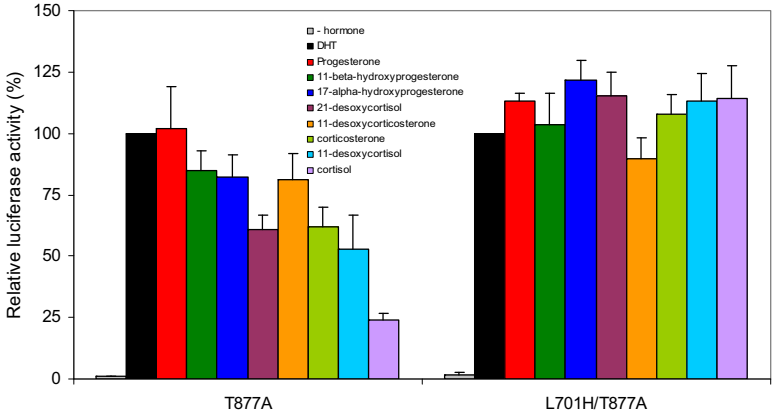

Figure S1. Transcription activation of AR T877A (left) and AR L701H/T877A (right) to the panel of related steroids. Experiments were performed as described in the legends to Figure 2. Hep3B cells were transiently transfected with expression vectors encoding the different AR constructs together with the $(ARE)_2$TATA-Luc reporter plasmid. Cells were incubated for 24 h with 100 nM DHT or 1 μM of the other steroids. For each receptor the transcriptional activity in response to DHT was set to 100%. The responses to the other steroids are relative to their respective DHT response. Bars represent the mean relative luciferase activities of three independent experiments performed in duplicate (+/- SEM).

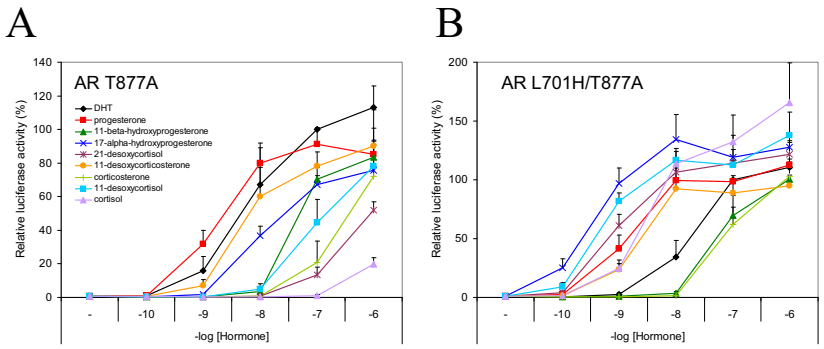

Figure S2. Dose-response curves of AR T877A (A) and AR L701H/T877A (B) to the panel of steroids. Hep3B cells were incubated for 24 h with different concentrations of ligands ranging from 0.1 nM to 1 µM. For each receptor the transcriptional activity in response to 100 nM DHT was set to 100%. Data represent the mean of three independent experiments performed in duplicate (+/- SEM).

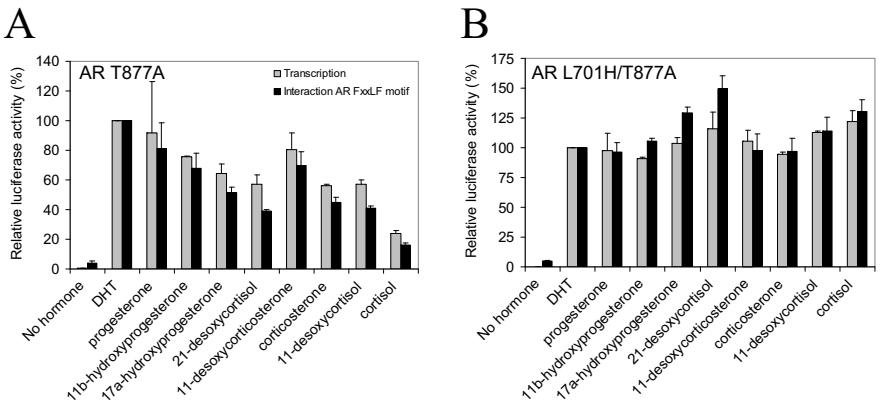

Figure S3. Transcriptional activaties (grey bars) of AR T877A (A) and AR L701H/T877A (B) correspond with AR FxxLF peptide interaction capacities (black bars). Cells were incubated for 24 h with 100 nM DHT or with 1 µM of the other steroids. For each receptor the transcriptional activity and the AR FxxLF peptide interaction in response to DHT was set to 100%. The responses and interactions in the presence of the other steroids are relative to their respective DHT response. Bars represent the mean relative luciferase activities of two independent experiments performed in duplicate (+/- SD).

CHAPTER 3

Novel FxxFF and FxxMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain

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ABSTRACT

Upon hormone binding, a hydrophobic coactivator-binding groove is induced in the androgen receptor (AR) ligand-binding domain (LBD). This groove serves as high affinity docking site for α-helical FxxLF motifs present in the AR N-terminal domain and in AR cofactors. Study of the amino acid requirements at position +4 of the AR FxxLF motif revealed that most amino acid substitutions strongly reduced or completely abrogated AR LBD interaction. Strong interactions were still observed following substitution of L+4 by F or M residues. L+4 to M or F substitutions in the FxxLF motifs of AR cofactors ARA54 and ARA70 were also compatible with strong AR LBD binding. Like the corresponding FxxLF motifs, interaction of FxxFF and FxxMF variants of AR and ARA54 motifs were AR specific, whereas variants of the less AR-selective ARA70 motif displayed increased AR specificity. A survey of currently known AR-binding proteins revealed the presence of an FxxFF motif in gelsolin and an FxxMF motif in PAK6. *In vivo* fluorescence resonance energy transfer (FRET) and functional protein-protein interaction assays showed direct, efficient and specific interactions of both motifs with AR LBD. Mutation of these motifs abrogated interaction of gelsolin and PAK6 proteins with AR. In conclusion, we demonstrate strong interaction of FxxFF and FxxMF motifs to the AR coactivator-binding groove thereby mediating specific binding of a subgroup of cofactors to the AR LBD.

INTRODUCTION

The androgen receptor (AR) is a key player in development and maintenance of male reproductive tissues (1, 2). AR is a ligand-inducible transcription factor of the nuclear receptor (NR) superfamily. Members of this family share a common structural and functional organization, including an N-terminal domain (NTD) harboring activation function 1 (AF-1), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) containing activation function 2 (AF-2) (3-5). Upon binding of its ligand, testosterone or 5α-dihydrotestosterone (DHT), AR LBD undergoes conformational changes leading to dissociation from heat-shock proteins and translocation to the nucleus (6). At the DNA, AR binds to specific androgen response elements to initiate target gene expression. Cofactors facilitate AR transcription function by histone modifications, chromatin remodeling, and bridging of the receptor to other components of the transcription initiation process, including general transcription factors and RNA polymerase II (7-9).

Although cofactors may functionally interact with all three NR domains, most extensive knowledge is available of LBD interaction. Crystal structures of NR LBDs have shown that ligand binding triggers repositioning of helix 12 (10-13). As a result a hydrophobic groove is formed, which serves as a docking site for amphipathic α-helical LxxLL motifs present in many cofactors. The specific affinity of LxxLL motifs for distinct NR LBDs depends on amino acid residues flanking the core L residues (10, 14-16). Until now, only a limited number of LxxLL motifs have been reported to interact with the AR LBD (17-20). Instead, AR LBD prefers binding of FxxLF motifs, one of which is located in the AR NTD (17, 21, 22). Although the function of the FxxLF motif-mediated interaction of AR NTD with AR LBD (N/C interaction) is not fully understood, it contributes to slowing of the androgen dissociation rate and selectively affects transcription of AR target genes (17, 22-25). Functional FxxLF motifs are also essential for interaction between AR LBD and cofactors ARA54, ARA70, and RAD9 (17, 26-28). However, for the majority of AR binding proteins the mode of interaction remains to be elucidated (29).

Alanine-scan mutagenesis of the AR FxxLF motif demonstrated that amino acid residues at positions +1, +4, and +5 are essential for interaction with the coactivator groove (21). Modeling and crystal structures of AR LBD in complex with FxxLF-like peptides, including AR and ARA70 FxxLF motifs, showed that amino acid residues at positions +1 and +5 are buried in the coactivator groove, rendering these residues entirely solvent inaccessible (17, 30-32). In contrast, the amino acid residue at position +4 rests in a shallow pocket on the periphery of the coactivator groove and is largely solvent exposed. Phage display screens for AR LBD interacting peptides and directed mutagenesis studies of the AR FxxLF motif demonstrated that not only F, but also M, Y, and W residues at positions +1 and +5 could be compatible with binding to the AR coactivator-binding groove, although F residues seem to be preferred (17, 18, 30).

Although it is presumed that the requirements for the amino acid residue at +4 in the FxxLF motif are less stringent than those at +1 and +5, our actual knowledge in this respect is limited. Here we performed a systematic functional analysis of the AR FxxLF motif mutated at +4. Yeast two-hybrid and mammalian one-hybrid experiments demonstrated that L to F and L to M substitutions in the AR FxxLF motif are compatible with high affinity and specific AR LBD interaction. Strong and specific interaction was also obtained if the same substitutions were introduced in the ARA54 and ARA70 FxxLF motifs. As assessed by *in vivo* fluorescence resonance energy transfer (FRET) analysis, functional protein-protein interaction assays and mutagenesis, the AR partners gelsolin and PAK6 were found to contain an FxxFF and FxxMF motif, respectively, necessary and sufficient for AR LBD interaction.

EXPERIMENTAL PROCEDURES

Plasmids

Yeast and mammalian expression plasmids encoding Gal_4AD-, Gal_4DBD-, and YFP-peptide fusion proteins were generated by in-frame insertion of double-stranded synthetic oligonucleotides with 5′-*Bam*HI and 3′-*Eco*RI cohesive ends into the corresponding sites of pACT2 (Takara Bio, Otsu, Shiga, Japan), pM-B/E (17), or in the *Bgl*II and *Eco*RI sites of pEYFP-C2 (Takara Bio), as described previously (17). Mutagenesis of position +4 in the AR FxxLF motif was performed in oligonucleotides encoding AR_{18-30}. Mutant oligonucleotides were inserted into pACT2 as described above. All peptide expression constructs were verified by sequence analysis.

Yeast expression construct pGalDBD-AR LBD ($AR_{661-919}$) has been described previously (33). Constructs encoding Gal_4DBD-fusions with LBDs of ERα, PR, and RXRα were generously provided by Michael Stallcup (34). Mammalian constructs expressing wild type AR (pCMVAR0) and F23L/F27L-AR (pCMVF23L/F27L-AR) have been described previously (17). pM-$PAK6_{12-681}$ was generated by subcloning a *Bgl*II-*Xba*I fragment from pSPORT6-PAK6 (IRAKp961I1968Q; RZPD, Berlin, Germany) into the *Bam*HI and *Xba*I sites of pM (Takara Bio). pM-gelsolin was obtained by subcloning an *Eco*RI-digested PCR fragment encoding amino acid residues 281-731 of gelsolin into pM. PCR was performed using primers 5′-GATC**GAATTC**TTCATCCTGGACCACG-3′ and 5′-GATC**GAATTC**CTCAGGCAGCCAGCTC-3′ (*Eco*RI sites in bold) on pOTB7-gelsolin (IMAGp958I211459Q; RZPD). FxxAA variants of pM-PAK6 and pM-gelsolin were generated by QuikChange (Stratagene, La Jolla, USA) using primer pair 5′-CTATTCCGAAGC**GC**G**GC**-CCTGTCCACTG-3′ and 5′-CAGTGGACAGG**GC**C**GC**GCTTCGGAATAG-3′ for PAK6 and 5′-CTGTTCAAGCAG**GC**C**GC**CAAGAACTGGCGG-3′ and 5′-CCGCCAGTTCTTG**GC**G**GC**CTGCTTGAACAG-3′ for gelsolin, respectively (substitutions in bold), according to the manufacturer's instructions. For generation of pCFP-ARLBD ($AR_{612-919}$) a *Bam*HI-digested PCR fragment from pAR0 (35) was cloned into the corresponding site of pECFP-C2 (Takara Bio). Primers used were 5′-AATTGG**GGATCC**GACCATCTTCTCGTCTTCGGAAATG-3′ and 5′-AATTGG**GGATCC**GATCACTGGGTGTG-

GAAATAGATG-3' (*Bam*HI sites in bold). pCYFP encoding the ECFP-EYFP chimera was kindly provided by Dr. Claude Gazin. The $(ARE)_2$TATA-LUC reporter construct has been previously described as (PRE)2-E1b-LUC (36). The $(UAS)_4$TATA-LUC reporter construct was kindly provided by Magda Meester. All constructs generated with PCR fragments and QuikChange mutagenesis were verified by sequence analysis.

Yeast culture, transformation, and β-galactosidase assay

Y190 yeast culture, transformation, and liquid culture β-galactosidase assays to quantify NR LBD-peptide interactions were performed as described previously (33, 37). Liquid culture β-galactosidase assays were performed in the presence of 1 μM DHT (for AR, Steraloids, Wilton, USA), 1 μM progesterone (PR, Steraloids), 100 nM estradiol (ERα) (Steraloids), 10 nM retinoic acid (RXRα) (Sigma, St. Louis, USA), or vehicle.

Mammalian cell culture, transient transfections, and luciferase activity

Hep3B cells were cultured and transfected as described previously (37). For one-hybrid assays, cells were transfected with 50 ng Gal_4DBD-peptide or Gal_4DBD-protein expression construct, 50 ng AR expression construct, and 150 ng $(UAS)_4$TATA-LUC construct, in the presence of 100 nM DHT or vehicle. Luciferase activity was determined as described previously (17, 37).

For FRET experiments, Hep3B cells were cultured overnight on glass cover slips in 9.5 cm^2 wells in α-minimal essential medium (α-MEM) supplemented with 5% FCS, L-glutamine and antibiotics. Four h prior to transfection the medium was substituted by 1 ml α-MEM containing 5% charcoal-stripped FCS. Cells were transfected with 1 μg pCYFP, or 1 μg pCFP-ARLBD and 0.5 μg YFP-peptide expression construct, together with 3 μl Fugene 6 (Roche Diagnostics, Mannheim, Germany) per μg DNA in 100 μl α-MEM. Four h after transfection the medium was substituted by 2 ml α-MEM containing 100 nM DHT. FRET assays were done the next day.

Western blot analysis

Yeast protein extraction and Western blot analysis for detection of Gal_4AD fusion proteins were performed as described previously (21, 33). Proteins were visualized using a monoclonal antibody against Gal_4AD (Takara Bio).

FRET measurement by acceptor photobleaching

Live cell imaging was performed using a Zeiss LSM510 confocal laser scanning microscope equipped with a Plan-Neofluar 40x/1.3 NA oil objective (Carl Zeiss, Jena, Germany) at a lateral resolution of 100 nm. CFP and YFP images were collected sequentially at 458 nm and 514 nm excitation, respectively, using a 458/514 nm dichroic beam splitter, a 515 nm beam splitter, and specific emission filters. CFP was excited with the 458 nm laser line of an Argon laser at moderate laser power and detected using a 470-500 nm band pass emission filter. YFP excitation was at 514 nm at moderate laser power and detected using a 560 nm emission filter.

After sequential collection of YFP and CFP images, YFP was bleached by scanning 25 times a nuclear region of ~100 μm^2, covering a large part of the nucleus, using the 514 nm argon laser line at high laser power. After acceptor photobleaching a second YFP and CFP image pair was collected. The apparent FRET efficiency was calculated after background subtraction as:

$$FRET = \frac{(CFPafter - CFPbefore) x YFPbefore}{(YFPbefore - YFPafter) x CFPafter}$$

where CFPbefore and YFPbefore are the average fluorescence intensities measured in the nuclei before bleaching and CFPafter and YFPafter the average fluoresence intensities after bleaching.

RESULTS

L to F and L to M substituted AR FxxLF motifs strongly interact with AR LBD

Although the importance of the core hydrophobic amino acid residues at positions +1 and +5 in FxxLF motifs has been described (17, 21, 26), little is known about the amino acid requirements at +4 for AR LBD binding. To study this, we tested every amino acid at this position in the context of the AR FxxLF motif using a yeast two-hybrid read-out system (Fig. 1A). In this assay, peptides were expressed as fusions to Gal$_4$AD and AR LBD was fused to Gal$_4$DBD. All assays were done in the presence of DHT (17). Western blot analysis of transformed yeast cells demonstrated that all Gal$_4$AD-peptide fusion proteins were appropriately expressed (Fig. 1B). The yeast two-hybrid screening showed that most L+4 substitutions completely abolished AR LBD interaction (Fig. 1B). Reduced interaction was observed with peptides containing a W, T, I, V, C, or Y residue at position +4 instead of an L. In contrast, AR LBD interactions were identical or even stronger than wild type motif if L+4 was substituted by F or M.

FxxFF and FxxMF variants of AR, ARA54, and ARA70 FxxLF motifs interact with AR LBD in mammalian cells

Next, we evaluated interaction capacities of FxxFF and FxxMF variants of AR FxxLF with full-length wild type AR in a mammalian one-hybrid assay (Fig. 2A and (17)). Interaction was assayed in Hep3B cells co-transfected with Gal$_4$DBD-peptide and full-length wild type AR expression constructs and a (UAS)$_4$TATA-LUC reporter. The results of this assay closely resembled the results obtained in yeast, as both FxxFF and FxxMF variants displayed hormone-dependent binding capacities comparable to the wild type motif (Fig. 2B). We also investigated the interaction of the peptides with full-length F23L/F27L-mutated AR (F23L/F27L-AR), which abrogates AR N/C interaction (17). This resulted in increased interactions of the FxxFF and FxxMF variants (Fig. 2C), indicating that both compete with the FxxLF motif in the AR NTD for AR LBD binding.

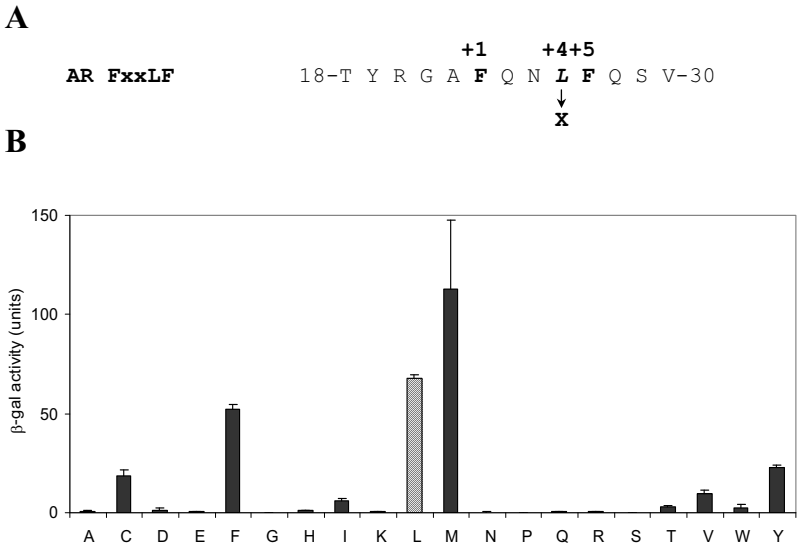

Figure 1. The effect of substitution of L+4 in the AR FxxLF motif on AR LBD interaction. (A) Amino acid sequence of the AR 18-30 peptide motif applied for mutagenesis of L+4. (B) Yeast two-hybrid analysis of L+4 substitution of the AR FxxLF motif for interaction with AR LBD. Y190 yeast cells were transformed with expression constructs encoding Gal_4DBD-AR LBD and Gal_4AD-peptide fusion proteins as described in Experimental Procedures. The amino acid single letter code of L+4 substitutions is indicated on the x-axis. Bars represent mean β-galactosidase activity of three independent experiments (+/- SD) in the presence of 1 μM DHT. No interactions were observed in the absence of hormone (data not shown). AR LBD interaction with wild type AR FxxLF motif is indicated with a hatched bar. The lower panel represents a Western blot visualizing the expression of Gal_4AD-peptide fusion proteins by GalAD-antibody staining.

Subsequently, interaction of F+4 and M+4 variants of ARA54 and ARA70 FxxLF motifs with AR LBD were assessed (Fig. 2A). The variants of both ARA54 (Fig. 2D) and ARA70 (Fig. 2F) interacted strongly with wild type AR. All variants showed increased interactions with F23L/F27L-AR, indicative of interaction with the coactivator-binding groove (Figs 2E and G). Summarizing, L+4 can be substituted by F or M residues in distinct FxxLF peptide motifs, thereby retaining AR LBD interaction.

Effects of F and M residues at position +4 on AR specificity

Previously, we and others have demonstrated that FxxLF motifs, including those of AR and ARA54, display high specificity for AR (17, 26, 38, 39). However, some FxxLF motifs, including the ARA70 motif, also interacted with PR (39). We studied in yeast two-hybrid experiments the effect of L to F and L to M substitutions at position +4 in the AR, ARA54, and ARA70

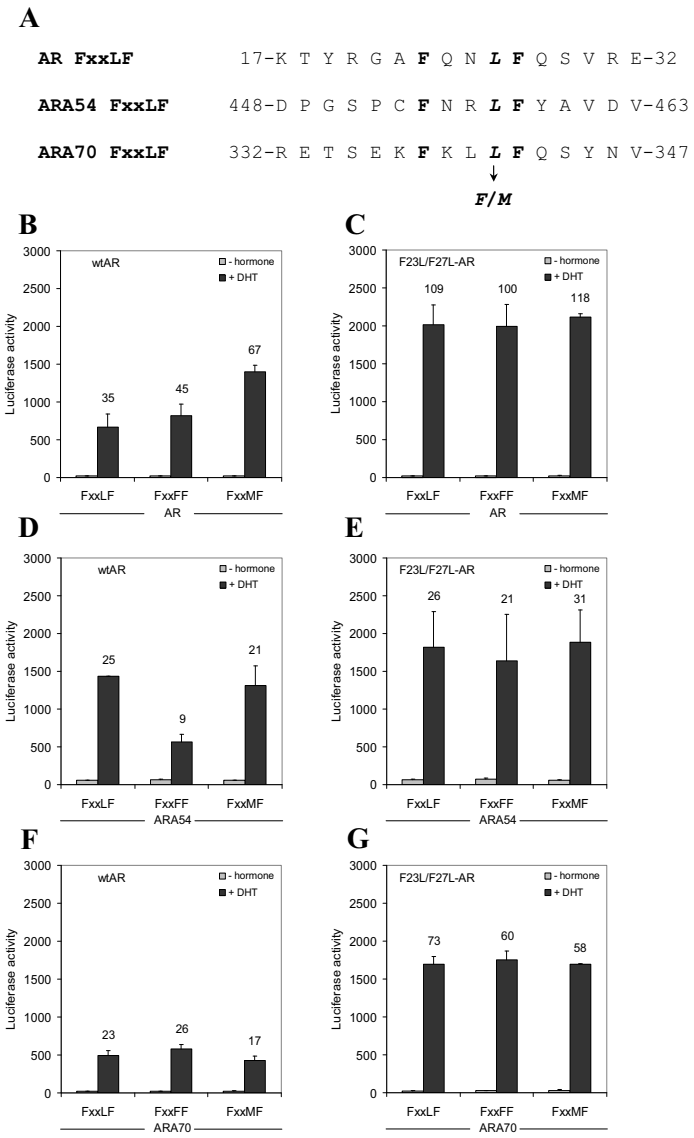

Figure 2. FxxFF and FxxMF variants of AR, ARA54, and ARA70 FxxLF motifs interact with AR LBD in mammalian cells. (A) Amino acid sequences of AR, ARA54, and ARA70 FxxLF peptides. (B to G) Mammalian one-hybrid analysis of L to F and L to M substituted FxxLF motifs of AR (B, C), ARA54 (D, E), and ARA70 (F, G) with full-length wild type AR (B, D, F) or F23L/F27L-AR (C, E, G). Hep3B cells were co-transfected with expression constructs encoding the indicated Gal_4DBD-peptide fusion protein and AR in the presence of the $(UAS)_4$TATA-LUC reporter. Interactions were determined in the absence and presence of 100 nM DHT. Each bar represents mean luciferase activity of two independent experiments (+/- SD). Mean fold inductions are shown above bars.

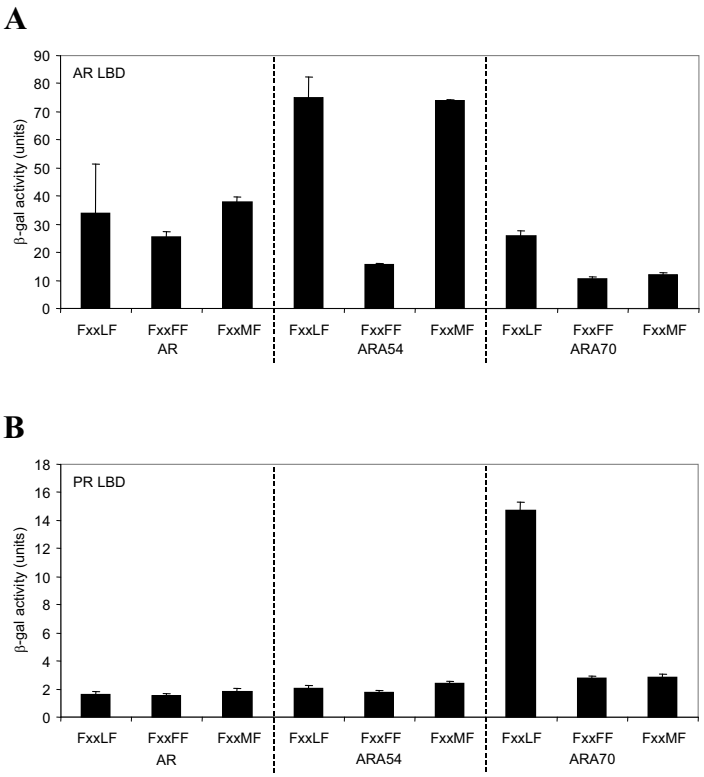

Figure 3. L to F and L to M substituted FxxLF motifs specifically interact with AR LBD. Yeast two-hybrid experiments were carried out to assess hormone-dependent interactions of L to F and L to M substituted FxxLF motifs of AR, ARA54, and ARA70 fused to Gal_4AD with the indicated NR LBDs fused to Gal_4DBD. Interaction was determined in the presence of 1 μM DHT for AR (A) and 1 μM progesterone for PR (B). Bars represent mean β-galactosidase activity of two independent experiments (+/- SD).

FxxLF motifs on AR specificity. Peptides were fused to Gal_4AD and LBDs of ERα, PR, and RXRα were fused to Gal_4DBD. Upon ligand binding all NR LBDs adopted a functional conformation since strong interaction with a control LxxLL peptide D11 was observed (39, 40). Contrary to a potent interaction with AR LBD (Fig. 3A), none of the FxxFF and FxxMF variant motifs interacted with LBDs of ERα, PR, or RXRα (Fig. 3B and data not shown). The specificity of the ARA70 FxxLF motif even increased upon L to F and L to M substitutions as no PR LBD interaction was observed with the variant ARA70 motifs (Fig. 3B). The weak β-galactosidase activities detected with PR LBD were due to the intrinsic activity of Gal_4DBD-PR LBD since similar values were obtained when this construct was expressed in the absence of a peptide expression construct (data not shown). These results demonstrate that L to F and L to M substitution variants of FxxLF motifs remain AR specific or become even more specific.

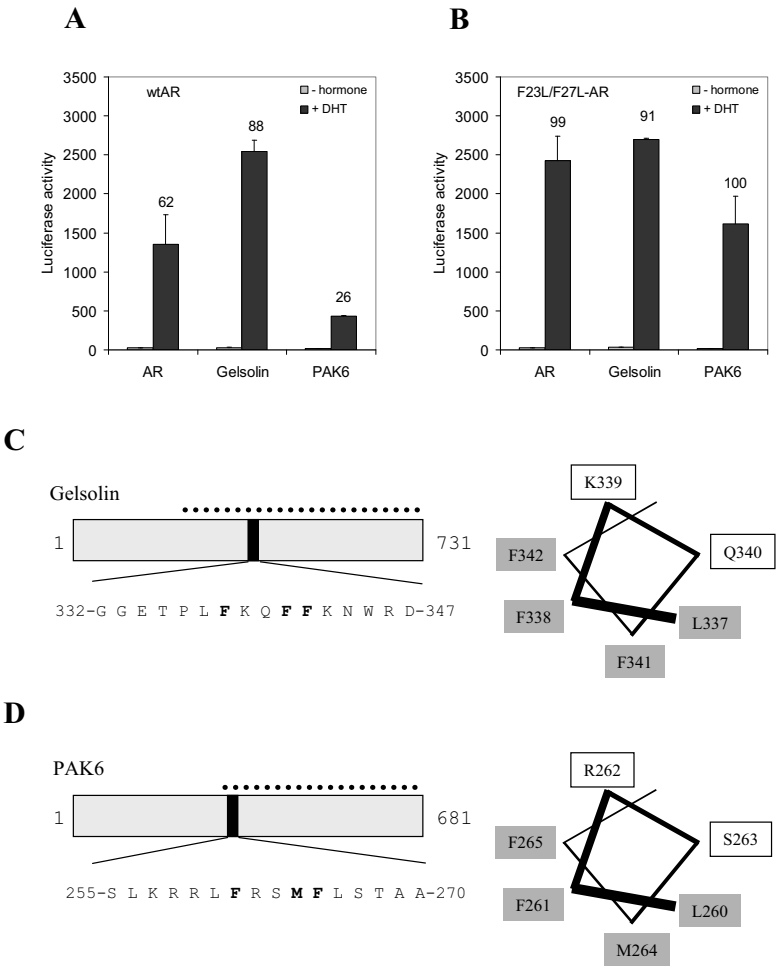

Figure 4. An FxxFF motif in gelsolin and an FxxMF motif in PAK6 interact with AR LBD. (A, B) Hep3B cells were co-transfected with expression constructs encoding Gal_4DBD-peptide (see for peptide sequences Figs 4C, D, left) and wild type (A) or F23L/F27L-substituted (B) full-length AR and a $(UAS)_4$TATA-LUC reporter plasmid. Interaction was determined in the absence and presence of 100 nM DHT. Bars represent mean luciferase activity of two independent experiments (+/- SD). Mean fold inductions are indicated above bars. (C, D, left) Schematic representation of gelsolin (C) and PAK6 (D) proteins. Positions of the FxxFF motif in gelsolin and the FxxMF motif in PAK6 and the corresponding peptide sequences tested for interaction with AR are indicated. The dotted lines represent gelsolin (C) and PAK6 (D) fragments originally identified in yeast two-hybrid screenings (43,48,49). (C, D, right) Helical wheel presentation of gelsolin FxxFF and PAK6 FxxMF motifs. Polar residues are indicated in white boxes and hydrophobic residues in grey boxes.

Naturally occurring AR-interacting FxxFF and FxxMF motifs

To assess a role of FxxFF and FxxMF motifs in cofactor-AR LBD interaction, we screened all AR interacting proteins present in the AR gene mutations database (www.mcgill.ca/androgendb; (29)) and in the human protein reference database (www.hprd.org) for the presence of these motifs. This yielded two proteins with an FxxFF (gelsolin and cdc37) and two with an FxxMF motif (PAK6 and supervillin). Mammalian one-hybrid experiments showed that the cdc37 FxxFF and supervillin FxxMF motifs weakly interacted with F23L/F27L-AR, but not with wild type AR (data not shown). In contrast, the gelsolin FxxFF and PAK6 FxxMF motifs displayed strong hormone-dependent interactions with both F23L/F27L-AR and wild-type AR (Figs 4A and B). AR N/C interaction did not affect gelsolin FxxFF binding to AR LBD, but reduced binding of the AR FxxLF and PAK6 FxxMF motifs, indicating that the gelsolin FxxFF motif had a higher affinity for AR LBD than the AR FxxLF and PAK6 FxxMF motifs. Both motifs are predicted to adopt an amphipathic α-helical structure (Figs 4C and D). FxxFF and FxxMF motifs present in AR cofactors gelsolin and PAK6 may thus be essential for interaction with AR.

To extend our knowledge on the interactions between AR LBD and gelsolin FxxFF and PAK6 FxxMF peptide motifs, *in vivo* FRET experiments were carried out (Fig. 5A). Hep3B cells were transiently co-transfected with constructs expressing CFP-tagged AR LBD and YFP-tagged peptide motifs. Close association of ligand-bound CFP-tagged AR LBD and YFP-tagged peptide results in energy transfer (FRET) by excitated CFP donor to YFP acceptor (Fig. 5A; left) (41). FRET efficiency was estimated by acceptor photobleaching (Figs 5A middle and right) (42, 43). FRET intensity was calculated based on the difference in CFP emission intensities before and after YFP photo destruction as described in Experimental Procedures.

FRET signals between AR LBD and FxxLF motifs of AR, ARA54, and ARA70 were readily detected in the presence of ligand (Fig. 5B). FRET signals between AR LBD and either gelsolin FxxFF or PAK6 FxxMF motifs were similar (Fig. 5C). These findings demonstrate direct *in vivo* interactions of AR LBD with gelsolin FxxFF and PAK6 FxxMF peptides.

Alanine scanning and AR LBD specificity of gelsolin FxxFF and PAK6 FxxMF motifs

To further characterize the gelsolin FxxFF and PAK6 FxxMF motifs we performed an alanine-scan by substituting consecutive doublet residues in both motifs into alanine residues (Fig. 6A). Mammalian one-hybrid results show that alanine substitutions encompassing the core hydrophobic residues at positions +1, +4, and +5 of both gelsolin and PAK6 completely abrogated AR interactions (Figs 6B and C). Residues at positions +6 and +7 of the PAK6 motif, but not of the gelsolin FxxFF motif, also appeared important for AR LBD interaction. All other alanine substitutions hardly interfered with AR binding.

As found for AR, ARA54, and ARA70 peptide motifs, gelsolin FxxFF and PAK6 FxxMF strongly bound to AR LBD, but hardly or not to the LBDs of ERα, PR, and RXRα (Fig. 7).

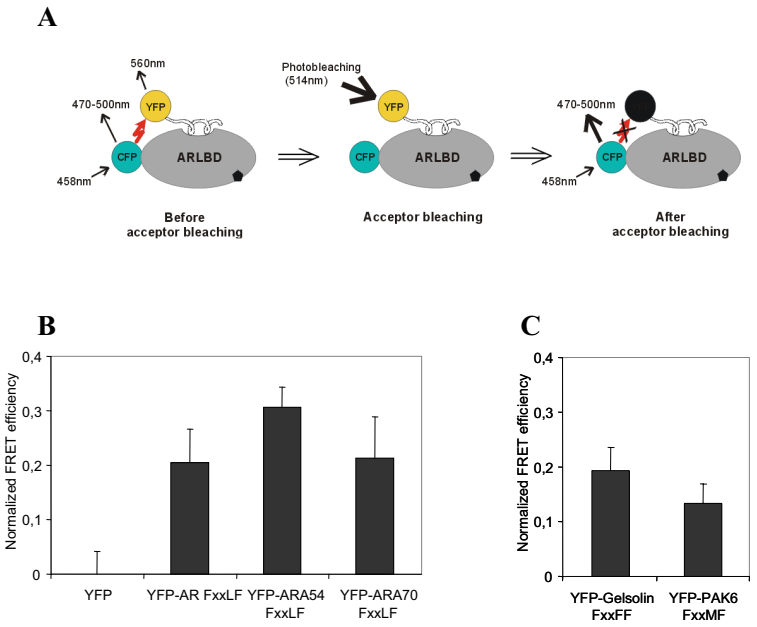

Figure 5. Direct *in vivo* interaction of AR LBD with FxxLF, FxxFF, and FxxMF motifs of AR NTD and AR cofactors. (A) Schematic representation of acceptor photobleaching FRET. Photo destruction of YPF of an interacting pair of CFP-tagged AR LBD and YFP-tagged peptide will result in enhanced CFP emission. (B, C) Direct interaction of AR LBD with the FxxLF motifs of AR, ARA54, and ARA70 (B) and with gelsolin FxxFF and PAK6 FxxMF motifs (C) as determined by *in vivo* FRET. Hep3B cells were transiently cotransfected with constructs expressing CFP-tagged AR LBD and YFP-tagged peptides. Western blot analysis demonstrated that all fusion proteins were expressed at the correct size (not shown). Confocal microscopy showed that both in the absence and presence of DHT CFP-AR LBD was localized in the nucleus, whereas the YFP-tagged peptides distributed over both nucleus and cytoplasm (data not shown). FRET was estimated based on emission intensities of CFP and YFP, before and after YFP photo destruction as described in Experimental Procedures. FRET efficiency is expressed relative to the values of co-expressed CFP-AR LBD and YFP without peptide (B) and normalized to CYFP values. FRET efficiencies of peptides were determined in three independent experiments in a total of 30 cells in the presence of 100 nM DHT. Error bars represent 2 x SEM.

AR LBD binding of cofactors gelsolin and PAK6 is FxxFF and FxxMF-mediated

Next, we investigated the importance of the FxxFF and FxxMF motifs for interaction of gelsolin and PAK6 with AR. PAK6 (aa 12-681) and gelsolin (aa 281-731) were fused to Gal_4DBD and allowed to interact with AR in the mammalian read-out system. As expected, both proteins interacted with wild type AR (Fig. 8A) and binding was increased if the competing FxxLF motif in AR NTD was inactivated (Fig. 8B). However, if the FxxFF motif in gelsolin and the FxxMF motif in PAK6 were mutated into FxxAA, interactions with both wild type AR and F23L/F27L-AR were abolished. Gelsolin and PAK6 protein expression levels were not affected by the

A

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Gelsolin Ala-I     332-G G E A A L F K Q F F K N W R D-347
Gelsolin Ala-II    332-G G E T P A A K Q F F K N W R D-347
Gelsolin Ala-III   332-G G E T P L F A A F F K N W R D-347
Gelsolin Ala-IV    332-G G E T P L F K Q A A K N W R D-347
Gelsolin Ala-V     332-G G E T P L F K Q F F A A W R D-347

PAK6 Ala-I         255-S L K A A L F R S M F L S T A A-270
PAK6 Ala-II        255-S L K R R A A R S M F L S T A A-270
PAK6 Ala-III       255-S L K R R L F A A M F L S T A A-270
PAK6 Ala-IV        255-S L K R R L F R S A A L S T A A-270
PAK6 Ala-V         255-S L K R R L F R S M F A A T A A-270
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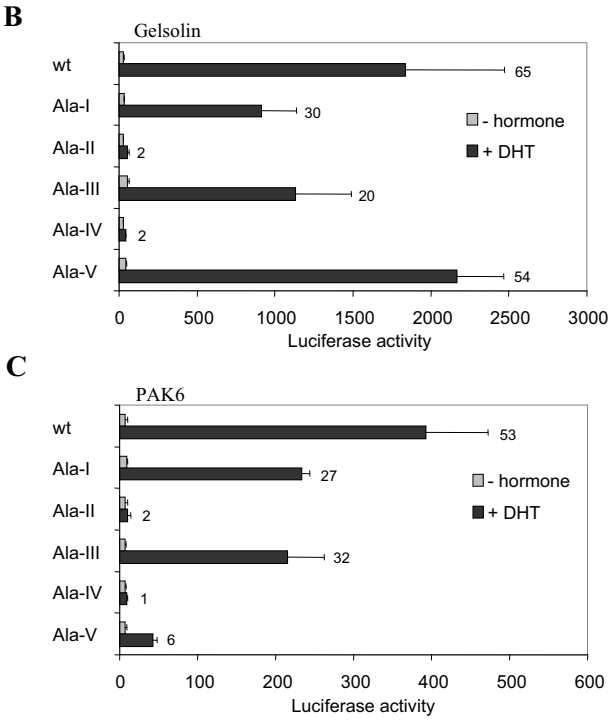

Figure 6. Alanine-scan of gelsolin FxxFF and PAK6 FxxMF motifs interacting with AR LBD. (A) Amino acid sequences of peptides used for the alanine scan of gelsolin FxxFF and PAK6 FxxMF motifs. (B, C) Gal_4DBD-fused gelsolin (B) and PAK6 (C) peptides were studied for interaction with F23L/F27L-AR using $(UAS)_4$TATA-LUC as reporter in transiently co-transfected Hep3B cells. Interaction was assayed in the absence and presence of 100 nM DHT. Bars represent mean luciferase activity of two independent experiments (+/- SD). Mean fold inductions are shown above bars.

mutations (data not shown). These data clearly demonstrate that the FxxFF and FxxMF motifs in gelsolin and PAK6, respectively, are necessary and sufficient for AR interaction.

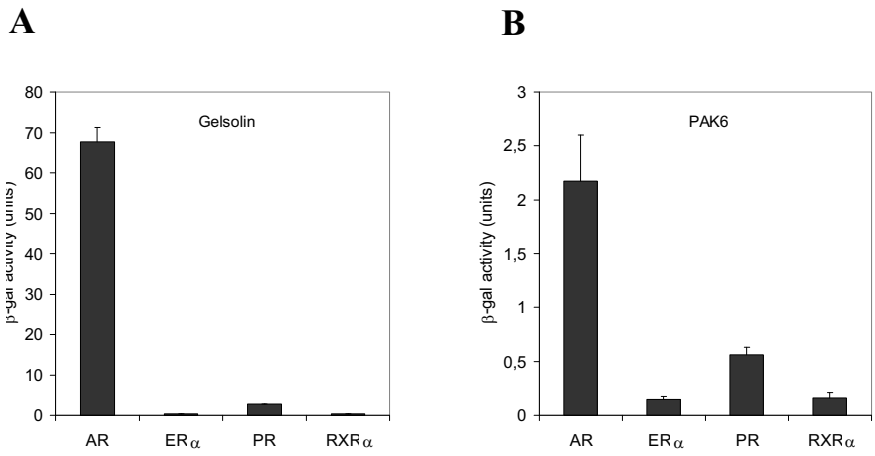

Figure 7. Gelsolin FxxFF and PAK FxxMF motifs specifically interact with AR LBD in a yeast two-hybrid assay. Y190 yeast cells were transformed with gelsolin FxxFF (A) and PAK6 FxxMF (B) motifs fused to Gal_4AD and LBDs of AR, ERα, PR, and RXRα fused to Gal_4DBD. Interaction was determined in the presence of 1 μM DHT, 100 nM 17β-estradiol, 1 μM progesterone, and 10 μM all-trans-retinoic acid, respectively. Bars represent mean β-galactosidase units of two independent experiments (+/- SD). A positive control, LxxLL peptide D11 (39), interacted with all NR LBDs ensuring proper LBD expression (data not shown).

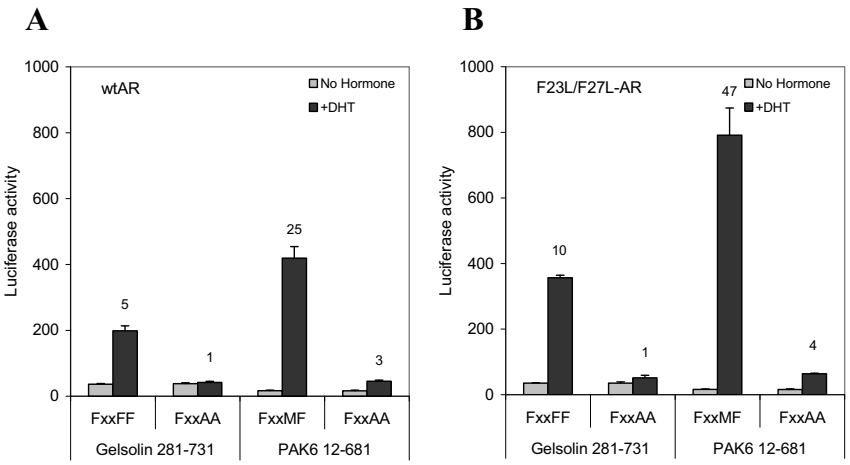

Figure 8. AR LBD binding of cofactors gelsolin and PAK6 is mediated by FxxFF and FxxMF motifs, respectively. (A, B) Hep3B cells were co-transfected with expression constructs for Gal_4DBD-PAK6 (aa12-681) or Gal_4DBD-gelsolin (aa 281-731) containing either wild type or FxxAA-mutated motifs and wild-type (A) or F23L/F27L-substituted (B) full length AR and a $(UAS)_4$TATA-LUC reporter plasmid. Interaction was assayed in the absence and presence of 100 nM DHT. Bars represent mean luciferase activity of two independent experiments (+/- SD). Mean fold inductions are indicated above bars.

DISCUSSION

Upon agonist binding the architecture of the AR LBD surface is rearranged to allow high affinity binding of FxxLF motifs present in AR NTD and in AR cofactors. Binding of these short amphipathic α-helical structures turned out to depend strongly on optimal docking of the F residues at +1 and +5 in the coactivator-binding groove of AR LBD (17, 21). Although the coactivator groove is sufficiently flexible to accommodate other large hydrophobic amino acid residues, F residues at +1 and +5 are preferred (17, 18, 30). Based on functional assays we here provide insight in the requirements of the amino acid at position +4 of peptide motifs for optimal AR LBD binding. We demonstrate that L+4 can be substituted by F and M residues in the AR, ARA54, and ARA70 FxxLF motifs, retaining strong and selective AR binding. Novel AR-interacting FxxFF and FxxMF motifs were identified in AR cofactors gelsolin and PAK6, respectively.

Systematic mutation screening of position +4 of the AR FxxLF motif resulted in the identification of three categories of peptides (Fig. 1). (1) The largest group of peptides does not interact with AR LBD. This group includes small hydrophobic, charged, or polar residues at +4; (2) Several peptides showed reduced interaction with AR LBD (C, I, T, V, W, and Y). Most of these variants have a hydrophobic residue at +4; and (3) strongly interacting variants containing bulky hydrophobic side chains (L, F, and M). Strong binding by L, F, and M residues indicates that hydrophobic contacts underlie the ability to interact with AR LBD. The inability or limited potency of most +4 variants to bind AR LBD can be due to destabilization of the peptide by distortion of the helical structure, active interference with LBD interaction caused by the charge or size of the side chains or the incapability to form sufficient hydrophobic contacts with the AR LBD. Our findings underscore the importance of the amino acid residue at +4 for optimal binding of peptide motifs to AR LBD, even though this amino acid residue is not deeply buried in the binding pocket (30, 31).

Phage display screens of random peptide libraries with full length AR or AR LBD as bait yielded different AR-interacting motifs containing F residues at positions +1 and +5 (30, 38). Besides the classical FxxLF sequences, FxxVF, FxxYF, and FxxFF motifs were identified in these screens. The FxxVF-containing peptide weakly interacted with AR, as is in agreement with our screening results, and strong interactions were observed with FxxYF and FxxFF sequences (38). In our +4 mutation screen of AR FxxLF, the FxxYF variant showed decreased interaction with AR LBD, suggesting that in this specific FxxYF motif the Y residue has a less optimal position for AR LBD binding. Similar data were found for ARA54 and ARA70 FxxLF-based FxxYF variants (data not shown). So, the requirement for the amino acid at +4 might depend on the further context of the motif. Chang and co-workers (38) demonstrated that most FxxYF and FxxFF peptide motifs picked up in phage display screens interacted with AR LBD not only in the presence but also in the absence of ligand. Repetition of these experiments in our

interaction assay indicated that ligand was essential for AR LBD interaction (data not shown). This apparent discrepancy might be due to differences in read-out systems.

Recently, crystal structures of AR LBDs in complex with the AR FxxLF and ARA70 FxxLF motifs and FxxLF, FxxFF, and FxxYF peptides selected by phage display have been resolved (30-32). Comparing LBDs with and without bound peptide showed that the side chains of amino acid residues in AR LBD that line the coactivator groove rearrange upon binding of the peptide motif. Largest conformational changes were observed for K720, M734, M894, and E897, leading to optimal binding sites for residues +1, +4, and +5 of interacting peptide motifs (30-32). The Fs at positions +1 and +5 are buried in a deep solvent inaccessible groove in AR LBD. This mode of interaction is largely conserved suggesting that these residues drive the interaction of the peptide motif (30). In contrast, the binding mode of the residue at position +4 seems less critical. This residue binds to a shallow hydrophobic depression formed by L712, V713, V716, and M894 in AR LBD (Fig. 9) (30-32). Based on the crystal structures, the side chains of the different amino acids at +4 studied so far (L, F, and Y) form hydrophobic contacts with V713, V716, and M894 in the groove with an additional contact formed between the FxxYF peptide and K717 of AR LBD. As shown in Figure 9, the FxxFF peptide has shifted in the coactivator groove towards the K720 residue as compared to the FxxLF and FxxYF peptide motifs (30). This shift together with a less optimal helical geometry of the peptide backbone makes that the F at +4 has a different orientation than an L or Y at this position (30). We have shown in this study that +4 of the peptide motif can also be an M. Because of the variability of the position of the +4 residue in the complex with AR LBD and because M has a highly flexible side chain, its precise positioning in the coactivator groove cannot be accurately predicted.

In contrast to LxxLL motifs, FxxLF motifs show a strong preference for AR (17, 26). Some FxxLF motifs, including the ARA70 FxxLF motif, also interact with PR (38, 39). The FxxFF and FxxMF motifs tested in the present study also specifically interacted with AR. L to F and L to M substitutions increased specificity of the ARA70 FxxLF motif. We hypothesize that M and F residues at position +4 select against binding to the coactivator-binding groove of PR. In agreement with this hypothesis, AR-interacting FxxLF and FxxFF peptides selected by phage display show a similar selectivity for AR: only 1 out of 5 FxxFF peptides interacted with PR LBD as compared to 4 out of 6 FxxLF-peptides (38). Of the residues in the AR coactivator-binding groove that contact the +4 side chains in FxxLF and FxxFF peptides (see Fig. 9) only V713 differs from the corresponding L727 residue in PR. As recently shown by He et al., V713L substitution in AR LBD reduced binding of the AR FxxLF motif. Vice versa, L727V substitution in PR LBD increased binding of the AR FxxLF motif (31). We presume that the size and orientation of L727 in PR LBD precludes binding of peptide motifs with bulky F and M residues at position +4.

Although the mode of interaction of the majority of cofactors with AR LBD is unknown, for several, including ARA54, ARA70, and RAD9, an essential FxxLF motif has been established (17, 26-28). Here we demonstrated that two other AR interacting proteins, gelsolin and PAK6,

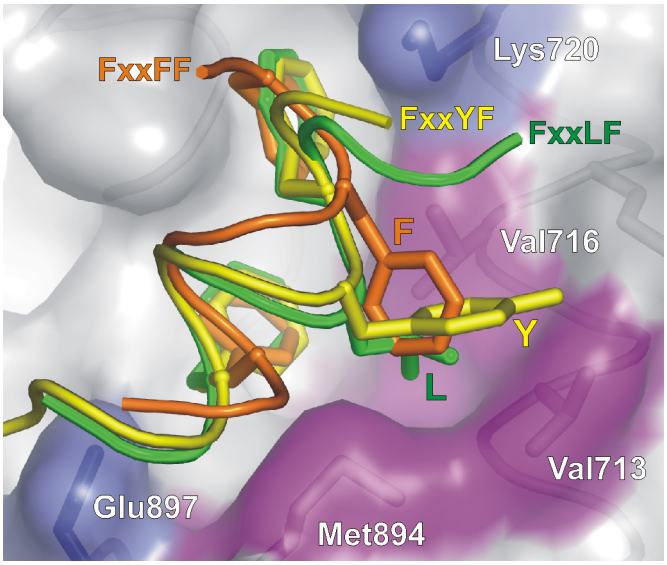

Figure 9. Variable binding modes of the +4 residue in FxxLF, FxxFF, and FxxYF peptide motifs to the coactivator groove. Surface representation of the coactivator groove region of the AR LBD (PDB entry: 1XOW). Residues that form the +4 binding site are in magenta. The binding mode of the FxxLF (green; 1XOW), FxxFF (orange, 1T73) and FxxYF (yellow, 1T7M) peptides are shown after global superposition of the various crystal structures. For clarity, only peptide side chains at positions +1, +4 and +5 are shown. AR side chains that line the +4 binding site are highlighted along with the two charge clamp residues (blue).

interact with AR LBD via an FxxFF and FxxMF motif, respectively. Gelsolin is an actin capping and severing protein, and is presumed to act as an AR cofactor by facilitating nuclear translocation (44). Interestingly, also other members of the gelsolin family, including flightless-1 and supervillin, have been identified as cofactors of AR and other NRs, suggesting an important role in NR function (45, 46). The gelsolin FxxFF motif is not only highly conserved among different species, but also among different members of the gelsolin family. Our preliminary data revealed that the conserved FxxFF motif present in adseverin also strongly binds AR LBD, suggesting that adseverin may act as an AR cofactor as well (data not shown). PAK6 is a member of the PAK family of serine/threonine kinases, which is, based on homology, divided into two subfamilies (Group I: PAK1, PAK2, and PAK3; Group II: PAK4, PAK5, and PAK6) (47). Although the FxxMF motif in PAK6 is conserved in other species, it is not conserved in any of the other members of the PAK family and so far PAK6 is the only member known to modulate NR function. PAK6 might repress AR function by phosphorylation of the DBD (48). Hormone-dependent interactions with AR LBD were observed in yeast two-hybrid and co-immunoprecipitation experiments, whereas GST pull-down experiments indicated that these LBD interactions were hormone-independent and also involved the hinge region (49, 50). Our results unambiguously demonstrated that the novel FxxMF motif is sufficient and necessary for hormone-dependent interaction of PAK6 with AR LBD.

The identification of the FxxFF and FxxMF motifs in AR cofactors raises the possibility that other so far unrecognized proteins interact with AR LBD via similar motifs. Based on these findings, it would be of interest to perform a proteome-wide *in silico* screen for Fxx(L/F/M)F peptide motifs combined with functional protein-protein interaction assays to identify new candidate AR partners.

Prostate cancer growth is dependent on the androgen-AR axis (51, 52). Nonetheless, endocrine treatment of metastatic prostate cancer by androgen withdrawal or blocking AR activity by antagonists is not curative, even though AR is still active in progressive disease in most cases (53). AR N/C interaction and cofactor interactions are important steps in AR activation. Disruption of these interactions might be a complementary or alternative approach to more efficiently inhibit AR function. Increased knowledge of the mode of AR LBD-peptide interaction will be instrumental in the design of small molecules that fit in the AR coactivator-binding groove and block protein interactions.

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REFERENCES

1. Lee DK, Chang C 2003 Endocrine mechanisms of disease: Expression and degradation of androgen receptor: mechanism and clinical implication. J Clin Endocrinol Metab 88:4043-4054
2. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16:271-321
3. Tsai MJ, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-486
4. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839
5. Warnmark A, Treuter E, Wright AP, Gustafsson JA 2003 Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. Mol Endocrinol 17:1901-1909
6. Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, Wang C, Mizokami A 1995 Androgen receptor: an overview. Crit Rev Eukaryot Gene Expr 5:97-125
7. Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. Mol Cell 9:601-610
8. Heinlein CA, Chang C 2002 Androgen receptor (AR) coregulators: an overview. Endocr Rev 23:175-200
9. Kinyamu HK, Archer TK 2004 Modifying chromatin to permit steroid hormone receptor-dependent transcription. Biochim Biophys Acta 1677:30-45
10. Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733-736
11. Greschik H, Moras D 2003 Structure-activity relationship of nuclear receptor-ligand interactions. Curr Top Med Chem 3:1573-1599
12. Pike AC, Brzozowski AM, Hubbard RE 2000 A structural biologist's view of the oestrogen receptor. J Steroid Biochem Mol Biol 74:261-268
13. Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 280:1747-1749
14. Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ, Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 12:3343-3356
15. McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Inostroza J, Torchia J, Nolte RT, Assa-Munt N, Milburn MV, Glass CK, Rosenfeld MG 1998 Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev 12:3357-3368
16. Needham M, Raines S, McPheat J, Stacey C, Ellston J, Hoare S, Parker M 2000 Differential interaction of steroid hormone receptors with LXXLL motifs in SRC-1a depends on residues flanking the motif. J Steroid Biochem Mol Biol 72:35-46
17. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J 2004 Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132-2150
18. Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C 2003 The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691-23698
19. Chang CY, McDonnell DP 2002 Evaluation of ligand-dependent changes in AR structure using peptide probes. Mol Endocrinol 16:647-660
20. Hall JM, Chang CY, McDonnell DP 2000 Development of peptide antagonists that target estrogen receptor beta-coactivator interactions. Mol Endocrinol 14:2010-2023

21. Steketee K, Berrevoets CA, Dubbink HJ, Doesburg P, Hersmus R, Brinkmann AO, Trapman J 2002 Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor. Eur J Biochem 269:5780-5791
22. He B, Kemppainen JA, Wilson EM 2000 FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986-22994
23. He B, Bowen NT, Minges JT, Wilson EM 2001 Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 276:42293-42301
24. He B, Lee LW, Minges JT, Wilson EM 2002 Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. J Biol Chem 277:25631-25639
25. Callewaert L, Verrijdt G, Christiaens V, Haelens A, Claessens F 2003 Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements. J Biol Chem 278:8212-8218
26. He B, Minges JT, Lee LW, Wilson EM 2002 The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226-10235
27. Hu YC, Yeh S, Yeh SD, Sampson ER, Huang J, Li P, Hsu CL, Ting HJ, Lin HK, Wang L, Kim E, Ni J, Chang C 2004 Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. J Biol Chem 279:33438-33446
28. Wang L, Hsu CL, Ni J, Wang PH, Yeh S, Keng P, Chang C 2004 Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol 24:2202-2213
29. Gottlieb B, Beitel LK, Wu JH, Trifiro M 2004 The androgen receptor gene mutations database (ARDB): 2004 update. Hum Mutat 23:527-533
30. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ 2004 Recognition and Accommodation at the Androgen Receptor Coactivator Binding Interface. PLoS Biol 2:E274
31. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM 2004 Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425-438
32. Estebanez-Perpina E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, Buehrer BM, Webb P, Fletterick RJ, Guy RK 2005 The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280:8060-8068
33. Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J 1997 Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Biochemistry 36:1052-1064
34. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, Stallcup MR 1998 Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 12:302-313
35. Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris C, Klaassen P, van der Korput JA, Voorhorst MM, van Laar JH, Mulder E, Trapman J 1989 The human androgen receptor: domain structure, genomic organization and regulation of expression. J Steroid Biochem 34:307-310
36. Jenster G, Spencer TE, Burcin MM, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor induction of gene transcription: a two-step model. Proc Natl Acad Sci U S A 94:7879-7884
37. Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO, Trapman J 2002 Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer 100:309-317
38. Chang CY, Abdo J, Hartney T, McDonnell DP 2005 Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol Endocrinol 19:2478-2490

39. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, Trapman J 2006 Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FxxLF and LxxLL motifs as determined by L/F swapping. Mol Endocrinol 20: 1742-1755
40. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19:8226-8239
41. Patterson GH, Piston DW, Barisas BG 2000 Forster distances between green fluorescent protein pairs. Anal Biochem 284:438-440
42. Bastiaens PI, Majoul IV, Verveer PJ, Soling HD, Jovin TM 1996 Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. Embo J 15:4246-4253
43. Bastiaens PI, Jovin TM 1996 Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: fluorescent-labeled protein kinase C beta I. Proc Natl Acad Sci U S A 93:8407-8412
44. Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, Chang HC, Yeh S, Miyamoto H, Shin M, Aozasa K, Okuyama A, Chang C 2003 Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res 63:4888-4894
45. Lee YH, Campbell HD, Stallcup MR 2004 Developmentally essential protein flightless I is a nuclear receptor coactivator with actin binding activity. Mol Cell Biol 24:2103-2117
46. Ting HJ, Yeh S, Nishimura K, Chang C 2002 Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci U S A 99:661-666
47. Jaffer ZM, Chernoff J 2002 p21-activated kinases: three more join the Pak. Int J Biochem Cell Biol 34:713-717
48. Schrantz N, da Silva Correia J, Fowler B, Ge Q, Sun Z, Bokoch GM 2004 Mechanism of p21-activated kinase 6-mediated inhibition of androgen receptor signaling. J Biol Chem 279:1922-1931
49. Lee SR, Ramos SM, Ko A, Masiello D, Swanson KD, Lu ML, Balk SP 2002 AR and ER interaction with a p21-activated kinase (PAK6). Mol Endocrinol 16:85-99
50. Yang F, Li X, Sharma M, Zarnegar M, Lim B, Sun Z 2001 Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6. J Biol Chem 276:15345-15353
51. Jenster G 1999 The role of the androgen receptor in the development and progression of prostate cancer. Semin Oncol 26:407-421
52. Trapman J 2001 Molecular mechanisms of prostate cancer. Eur J Cancer 37 Suppl 7:S119-125
53. Balk SP 2002 Androgen receptor as a target in androgen-independent prostate cancer. Urology 60:132-138

CHAPTER 4

Functional screening of FxxLF-like peptide motifs identifies SMARCD1/BAF60a as an androgen receptor cofactor that selectively modulates TMPRSS2 expression

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ABSTRACT

Androgen receptor (AR) transcriptional activity is tightly regulated by interacting cofactors and cofactor complexes. The best described cofactor interaction site in the AR is the hormone-induced coactivator binding groove in the ligand binding domain (LBD), which serves as high affinity docking site for FxxLF-like motifs. This study aimed at identifying novel AR cofactors by *in silico* selection and functional screening of FxxLF-like peptide motifs. Candidate interacting motifs were selected from a proteome-wide screening and from a supervised screening focusing on components of protein complexes involved in transcriptional regulation. Out of the 104 peptides tested, 12 displayed moderate to strong *in vivo* hormone-dependent interactions with AR. For three of these, ZBTB16/PLZF, SMARCA4/BRG1, and SMARCD1/BAF60a, the full-length protein was tested for interaction with AR. Out of these, BAF60a, a subunit of the SWI/SNF chromatin remodelling complex, displayed hormone-dependent interactions with AR through its FxxFF motif. BAF60a depletion by siRNA in LNCaP cells demonstrated differential effects on expression of endogenous AR target genes. AR-driven expression of *TMPRSS2* was almost completely blocked by BAF60a siRNA. In summary, our data demonstrate that BAF60a directly interacts with the AR LBD via its FxxFF motif, thereby selectively activating specific AR-driven promoters.

INTRODUCTION

The androgen receptor (AR) is a ligand-dependent transcription factor that is essential for normal male sexual development and for maintaining function of male-specific organs (1, 2). AR and other steroid receptors are members of the nuclear receptor (NR) superfamily (3). The NR family is characterized by a structural and functional organization, that includes a variable N-terminal domain (NTD) containing activation function 1 (AF-1), a highly conserved DNA-binding domain (DBD), and a moderately conserved C-terminal ligand-binding domain (LBD) containing activation function 2 (AF-2) (3, 4).

In eukaryotic cells, gene expression usually is in a repressed state due to a compact chromatin structure (5). A cascade of events is necessary to allow AR induced transcription of target genes. The initiating step is binding of testosterone or the more active metabolite dihydrotestosterone (DHT), leading to a conformational change in the AR LBD, dissociation of heat-shock proteins, and translocation of the AR from the cytoplasm to the nucleus. Here, AR forms homodimers and subsequently recognizes androgen response elements (AREs) located within enhancer and promoter regions of AR target genes, followed by recruitment of cofactors (6-11). Although for the majority of cofactors the mechanism by which they modulate AR activity still needs to be determined, for several, including CARM1, PRMT1, and members of the p160 family of cofactors (SRC1, TIF2, SRC3), it is known that they possess intrinsic histone modifying properties or serve as bridging factor for more potent histone acetyltransferases, such as CBP/p300 and p/CAF (12-15). Multi-subunit cofactor complexes recruited by AR include ATP-dependent chromatin remodelling complexes, such as SWI/SNF. The Mediator/TRAP/DRIP complex bridges the receptor to general transcription factors and RNA polymerase II (8, 10, 16-19). Complex interplay between individual cofactors and cofactor complexes at enhancer and promoter regions creates a dynamic chromatin environment allowing tight regulation of AR target gene expression.

For the majority of the more than 130 interacting proteins known to interact with AR, not only the physiological relevance still needs to be determined, but even how they interact with the AR. So far, the coactivator binding groove formed in the LBD after hormone binding is the only well-described protein-interaction surface of AR (20, 21). Whereas in most NRs this groove serves as high affinity docking site for α-helical LxxLL motifs, the coactivator groove in the AR is rather unique as it prefers binding of FxxLF motifs which drive the interaction of AR cofactors ARA54, ARA70, and hRAD9 (22-30). Another unique feature of the AR is that an FxxLF motif in the NTD is also able to bind strongly to the AR coactivator groove (31, 32). Although the physiological function of this FxxLF-mediated interaction of AR NTD with AR LBD (N/C interaction) is not fully understood, it is believed to decrease ligand dissociation rate and to selectively affect gene transcription (25, 33-36). Recently, we demonstrated that N/C interaction takes place in the mobile AR fraction. After binding of AR to DNA, N/C interaction is relieved and the coactivator groove becomes accessible for cofactors (37).

Previous direct mutagenesis studies of the AR FxxLF motif and phage display screenings with random and focused sequences using AR LBD or full-length AR as bait yielded several novel motif variants, including FxxFF, FxxMF and FxxYF, that are able to bind to the AR coactivator groove (21, 38, 39). A survey of known AR cofactors yielded two proteins, gelsolin and PAK6, that were fully dependent on the FxxFF and FxxMF motif, respectively, for interaction with AR (39). The present study aims at identifying novel AR cofactors on basis of FxxLF-like motifs. Based on an *in silico* proteome-wide screening and a supervised screening that focused on members of protein complexes involved in transcriptional regulation, over one hundred FxxLF-like motifs were selected to be assayed for interaction with AR. Our screenings yielded twelve peptides that displayed moderate to strong hormone-dependent interactions with AR. The FxxFF motif of SMARCD1/BAF60a, a subunit of the SWI/SNF chromatin remodelling complex, also interacted with AR if tested in the context of the full-length protein. Interactions between AR and BAF60a were dependent on the presence of hormone and required the BAF60a FxxFF motif. siRNA experiments demonstrated that BAF60a functions as a promoter-selective AR cofactor essential for the expression of the *TMPRSS2* gene.

MATERIALS AND METHODS

Plasmids

Mammalian expression plasmids encoding Gal_4DBD-peptide fusion proteins were generated by in-frame insertion of double-stranded synthetic oligonucleotides with 5'-*Bam*HI and 3'-*Eco*RI cohesive ends into the corresponding sites of the pM-B/E vector (25). Peptide expression constructs were sequenced to verify correct reading frames. In addition, proteins encoded by these plasmids were analyzed for size and expression by Western blotting.

pM-B was constructed by cutting pM (Takara Bio, Otsu, Shiga, Japan) with *Eco*RI after which the cohesive ends were filled up with Klenow enzyme and religated. pM-SMARCA4/BRG1 was generated by subcloning a *Hin*dIII fragment encoding amino acids 917-1599 of SMARCA4/BRG1 from pBJ-BRG1 (kindly provided by Dr. Gerald Crabtree, Stanford University Medical School) into the *Hin*dIII site of pM-B. pM-B/E-ZBTB16 was obtained by subcloning a *Bam*HI/*Eco*RI-digested PCR fragment encoding amino acid residues 2-673 of ZBTB16 into pM-B/E. PCR was performed using primer pair 5'-GATC**GGATCC**TCGATCTGACAAAAATGGGCATG-3' and 5'-GATC**GAATTC**TCACACATAGCACAGGTAGAGG-3' (*Bam*HI and *Eco*RI sites in bold) on pSPORT6-ZBTB16 (RZPD, Berlin, Germany). pM-BAF60a was obtained by subcloning a *Bam*HI/*Xho*I fragment encoding amino acid residues 20-476 of BAF60a from pcDNA-BAF60a (kindly provided by Dr. Nick Koszewski, University of Kentucky, and described previously (40)) into the *Bam*HI/*Sal*I sites of pM. FxxAA variant of pM-BAF60a was generated by Quikchange (Stratagene, La Jolla, Ca) using primer pair 5'-GGAAGTTCTCTTCC**GC**T**GC**TAAGTCCTTGG-3' and 5'-CCAAGGACTTA**GC**A**GC**GGAAGAGAACTTCC-3' (base substitutions in bold) according to the

manufacturer's instructions. All constructs generated by PCR and Quikchange mutagenesis were verified by sequence analysis, whereas protein size and expression were determined by Western blotting.

Mammalian constructs expressing wild type AR (pCMVAR0; (25), F23L/F27L-mutated AR (pCMVF23L/F27L-AR; (25) and rat glucocorticoid receptor (GR; (41)) have been described previously. The expression construct for progesterone receptor (PR) and the $(UAS)_4$TATA-Luc reporter construct were kindly provided by Dr. Leen Blok and Magda Meester (Erasmus MC, Rotterdam, The Netherlands), respectively.

Mammalian cell culture

Hep3B cells were cultured in αMEM (Bio-Whittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) and antibiotics, whereas LNCaP cells were maintained in DMEM (Bio-Whittaker) supplemented with 5% FCS and antibiotics.

Mammalian one-hybrid assay

Hep3B cells were plated at a density of $5x10^4$ cells per well of a 24-well plate and were allowed to grow for 24 hours. Four hours prior to transfection, the medium was replaced by 250 µl α-MEM supplemented with 5% charcoal-stripped FCS, antibiotics, and hormone or vehicle. Transfections were performed in 25 µl α-MEM containing 1 µl Fugene 6 (Roche Diagnostics, Mannheim, Germany), 50 ng Gal_4DBD-peptide or Gal_4DBD-protein expression construct (pM), 50 ng AR, PR, or GR expression construct, and 150 ng $(UAS)_4$TATA-Luc reporter per well. Luciferase activities were measured 24 hours after transfection as described previously (25).

Western blot analysis

Hep3B cells were transfected with 250 ng Gal_4DBD-peptide or Gal_4DBD-protein expression construct as described above. Twenty-four hours after transfection, cells were lysed in 100 µl Laemmli buffer (50 mM Tris, 10 mM DTT, 10% glycerol, 2% SDS and 0.001% Bromophenol Blue). Lysates were boiled and subjected to electrophoresis on a 10% SDS polyacrylamide gel, after which proteins were transferred to a nitrocellulose membrane. Blots were incubated with monoclonal antibodies directed against Gal_4DBD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and subsequently with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark). Proteins were visualized using SuperSignal West Pico Chemiluminescent blotting substrate from Pierce (Rockford, IL), followed by exposure to X-ray film.

siRNA transfection

LNCaP cells were seeded in 25 cm^2 culture flasks in DMEM supplemented with 5% FCS. After 72 h, the medium was replaced with DMEM supplemented with 5% charcoal-stripped FCS. Four hours later, siRNAs were transfected according to the manufacturer's protocol using

Dharmafect 3 (Dharmacon, Lafayette, CO) and allowed to grow for another 48 h. Cells were then treated with 1 nM R1881 or vehicle for 8 h, after which they were trypsinized and harvested. Cell pellets were stored at -80°C until RNA isolation. BAF60a, BAF60b, and BAF60c siRNAs as well as control non-targeting siRNA were obtained as pre-designed siRNA pools from Dharmacon. siRNA against AR (5'-GCAGUAUCCGAAGGCAGCA-3') was ordered as annealed double stranded siRNA from Qiagen (Valencia, CA).

RNA isolation, cDNA preparation, and quantitative PCR

Analysis of AR target gene mRNA expression in absence or presence of siRNA was performed by quantitative PCR (QPCR) using the SYBR Green method (Applied Biosystems, Foster City, CA). Total RNA was isolated using the RNeasy kit (Qiagen). Synthesis of cDNA and performance of the QPCR have been described previously (42). Amounts of specific mRNAs for each sample were determined relative to porphobilinogen deaminase (*PBGD*) by the standard curve method. Primer combinations used are indicated in Supplementary Table I.

RESULTS

In silico selection of candidate AR binding partners on basis of FxxLF-like motifs

In order to identify novel AR cofactors, two different *in silico* screenings were performed to select for FxxLF-like motif harboring proteins. The first was based on a proteome-wide screening (Fig. 1A). Using the Reference Sequence (RefSeq) protein database from NCBI (release 15), all human proteins containing an FxxFF, FxxMF, or FxxYF motif were selected. Because of the high number of motifs retrieved (>27,000), additional selection criteria were set. Using the Homologene database, those motifs were selected that are conserved between human and both mouse and rat orthologues. Based on Gene Ontology, motifs were selected which are present in proteins that reside in the same cellular compartments as AR, i.e. cytoplasm and nucleus, or of which the subcellular localization was unknown. Proteins present in other cellular compartments as well as secreted proteins were excluded. Finally, proteins were selected which are expressed in at least one of the same tissues as AR, including prostate, epididymus, seminal vesicles, and testis, or of which the expression pattern was unknown. Based on these criteria 33 FxxFF, 27 FxxMF, and 11 FxxYF motifs in these proteins were assayed as peptide for interaction with AR (Supplementary Table II). In addition, based on similar criteria, but using the SwissProt database, 18 FxxLF motifs were selected and tested for AR interaction (Supplementary Table II).

A second screening was based on a focused selection method (Fig. 1B). Because the AR is a transcription factor, proteins were selected that are part of two multi-subunit complexes involved in the transcription process: SWI/SNF and Mediator/TRAP/DRIP. SWI/SNF complexes consist of a core ATPase, either SMARCA2/BRM or SMARCA4/BRG1, and 10 to 12 BRG1

associated factors (BAFs). Mediator/TRAP/DRIP may consist of more than 16 components. Because the number of FxxLF, FxxFF, FxxMF, and FxxYF motifs in these proteins is limited, we extended the selection criteria by inclusion of related motifs, like FxxWF, FxxIF, FxxYL and FxxLY, which also had to be conserved in mouse and rat orthologues. These criteria yielded 15 motifs (Supplementary Table III). Together with the 89 motifs selected in the first screening, a total of 104 motifs were assayed for AR interaction.

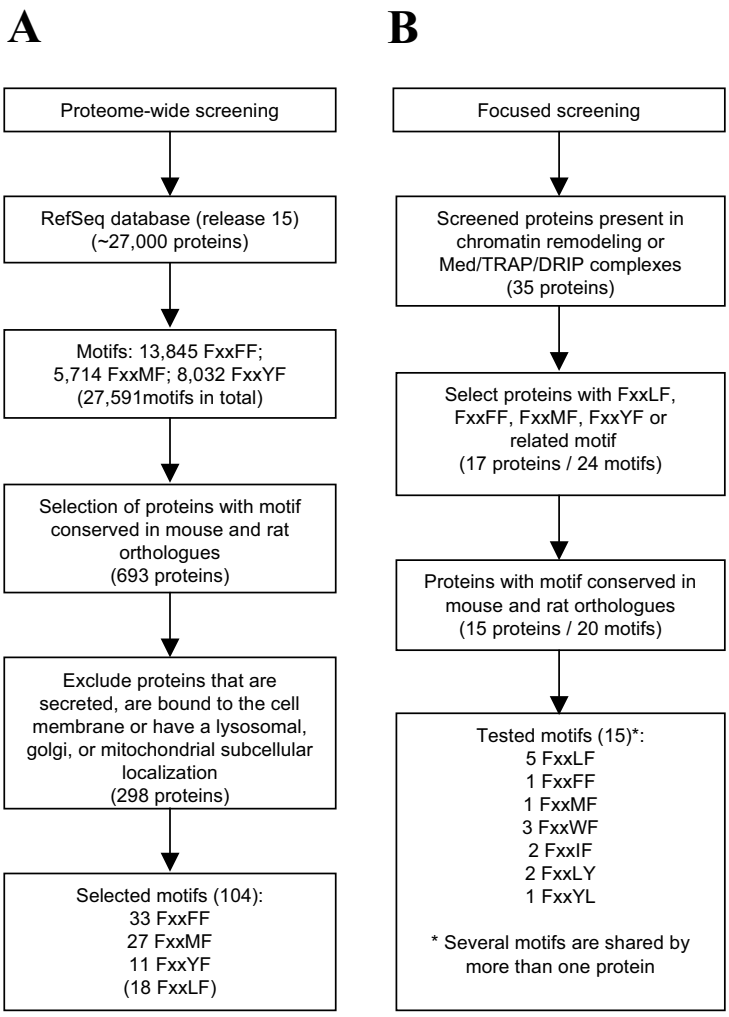


Figure 1. Selection procedures of motifs to test for interaction with AR. Flow-charts showing selection criteria for the proteome-wide screening (A) and the supervised screening focusing on proteins present in large protein complexes involved in the transcriptional process (B). Numbers of remaining peptides and/or proteins after each selection are indicated.

Functional screening yields 12 AR-interacting motifs

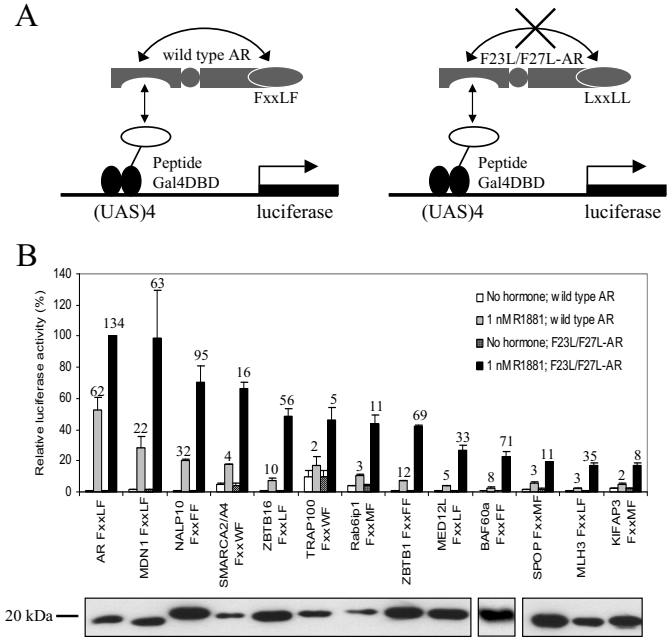
The 104 selected motifs were tested for interaction with AR in a mammalian one-hybrid assay as shown schematically in Figure 2A. Vectors were constructed expressing the motifs linked to Gal_4DBD and these fusion proteins were assayed for interaction with full-length wild type AR (Fig. 2A, left) or a mutant AR in which the competing FxxLF motif in the NTD was substituted by inactive LxxLL (F23L/F27L-AR; Fig. 2A, right). If the AR is recruited by Gal_4DBD-peptide, the AR will transactivate the $(UAS)_4$TATA-Luc reporter in the presence of 1 nM R1881, but not in the absence of hormone.

Most selected peptides displayed weak interactions with F23L/F27L-AR (<20% interaction capacity as compared to AR FxxLF motif or less than 5-fold hormone induction) or did not interact at all (data not shown). Twenty peptides displayed elevated luciferase activities even in the absence of hormone (between 5 and 20% luciferase activity as compared to the hormone-dependent interaction of AR FxxLF peptide with F23L/F27L-AR). Eight peptides displayed high basal activities (>20% activity compared to the AR FxxLF interaction with F23L/F27L-AR; Supplementary Tables II and III). These elevated and high basal activities were caused by the peptides themselves as similar luciferase values were obtained in the absence of AR (data not shown). In general, peptides that showed elevated or high basal activities were characterized by a relatively high number of acidic residues, which are known to contain intrinsic activity (43).

Our screenings yielded twelve peptides that showed modest to strong interactions with F23L/F27L-AR (>20% interaction capacity as compared to the AR FxxLF motif and more than 5-fold hormone induction; Fig. 2B). Interactions were reduced when assayed with wild type AR, demonstrating competition between the peptide and the FxxLF motif in the AR NTD for binding to the coactivator binding groove in the LBD. Eight peptides (MDN1, NALP10, ZBTB16, Rab6IP1, ZBTB1, SPOP, MLH3, KIFAP3) were derived from the proteome-wide screening. Four peptides (SMARCA2/A4, TRAP100, MED12L, SMARCD1/BAF60a) were derived from the supervised screening. Of these interacting peptides, four contained an FxxLF, three an FxxFF, and three an FxxMF motif (Fig. 2C). SMARCA2/A4 and TRAP100 interacted with the AR via a novel FxxWF motif. Western blot analysis demonstrated expression levels of the twelve interacting peptides (Fig. 2B).

Interaction of full-length BAF60a with AR is dependent on an intact FxxFF motif

Of the 12 peptide motifs interacting with AR, three were selected for interaction with AR in their respective full-length protein context. Although MDN1 FxxLF and NALP10 FxxFF were the strongest AR-interacting peptides (Fig. 2B), they were not further analyzed yet because of the size of the protein (MDN1: 5596 amino acid residues) or less likely AR mediated function (NALP10: negative regulator of inflammatory and apoptotic signal transduction), respectively. SMARCA4/BRG1, one of the two core ATPases of the SWI/SNF chromatin remodeling complex, was selected for further analysis because of the strong interaction and its novel FxxWF motif.



C

AR	KTYRGA **F**QN**LF** QSVRE
MDN1	KDTSPV **F**QR**LF** LESSD
NALP10	SFRHIS **F**QD**FF** HAMSY
SMARCA2/4	FKSCST **F**EQ**WF** NAPFA
ZBTB16	ACTSKM **F**EI**LF** HRNSQ
TRAP100	AGPHTQ **F**VQ**WF** MEECV
RAB6IP1	EVFANR **F**TQ**MF** ADYEV
ZBTB1	AACSSY **F**RM**FF** MNHQH
MED12L	SHLRSK **F**GD**LF** SSACS
BAF60a	TKQKRK **F**SS**FF** KSLVI
SPOP	AARSPV **F**SA**MF** EHEME
MLH3	NRTMKT **F**VK**LF** QSGKA
KIFAP3	ISMDDR **F**KS**MF** AYTDC

Figure 2. Mammalian one-hybrid analysis yields 12 AR-interacting peptides. (A) Schematic representation of the mammalian one-hybrid assay. Peptides were fused to Gal_4DBD and served as bait for full-length wild type AR (A, left) or F23L/F27L-AR in which the competitive FxxLF motif in the NTD has been substituted by LxxLL (A, right). In case of interaction between peptide and AR LBD, the AR NTD serves as transcription activation domain. Interactions were determined in absence and presence of hormone using Hep3B cells. (B) Twelve peptides interact with AR. Only peptides are shown of which the interaction capacity was >20% as compared to the interaction of AR FxxLF motif with F23L/F27L-AR or have a more than 5-fold hormone induced interaction with F23L/F27L-AR. White and grey bars represent interactions with wild type AR in the absence or presence of R1881, whereas hatched and black bars represent interactions with F23L/F27L-AR in absence or presence of R1881. Each bar represents mean relative luciferase activity of two independent experiments (+/- SD). Interaction of the AR FxxLF motif with F23L/F27L-AR was set to 100% and mean –fold inductions are shown above the bars. Expression of the Gal_4DBD-peptide fusion proteins was visualized in a Western blot using antibodies against Gal_4DBD and is shown below the figure.

ZBTB16, ZBTB1, and SPOP are related members of the family of BTB/POZ domain-containing proteins. ZBTB16, a transcriptional repressor also known as PLZF, was selected for further analysis because it showed strongest interactions with AR (Fig. 2B). Of the remaining motifs the BAF60a subunit of the SWI/SNF complex was selected for further analysis as it displayed highest hormone inductions with both wild type AR and F23L/F27L-AR.

Figure 3A schematically shows the positions of the motifs in the proteins and the ZBTB16, SMARCA4, and BAF60a fragments assayed for AR interaction. Protein fragments were fused to Gal_4DBD and evaluated for interaction with AR in a mammalian one-hybrid assay as described above. Although ZBTB16 FxxLF and SMARCA4 FxxWF interacted with AR as peptides, no interactions were observed if both motifs were in a larger protein fragment (Fig. 3B). In contrast, hormone induced AR interaction was observed with BAF60a protein (Fig. 3B). Interactions of BAF60a with AR were completely dependent on an intact motif, as a mutant BAF60a, in which the FxxFF motif was mutated into FxxAA, did not interact (Fig. 3B). Western blot analysis confirmed the expression and size of all fusion-proteins (Fig. 3C).

BAF60a preferentially interacts with AR

Previously, we demonstrated that several FxxFF motifs selectively bind AR LBD (39). To investigate whether this is also true for BAF60a peptide and protein, mammalian one-hybrid assays were carried out with PR and GR.

The NR interaction profiles of BAF60a peptide (Fig. 4A) and protein (Fig. 4B) were highly similar. Both BAF60a peptide and protein displayed strong hormone-dependent interaction with AR, but not at all with PR. Weak interactions were observed with GR. A control peptide, D11 LxxLL (44), displayed strong interactions with all three NRs (data not shown). Mutating the FxxFF motif in the peptide or the protein into FxxAA resulted in strongly reduced interactions of BAF60a with AR. So, the FxxFF motif is essential for AR interaction.

BAF60a is essential for AR-dependent TMPRSS2 expression

To study whether BAF60a plays a functional role in AR target gene expression, siRNA experiments were carried out. BAF60a has two highly homologous family members, SMARCD2/BAF60b and SMARCD3/BAF60c, with conserved FxxFF motifs. Therefore, also the contribution of these two family members on AR regulated transcription was investigated.

LNCaP cells were transfected with siRNA against each of the three BAF60 members, after which the mRNA levels were measured by quantitative PCR (QPCR). All three BAF60s were expressed in LNCaP cells and expression of each individual BAF60 member was specifically inhibited by the corresponding siRNA (Supplementary Figure 1). Next, we investigated the role of the different BAF60s in regulating transcription from endogenous androgen responsive genes in prostate cancer cells. LNCaP cells were transfected with control siRNA (siControl), siRNA against AR (siAR), or siRNA against the three individual BAF60 members, followed by incubation with R1881 or vehicle for 8 hours. Expression of *SGK*, *SARG*, *NDRG1*,

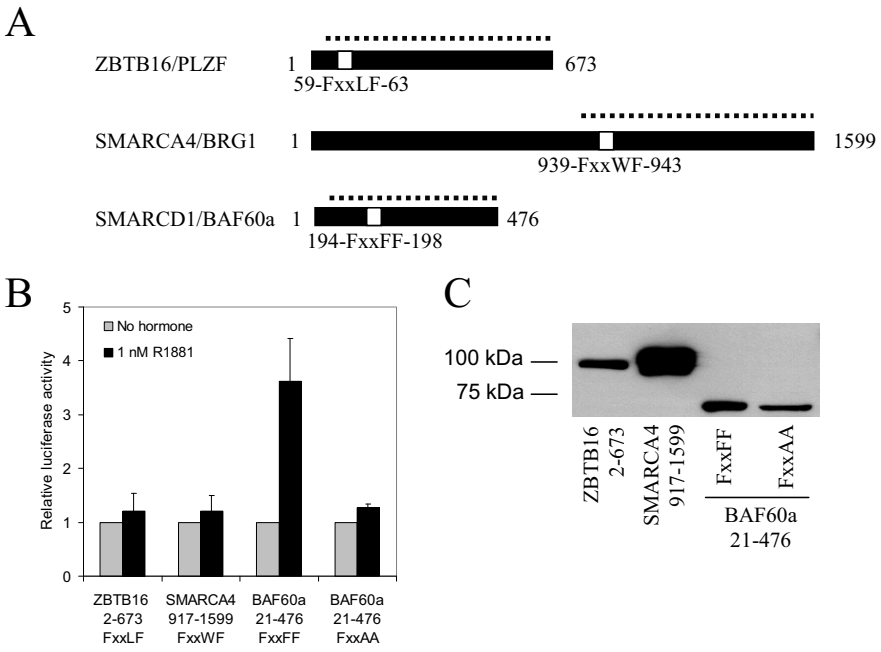


Figure 3. Full-length BAF60a displays hormone– and motif-dependent interactions with AR. (A) Schematic representation of proteins tested for interaction with AR. Shown are the relative position of the motif in the protein (white box) and the fragment of ZBTB16, SMARCA4, and BAF60a used for interaction with AR (dotted line). (B) Mammalian one-hybrid analysis of proteins tested for interaction with AR. Experimental setup was similar to that described in the legends of Fig. 2A and B. Data shown are the relative interactions. Interaction of each protein with AR in absence of hormone was set to 1. Bars represent the mean of two independent experiments (+/- SD). (C) Western blot visualizing the expression of Gal_4DBD-protein fusions by Gal_4DBD antibody staining.

PSA, *KLK2*, and *TMPRSS2* mRNA was measured by QPCR (Fig. 5). Expression of all target genes was strongly induced in the presence of hormone (siControl) and was dependent on AR as inhibition of AR expression with siRNA (siAR) strongly inhibited target gene expressions. Depletion of BAF60a, BAF60b, and BAF60c with siRNA did not affect or weakly decreased the hormone-dependent expression of *SGK*, *SARG*, and *NDRG1* (Fig. 5A-C), whereas *PSA* and *KLK2* expression were reduced to about 50% in the presence of each individual siRNA (Fig. 5D and E). In contrast, a differential effect was observed on *TMPRSS2* expression (Fig. 5F). Expression of *TMPRSS2* was weakly reduced (about 30%) after depletion of BAF60b and BAF60c, but was almost completely abolished after BAF60a depletion. These data show that BAF60a is essential for high AR-dependent expression of *TMPRSS2*.

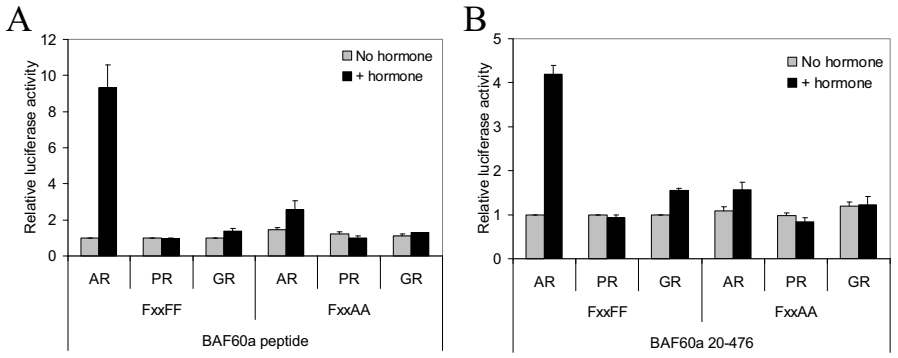


Figure 4. BAF60a preferentially interacts with AR. Mammalian one-hybrid assay showing the interactions of BAF60a FxxFF and FxxAA peptides (A) or proteins (B) with full-lengths AR, PR and GR. Experimental setup is similar to that described in the legends of Fig. 2A and B. Bars represent mean relative luciferase activities of three independent experiments (+/- SEM) in absence (grey bars) or presence (black bars) of hormone. Interaction of the wild type motif-harbouring peptide or protein with a NR in absence of hormone was set to 1. Hormones used were 1 nM R1881 for AR and PR, and 10 nM dexamethasone for GR.

DISCUSSION

This study aimed at identifying novel AR cofactors that directly interact with AR on the basis of FxxLF-like motifs binding to the ligand-induced coactivator-binding groove in the AR LBD. *In silico* peptide motif selections followed by functional interaction assays yielded SMARCD1/BAF60a, a component of the SWI/SNF chromatin remodelling complex, as a novel AR cofactor that directly and hormone-dependently interacts with AR via its FxxFF motif. We further demonstrated that BAF60a differentially affected AR target gene expression and that BAF60a was essential for high *TMPRSS2* expression.

Our search for novel AR cofactors started with a proteome-wide *in silico* screening to select for potentially AR-interacting motifs, based on sequences previously demonstrated to bind strongly AR LBD, such as FxxLF, FxxFF, FxxMF, and FxxYF (21, 38, 39). Selected motifs were then tested as peptides in mammalian one-hybrid assays to determine their interaction capacity with AR. Although this approach is semi-high-throughput, it has several advantages over conventional screening methods, such as yeast two-hybrid and phage display. A major advantage is that selection criteria can be defined, like conservation, tissue of expression, and subcellular localization. In addition, motifs can be selected that otherwise could have been missed by screening of cDNA libraries, for example because the motif is located at the very N-terminus of a protein or is present in a sequence underrepresented in libraries. On the other hand, *in silico* screenings are dependent on database information. Because most databases are incomplete, it can be predicted that potentially interacting motifs were missed. We

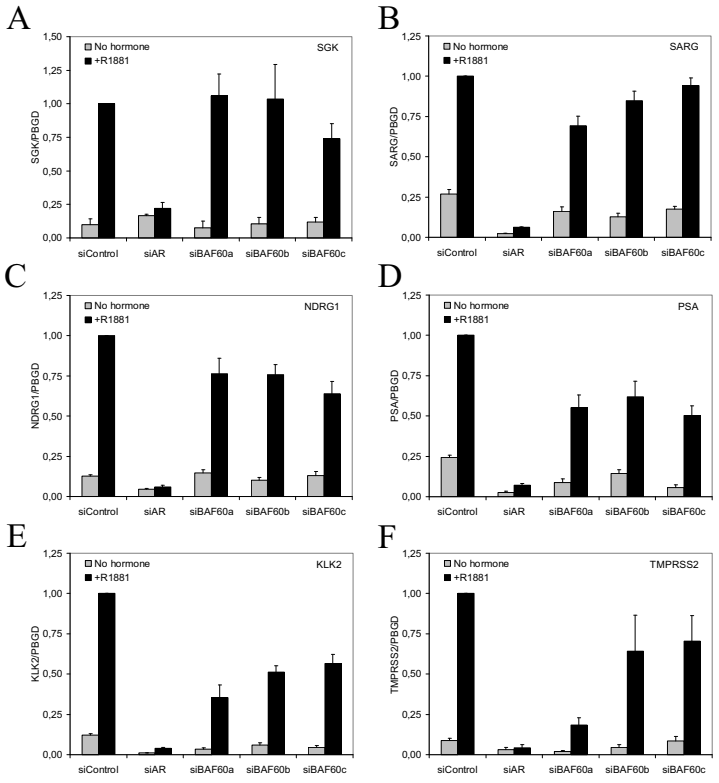


Figure 5. BAF60a selectively affects the hormone-dependent expression of the AR target gene TMPRSS2. LNCaP cells were transfected with non-targeting siRNA (siControl), siRNA directed to AR (siAR), or with siRNA against BAF60a, BAF60b, or BAF60c as described in the Experimental procedures. mRNA levels of AR target genes *SGK* (A), *SARG* (B), *NDRG1* (C), *PSA* (D), *KLK2* (E), and *TMPRSS2* (F) were determined by QPCR in the absence or presence of 1 nM R1881. Results shown are the average of 3-6 QPCR experiments (+/- SEM) divided over two individual siRNA experiments. Expressions were normalized to *PBGD* values and are relative to the hormone-dependent expression in the presence of siControl.

also focused on a limited number of potentially AR-interacting FxxLF-like motifs. It cannot be excluded that as yet unidentified cofactors exist that interact via a different type of FxxLF-like motif not included in our search.

Of the 104 selected FxxLF-like motifs assayed for interaction with AR, 12 displayed moderate to strong interactions. From this low number it is again clear that the three core residues of FxxLF-like motifs are not the sole interaction determinants (24, 32, 45). It may be that the non-interacting peptides do not form an α-helical structure or that residues flanking the FxxLF-like motif prevent stable interactions with the AR LBD surface. Although comparison of the amino acid sequences of the interacting peptides did not reveal a clear consensus in residues flanking the FxxLF-like motifs, all interacting peptides (except for ZBTB1) contained

positively charged residues N-terminally to the core motif; a tendency for charged residues at the C-terminus was less clear (Fig. 2C). This correlates with previously published data demonstrating the importance of charged residues flanking the core motif which form electrostatic interactions with oppositely charged residues on the AR LBD surface (45). However, charged residues are not the only determinant for peptide interaction, as many non-interacting peptides from our screening contain a similar charge distribution.

We identified two peptides, SMARCA2/SMARCA4 and TRAP100, that interacted with AR via a novel type of motif: FxxWF. Previous screenings for novel AR-interacting motifs already demonstrated that W (Trp) residues could be compatible with binding to the AR coactivator binding groove when present at positions +1 and +5 (21, 25, 46). Our previous screening at position +4 of the AR FxxLF motif demonstrated that substitution of L (Leu) for W (Trp) resulted in a peptide weakly interacting with the AR LBD (39). The data obtained in this study show that SMARCA2/A4 and TRAP100 FxxWF motifs do interact with AR, suggesting that in these amino acid contexts W+4 is able to obtain a conformation that is favourable for interaction with the AR coactivator groove.

Three motifs were selected for further analysis in full-length proteins. Of the twelve AR-interacting peptide motifs, eight were derived from the proteome-wide screening. With current knowledge, the molecular functions of three of these (NALP10, Rab6IP1, and KIFAP3) could not be linked to AR transcription regulation and these motifs were therefore not selected. Two peptides (MDN1 and MLH3) were not selected because of protein size (MDN1 consists of almost 5600 amino acid residues) or because of relative weak interaction. However, both proteins remain interesting candidates for AR binding and will be part of future study. Three peptides (ZBTB1, ZBTB16, and SPOP) are present in members of the family of BTB/POZ domain containing proteins. Because the motifs are located at similar positions within the BTB/POZ domain, ZBTB16 was selected as it showed strongest interactions with AR. The remaining four peptides were derived from the screening focusing on complexes involved in transcriptional processes. Two of these (BAF60a and SMARCA4) were selected for further analysis. Of the other two, TRAP100 was previously shown to coimmunoprecipitate with AR and to enhance AR-dependent transcription (18). However, whether this interaction is directly through its FxxWF motif remains to be determined. Also the involvement of MED12L in AR-dependent transcription remains to be investigated.

Although SMARCA4 FxxWF and ZBTB16 FxxLF interacted with AR as peptides, no interactions were observed if these motifs were tested in large protein fragments. This suggests that in these fragments the helical structure of the motif is disturbed or that the motif is hidden in the three-dimensional structure of the protein. Analyzing crystal structures of the BTB-POZ domain in ZBTB16 revealed that the FxxLF motif is oriented towards the interior of this domain, which may explain why interaction was only found for the peptide. Although BTB/POZ domains are poorly conserved among the different family members, the residues N-terminally to the motif are highly conserved (47). Together with the observation that the

FxxLF-like motifs are located at similar positions within the BTB/POZ domains, it seems unlikely that other members of this family do interact with AR via this domain.

Both SMARCA4/BRG1 and BAF60a are components of SWI/SNF chromatin remodelling complexes. SWI/SNF core complexes consist of the ATPase BRM or BRG1, and the core subunits BAF47/INI1/SNF5, BAF155, and BAF170 (48). Other subunits commonly found include BAF53, BAF57, BAF60, BAF180, and BAF250 (49-53). Recruitment of SWI/SNF complexes is crucial for transcriptional activity by essentially all NRs (49, 54-57). More recently it has been demonstrated that chromatin bound AR recruits the SWI/SNF core ATPases BRM and BRG1, and requires a functional SWI/SNF complex for its transcriptional activity (16, 17, 58). Although there was a preference for the recruitment of SWI/SNF complexes containing BRM (17), in vitro interaction assays failed to detect a direct interaction between BRM and AR (59). Similarly, no direct interactions were observed between BRG1 and GR or ERα (60, 61). Our results, which failed to detect an interaction between BRG1 and AR, are consistent with these previous observations and suggest that recruitment of SWI/SNF complexes to NRs, including AR, is not via the core ATPases, but via the other subunits. Recently, it was demonstrated that BAF57 displayed hormone-independent interactions with AR and was essential for hormone-dependent AR transcriptional activity (59). Here we show that BAF60a serves as a good candidate bridging factor for a hormone-dependent interaction of SWI/SNF with AR.

The BAF60 family of proteins consists of BAF60a, BAF60b, and BAF60c, which appear to have ubiquitous expression patterns (52, 62, 63). Highest levels of BAF60b are found in pancreas and lung, whereas BAF60c is preferably expressed in brain and muscle tissue. Depletion in mice demonstrated that BAF60c was essential for development of the heart and for left-right asymmetry (64, 65). In addition, BAF60c was demonstrated to interact with the NRs ERα, PPARγ, RAR, RXRα, FXR, and LRH-1 (62, 66). Also BAF60a has been found to interact with NRs, including GR, PR, ERα, FXR, and PPARγ (61). Although no interactions were observed with VDR and RXRα as individual proteins (61), BAF60a did interact with these proteins when present as heterodimer (40). GST-pulldown and coimmunoprecipitation experminents demonstrated that BAF60a and BAF60c interacted with NRs in the absence of hormone and interactions were not enhanced by ligand. Furthermore, it was demonstrated that BAF60a harbours two discrete protein interaction surfaces (61). An N-terminal fragment was necessary for the interaction with BRG1, GR DBD, and other NRs, whereas a C-terminal fragment was essential for interaction with the SWI/SNF core components BAF155 and BAF170. These observations are in contrast with our findings regarding AR. First of all, we found that interactions between BAF60a and AR were hormone-dependent. Secondly, we found that the interactions were dependent on the FxxFF motif, demonstrating that the interaction with AR occurs via the coactivator groove in the LBD. And thirdly, the FxxFF motif is present in the C-terminal fragment of BAF60a. These observations indicate a unique mode of interaction between BAF60a and AR as compared to other NRs.

Conflicting data exist on the presence of BAF60 proteins in SWI/SNF complexes. Wang et al. showed that at least BAF60a and BAF60b are present in separate SWI/SNF complexes (52), whereas Lemon et al. found BAF60a and BAF60b in the same complex (49). Our results showed that depletion of the different BAF60 members by siRNA had no or limited effect on AR dependent expression of *SGK*, *SARG*, and *NDRG1* (Fig. 5A-C), suggesting that there is redundancy between the different BAF60 members, that other subunits in the same complex determine recruitment by AR, or that expression of these genes is independent of BAF60 containing complexes. Each of the three BAF60 members was essential for optimal expression of *PSA* and *KLK2* (Figs. 5D and E). In contrast, involvement of BAF60 members in expression of *TMPRSS2* revealed that only BAF60a was essential for expression or *TMPRSS2* suggesting that there is little redundancy (Fig. 5F). This is the first report in which a functional difference for BAF60 proteins on different target promoters is described. Recently, it was reported that the *TMPRSS2* gene was frequently fused to members of the Ets family of transcription factors in prostate cancers (42, 67, 68). Because of the gene fusion, expression of ERG and other Ets factors is now under control of the AR-responsive *TMPRSS2* promoter, leading to aberrant androgen-regulated overexpression of Ets factors. Our data indicate that BAF60a may serve as a target in these types of tumors by affecting AR transcription involving the *TMPRSS2* promoter.

It is currently not known why there is a specific effect of BAF60a on *TMPRSS2* expression. AR regulates expression of genes involved in different cellular processes such as proliferation, differentiation and cell survival. So, maybe BAF60a is essential for expression of genes in specific processes. However, further experiments need to be performed to clarify this.

Our results and previous observations demonstrated that SWI/SNF recruitment to AR involves at least two different BAF subunits: BAF57 and BAF60a. Similar multi-subunit interactions with SWI/SNF has also been observed for GR. Whereas BAF57 and BAF60a interacted hormone-independently with GR, BAF250 was found to interact in a hormone-dependent way (50, 61). These results demonstrate that also hormone-dependent interactions are likely to be essential for the recruitment SWI/SNF to liganded NRs. Recently we showed that after DNA binding, AR N/C interaction is relieved and the coactivator binding groove is accessible for FxxLF-like motifs present in cofactors (37). We hypothesize that upon binding to enhancer and/or promoter regions of specific target genes, like *TMPRSS2*, AR recruits the SWI/SNF complex via BAF60a through a direct interaction between its FxxFF motif and the AR coactivator groove, and via BAF57 via a different interaction outside the AR LBD, leading to chromatin remodelling and subsequent target gene expression.

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REFERENCES

1. Lee DK, Chang C 2003 Endocrine mechanisms of disease: Expression and degradation of androgen receptor: mechanism and clinical implication. J Clin Endocrinol Metab 88:4043-4054
2. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16:271-321
3. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839
4. Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT 2007 Nuclear receptor structure: implications for function. Annu Rev Physiol 69:201-220
5. Narlikar GJ, Fan HY, Kingston RE 2002 Cooperation between complexes that regulate chromatin structure and transcription. Cell 108:475-487
6. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM 2007 Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 120:719-733
7. Heinlein CA, Chang C 2002 Androgen receptor (AR) coregulators: an overview. Endocr Rev 23:175-200
8. Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. Mol Cell 9:601-610
9. Louie MC, Yang HQ, Ma AH, Xu W, Zou JX, Kung HJ, Chen HW 2003 Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc Natl Acad Sci U S A 100:2226-2230
10. Wang Q, Carroll JS, Brown M 2005 Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 19:631-642
11. Verrijdt G, Haelens A, Claessens F 2003 Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. Mol Genet Metab 78:175-185
12. Lee DY, Teyssier C, Strahl BD, Stallcup MR 2005 Role of protein methylation in regulation of transcription. Endocr Rev 26:147-170
13. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389:194-198
14. Wang H, Huang ZQ, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, Briggs SD, Allis CD, Wong J, Tempst P, Zhang Y 2001 Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. Science 293:853-857
15. Stallcup MR, Kim JH, Teyssier C, Lee YH, Ma H, Chen D 2003 The roles of protein-protein interactions and protein methylation in transcriptional activation by nuclear receptors and their coactivators. J Steroid Biochem Mol Biol 85:139-145
16. Huang ZQ, Li J, Sachs LM, Cole PA, Wong J 2003 A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. Embo J 22:2146-2155
17. Marshall TW, Link KA, Petre-Draviam CE, Knudsen KE 2003 Differential requirement of SWI/SNF for androgen receptor activity. J Biol Chem 278:30605-30613
18. Wang Q, Sharma D, Ren Y, Fondell JD 2002 A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression. J Biol Chem 277:42852-42858
19. Vijayvargia R, May MS, Fondell JD 2007 A coregulatory role for the mediator complex in prostate cancer cell proliferation and gene expression. Cancer Res 67:4034-4041
20. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM 2004 Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425-438

21. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ 2004 Recognition and Accommodation at the Androgen Receptor Coactivator Binding Interface. PLoS Biol 2:E274
22. Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ, Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 12:3343-3356
23. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, Stallcup MR 1998 Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 12:302-313
24. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, Trapman J 2006 Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping. Mol Endocrinol 20:1742-1755
25. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J 2004 Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132-2150
26. Hall JM, Chang CY, McDonnell DP 2000 Development of peptide antagonists that target estrogen receptor beta-coactivator interactions. Mol Endocrinol 14:2010-2023
27. He B, Minges JT, Lee LW, Wilson EM 2002 The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226-10235
28. Hu YC, Yeh S, Yeh SD, Sampson ER, Huang J, Li P, Hsu CL, Ting HJ, Lin HK, Wang L, Kim E, Ni J, Chang C 2004 Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. J Biol Chem 279:33438-33446
29. McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Inostroza J, Torchia J, Nolte RT, Assa-Munt N, Milburn MV, Glass CK, Rosenfeld MG 1998 Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev 12:3357-3368
30. Wang L, Hsu CL, Ni J, Wang PH, Yeh S, Keng P, Chang C 2004 Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol 24:2202-2213
31. He B, Kemppainen JA, Wilson EM 2000 FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986-22994
32. Steketee K, Berrevoets CA, Dubbink HJ, Doesburg P, Hersmus R, Brinkmann AO, Trapman J 2002 Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor. Eur J Biochem 269:5780-5791
33. Askew EB, Gampe RT, Jr., Stanley TB, Faggart JL, Wilson EM 2007 Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. J Biol Chem 282:25801-25816
34. Callewaert L, Verrijdt G, Christiaens V, Haelens A, Claessens F 2003 Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements. J Biol Chem 278:8212-8218
35. He B, Bowen NT, Minges JT, Wilson EM 2001 Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 276:42293-42301
36. He B, Lee LW, Minges JT, Wilson EM 2002 Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. J Biol Chem 277:25631-25639
37. van Royen ME, Cunha SM, Brink MC, Mattern KA, Nigg AL, Dubbink HJ, Verschure PJ, Trapman J, Houtsmuller AB 2007 Compartmentalization of androgen receptor protein-protein interactions in living cells. J Cell Biol 177:63-72
38. Chang CY, Abdo J, Hartney T, McDonnell DP 2005 Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol Endocrinol 19:2478-2490

39. van de Wijngaart DJ, van Royen ME, Hersmus R, Pike AC, Houtsmuller AB, Jenster G, Trapman J, Dubbink HJ 2006 Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain. J Biol Chem 281:19407-19416
40. Koszewski NJ, Henry KW, Lubert EJ, Gravatte H, Noonan DJ 2003 Use of a modified yeast one-hybrid screen to identify BAF60a interactions with the Vitamin D receptor heterodimer. J Steroid Biochem Mol Biol 87:223-231
41. Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J 1996 Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 271:6379-6388
42. Hermans KG, van Marion R, van Dekken H, Jenster G, van Weerden WM, Trapman J 2006 TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. Cancer Res 66:10658-10663
43. Giniger E, Ptashne M 1987 Transcription in yeast activated by a putative amphipathic alpha helix linked to a DNA binding unit. Nature 330:670-672
44. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19:8226-8239
45. He B, Wilson EM 2003 Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs. Mol Cell Biol 23:2135-2150
46. Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C 2003 The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691-23698
47. Kelly KF, Daniel JM 2006 POZ for effect--POZ-ZF transcription factors in cancer and development. Trends Cell Biol 16:578-587
48. Phelan ML, Sif S, Narlikar GJ, Kingston RE 1999 Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. Mol Cell 3:247-253
49. Lemon B, Inouye C, King DS, Tjian R 2001 Selectivity of chromatin-remodelling cofactors for ligand-activated transcription. Nature 414:924-928
50. Nie Z, Xue Y, Yang D, Zhou S, Deroo BJ, Archer TK, Wang W 2000 A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol Cell Biol 20:8879-8888
51. Wang W, Cote J, Xue Y, Zhou S, Khavari PA, Biggar SR, Muchardt C, Kalpana GV, Goff SP, Yaniv M, Workman JL, Crabtree GR 1996 Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. Embo J 15:5370-5382
52. Wang W, Xue Y, Zhou S, Kuo A, Cairns BR, Crabtree GR 1996 Diversity and specialization of mammalian SWI/SNF complexes. Genes Dev 10:2117-2130
53. Yan Z, Cui K, Murray DM, Ling C, Xue Y, Gerstein A, Parsons R, Zhao K, Wang W 2005 PBAF chromatin-remodeling complex requires a novel specificity subunit, BAF200, to regulate expression of selective interferon-responsive genes. Genes Dev 19:1662-1667
54. Belandia B, Orford RL, Hurst HC, Parker MG 2002 Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. Embo J 21:4094-4103
55. Dilworth FJ, Fromental-Ramain C, Yamamoto K, Chambon P 2000 ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro. Mol Cell 6:1049-1058
56. Fryer CJ, Archer TK 1998 Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. Nature 393:88-91
57. Ichinose H, Garnier JM, Chambon P, Losson R 1997 Ligand-dependent interaction between the estrogen receptor and the human homologues of SWI2/SNF2. Gene 188:95-100

58. Klokk TI, Kurys P, Elbi C, Nagaich AK, Hendarwanto A, Slagsvold T, Chang CY, Hager GL, Saatcioglu F 2007 Ligand-specific dynamics of the androgen receptor at its response element in living cells. Mol Cell Biol 27:1823-1843
59. Link KA, Burd CJ, Williams E, Marshall T, Rosson G, Henry E, Weissman B, Knudsen KE 2005 BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. Mol Cell Biol 25:2200-2215
60. DiRenzo J, Shang Y, Phelan M, Sif S, Myers M, Kingston R, Brown M 2000 BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. Mol Cell Biol 20:7541-7549
61. Hsiao PW, Fryer CJ, Trotter KW, Wang W, Archer TK 2003 BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. Mol Cell Biol 23:6210-6220
62. Debril MB, Gelman L, Fayard E, Annicotte JS, Rocchi S, Auwerx J 2004 Transcription factors and nuclear receptors interact with the SWI/SNF complex through the BAF60c subunit. J Biol Chem 279:16677-16686
63. Nomoto K, Nakazato S, Kazahari K, Ono M 1997 Gene structure of rat BAF60b, a component of mammalian SW1/SNF complexes, and its physical linkage to the growth hormone gene and transcription factor SUG/proteasome p45 gene. Gene 202:157-165
64. Lickert H, Takeuchi JK, Von Both I, Walls JR, McAuliffe F, Adamson SL, Henkelman RM, Wrana JL, Rossant J, Bruneau BG 2004 Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. Nature 432:107-112
65. Takeuchi JK, Lickert H, Bisgrove BW, Sun X, Yamamoto M, Chawengsaksophak K, Hamada H, Yost HJ, Rossant J, Bruneau BG 2007 Baf60c is a nuclear Notch signaling component required for the establishment of left-right asymmetry. Proc Natl Acad Sci U S A 104:846-851
66. Flajollet S, Lefebvre B, Cudejko C, Staels B, Lefebvre P 2007 The core component of the mammalian SWI/SNF complex SMARCD3/BAF60c is a coactivator for the nuclear retinoic acid receptor. Mol Cell Endocrinol 270:23-32
67. Tomlins SA, Mehra R, Rhodes DR, Smith LR, Roulston D, Helgeson BE, Cao X, Wei JT, Rubin MA, Shah RB, Chinnaiyan AM 2006 TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res 66:3396-3400
68. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM 2005 Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644-648

SUPPLEMENTARY INFORMATION

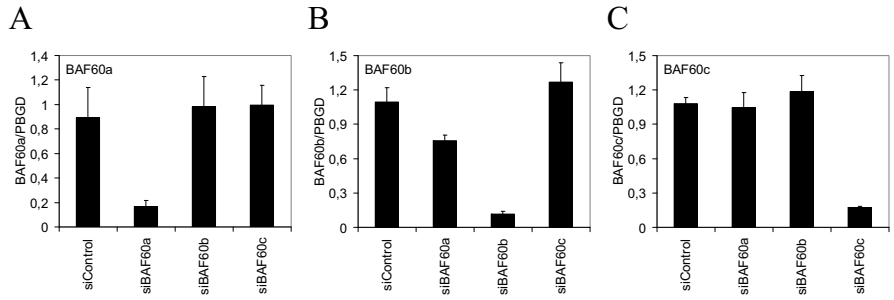

Figure S1. siRNAs against BAF60a, BAF60b, and BAF60c are specific for their target mRNA. LNCaP cells were transfected with non-targeting siRNA (siControl) or with siRNA against BAF60a, BAF60b, or BAF60c as described in the Experimental procedures. mRNA levels of BAF60a (A), BAF60b (B), or BAF60c (C) were then determined by QPCR and normalized to *PBGD* values. Results shown are based four individual values (+/- SEM).

Supplemental Table I: Sequences of QPCR primers used in this study

Target	Sequence
PBGD FW	5'-CATGTCTGGTAACGGCAATG-3'
PBGD REV	5'-GTACGAGGCTTTCAATGTTG-3'
SMARCD1 FW	5'-CCTTGAAGACCCAGATGAAT-3'
SMARCD1 REV	5'-AGTAGAAGTATCGGCACACA-3'
SMARCD2 FW	5'-TGAAGGCCCAAATGAGCAAT-3'
SMARCD2 REV	5'-CAAAGATGTGCCTGCCTACT-3'
SMARCD3 FW	5'-TTAAAGGGGCAGATGAGCAG-3'
SMARCD3 REV	5'-AGTAGAAGTAGCGACTGACG-3'
TMPRSS2 FW	5'-CCTCTGGTCACTTCGAAGAAC-3'
TMPRSS2 REV	5'-GTAAAACGACGTCAAGGACG-3'
SGK FW	5'-GCAGAAGGACAGGACAAAGC-3'
SGK REV	5'-GTCAAAGTGCCGTAGCTCGT-3'
SARG FW	5'-TACGACTTCCTGTCCACTGA-3'
SARG REV	5'-GAGCTGGACTCAGTTACTGTC-3'
PSA FW	5'-ACGTGTGTGCAAGTTCACC-3'
PSA REV	5'-TGTACAGGGAAGGCCTTTCG-3'
NDRG1 FW	5'-GACCCAACAAAGACCACTCTC-3'
NDRG1REV	5'-TGCCATCCAGAGAAGTGACG-3'
KLK2 FW	5'-TCCAATGACATGTGTGCTAG-3'
KLK2 REV	5'-CACCATTACAGACAAGTGGA-3'

Supplemental Table II: Peptide motifs selected for interaction with AR after proteome-wide in silico screening

	Gene symbol	Description	Protein size (aa)	Motif starts at aa	Peptide sequence	Basal activity[b]
	AR	Androgen receptor	919	23	KTYRGA**F**QN**LF**QSVRE	
1	AQR	Aquarius homolog	1521	970	VSTFFP**F**HE**YF**ANAPQ	Elevated
2	ARNT2	Aryl-hydrocarbon receptor nuclear translocator 2	706	484	QERDPR**F**AE**MF**AGISA	
3	ARV1	ARV1	271	140	YAKEWD**F**YR**MF**AIAAL	
4	ASB11	Ankyrin repeat and SOCS box protein 11	323	14	YGFKNI**F**IT**MF**ATFFF	
5	ASCC1	Activating signal cointegrator 1 complex subunit 1	400	164	KQPFTH**F**LA**FF**LNEVE	
6	ATM	Ataxia telangiectasia mutated	3056	209	DGLNSK**F**LD**FF**SKAIQ	
7	BRD2	Bromodomain-containing protein 2	801	145	SECMQD**F**NT**MF**TNCYI	Elevated
8	CBL	E3 ubiquitin-protein ligase CBL	906	244	VFEFDI**F**TR**LF**QPWSS	
9	CCNE2[a]	Cyclin E2	404	92	RFTNYR**F**KN**LF**INPSP	
10	CDK6	Cyclin-dependent kinase 6	326	209	WSVGCI**F**AE**MF**RRKPL	
11	CEBPD[a]	CCAAT/enhancer-binding protein delta	269	82	LCHDEL**F**AD**LF**NSNHK	
12	CPEB4	Cytoplasmic polyadenylation element binding protein 4	729	679	ARCGGK**F**AP**FF**CANVT	
13	CRY2	Cryptochrome 2	461	425	LSCSAF**F**QQ**FF**HCYCP	
14	CTDSP2	Nuclear LIM interactor-interacting factor 2	271	38	PRGRNI**F**KA**LF**CCFRA	
15	DET1	De-etiolated homolog 1	550	319	AMAKRR**F**FQ**YF**DQLRQ	
16	DHX29	DEAH box protein 29	1369	742	TVDSEK**F**ST**YF**THCPI	
17	DHX34	DEAH box protein 34	576	60	SEECQK**F**WT**FF**ERLQR	
18	DHX35	DEAH box protein 35	703	216	TLDADK**F**RD**FF**NQNET	
19	DHX38	DEAH box protein 38	1227	692	TMDAEK**F**AA**FF**GNVPI	Elevated
20	DNAJB9	DnaJ homolog,subfamily B,member 9	223	166	SFGGGL**F**DD**MF**EDMEK	High
21	DNMT1	DNA (cytosine-5-)-methyltransferase 1	1616	628	KLVYQI**F**DT**FF**AEQIE	
22	EIF3S3	Eukaryotic translation initiation factor 3, subunit 3	352	194	KKANIT**F**EY**MF**EEVPI	Elevated
23	EIF4ENIF1	eukaryotic translation initiation factor 4E nuclear import factor 1	985	307	SPGDFD**F**NE**FF**NLDKV	High
24	ENC1	Ectoderm-neural cortex 1 protein	589	71	AACSRY**F**EA**MF**SGGLK	
25	ERCC6[a]	DNA excision repair protein ERCC6	1493	504	KVPGFL**F**KK**LF**KYQQT	
26	FOXP1	Forkhead box protein P1	677	495	NEIYNW**F**TR**MF**AYFRR	
27	FRK	Fyn-related kinase	505	47	QRHGHY**F**VA**LF**DYQAR	
28	GPR177	G protein-coupled receptor 177	487	432	MWNLYV**F**AL**MF**LYAPS	
29	HNRPK	Heterogeneous nuclear ribonucleoprotein K	463	239	TYDYGG**F**TM**MF**DDRRG	Elevated
30	HOXA11	Homeobox protein A11	313	140	GVLPQA**F**DQ**FF**ETAYG	Elevated
31	HOXC11	Homeobox protein C11	304	151	SVLPQA**F**DR**FF**DNAYC	
32	HRMT1L3	Heterogeneous nuclear ribonucleoprotein methyltransferase-like protein 3	531	455	TAIAGY**F**DI**YF**EKNCH	Elevated
33	IRF7	Interferon regulatory factor 7	503	407	DTPIFD**F**RV**FF**QELVE	Elevated
34	KBTBD4	Kelch repeat and BTB domain-containing protein 4	518	70	SAQSCF**F**RS**MF**TSNLK	
35	KIAA0020	KIAA0020	648	332	SLVHKV**F**LD**FF**TYAPP	
36	KIAA0652	KIAA0652	517	16	RKDLDK**F**IK**FF**ALKTV	
37	KIF23	Kinesin-like protein 23	856	497	SKLTHL**F**KN**YF**DGEGK	

38	KIFAP3	kinesin-associated protein 3	792	416	ISMDDR**F**KS**MF**AYTDC	
39	KLHL12	Kelch-like protein 12	568	58	AACSDY**F**CA**MF**TSELS	Elevated
40	LASS5	LAG1 longevity assurance homolog 5	350	214	DIKRKD**F**LI**MF**VHHLV	
41	LSM2	U6 snRNA-associated Sm-like protein	95	3	ML**F**YS**FF**KSLVG	
42	MCM3	DNA replication licensing factor	808	479	DSLLSR**F**DL**LF**IMLDQ	
43	MCM3AP	MCM3-associated protein	1980	852	ALNSNN**F**VR**FF**KLVQS	
44	MCM6	DNA replication licensing factor	821	530	APIMSR**F**DL**FF**ILVDE	
45	MCM7	DNA replication licensing factor	719	134	AELMRR**F**EL**YF**QGPSS	
46	MCM8	DNA replication licensing factor	840	99	IEKIQA**F**EK**FF**TRHID	
47	MDN1	Midasin homolog	5596	123	KDTSPV**F**QR**LF**LESSD	
48	MKI67[a]	Antigen KI-67	3256	1227	LEDLAG**F**KE**LF**QTPGH	
49	MKRN4	Makorin, ring finger protein, 4	485	454	GQQRNH**F**WE**FF**EEGAN	Elevated
50	MLF2	Myeloid leukemia factor 2	248	76	LGMSGG**F**MD**MF**GMMND	High
51	MLH3	MutL protein homolog 3	1453	120	NRTMKT**F**VK**LF**QSGKA	
52	MSH2	MutS protein homolog 2	934	19	SAAEVG**F**VR**FF**QGMPE	
53	NALP10	NACHT, leucine rich repeat and PYD containing 10	655	469	SFRHIS**F**QD**FF**HAMSY	
54	NAP1L1	Nucleosome assembly protein 1-like 1	391	297	TVSNDS**F**FN**FF**APPEV	Elevated
55	NARG1	NMDA receptor-regulated protein 1	866	679	ETHLFA**F**EI**YF**RKEKF	
56	NR5A1	Nuclear receptor subfamily 5, group A, member 1	461	373	EFVCLK**F**II**LF**SLDLK	
57	PCID2	PCI domain-containing protein 2	399	176	FLVNQL**F**KI**YF**KINKL	
58	PLK1	Polo-like kinase 1	603	116	HQHVVG**F**HG**FF**EDNDF	Elevated
59	PLK2	Polo-like kinase 2	685	145	HKHVVQ**F**YH**YF**EDKEN	
60	POLR2B	DNA-directed RNA polymerase II 140 kDa polypeptide	1174	404	PLLAFL**F**RG**MF**KNLLK	
61	PPP1R13B	Protein phosphatase 1 regulatory subunit 13B	1090	217	SAEIER**F**SA**MF**QEKKQ	
62	PPP2R3B	protein phosphatase 2, regulatory subunit B	414	307	KLANVF**F**DT**FF**NIEKY	
63	PPP4R2	Protein phosphatase 4, regulatory subunit 2	495	123	MIQWSQ**F**KG**YF**IFKLE	
64	PRPF8	PRP8 pre-mRNA processing factor 8 homolog	2335	2267	MLLSDR**F**LG**FF**MVPAQ	
65	RAB6IP1	RAB6 interacting protein 1	1287	524	EVFANR**F**TQ**MF**ADYEV	
66	RFP2	Ret finger protein 2	407	142	AQERDA**F**ES**LF**QSFET	
67	SALL3	Sal-like protein 3	1300	1192	GGDALK**F**SE**MF**QKDLA	
68	SEC14L2	SEC14-like protein 2	403	174	VEAYGE**F**LC**MF**EENYP	High
69	SENP1	SUMO1/sentrin specific peptidase 1	643	493	LPSVHA**F**NT**FF**FTKLK	
70	SETDB1	Histone H3-K9 methyltransferase 4	1291	293	VKNKLR**F**LI**FF**DDGYA	
71	SIN3A	SIN3 homolog A, transcription regulator	1273	1064	MSDENC**F**KL**MF**IQSQG	
72	SMC5	Structural maintenance of chromosomes 5	1101	943	EKINEK**F**SN**FF**SSMQC	
73	SPOP	Speckle-type POZ protein	374	225	AARSPV**F**SA**MF**EHEME	
74	SRMS	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	488	11	LRRRLA**F**LS**FF**WDKIW	
75	STAT5B[a]	Signal transducer and activator of transcription 5B	787	440	SVTEEK**F**TI**LF**ESQFS	
76	STK4	Serine/threonine-protein kinase 4	487	395	QPAKPS**F**LE**YF**EQKEK	

77	SUPT16H	Chromatin-specific transcription elongation factor 140 kDa subunit	1047	194	SITSEV**F**NK**FF**KERVM	
78	TBC1D1	TBC1 domain family member 1	1168	970	LYAAPW**F**LT**MF**ASQFP	
79	TCF3	Transcription factor 3	654	22	LSDLLD**F**SM**MF**PLPVT	High
80	TNPO1	Transportin 1	890	179	NIMIPK**F**LQ**FF**KHSSP	
81	UBE1L[a]	Ubiquitin-activating enzyme E1-like	1011	618	QWARHE**F**EE**LF**RLSAE	
82	ULK1	Unc-51-like kinase 1	1050	849	ILRGLR**F**TL**LF**VQHVL	
83	WBSCR22	Williams-Beuren syndrome chromosome region 22 protein	281	146	KRLYCF**F**AS**LF**SVLVR	
84	XRCC5[a]	DNA-repair protein XRCC5	731	497	KIPNPR**F**QR**LF**QCLLH	
85	ZBTB1	Zinc finger and BTB domain-containing protein 1	713	49	AACSSY**F**RM**FF**MNHQH	
86	ZBTB16	Zinc finger and BTB domain-containing protein 16	673	59	ACTSKM**F**EI**LF**HRNSQ	
87	ZBTB24	Zinc finger and BTB domain-containing protein 24	697	62	AASSEY**F**SM**MF**AEEGE	High
88	ZDHHC2	zinc finger, DHHC-type containing 2	367	209	PDTQAK**F**HI**MF**LFFAA	
89	ZNF364	Zinc finger protein 364	304	149	GILQHI**F**AG**FF**ANSAI	

[a] Indicates that this peptide has only been tested for interaction with AR LBD in a yeast two-hybrid assay as described previously (39).

[b] Peptides have an elevated basal activity if the relative values in absence of hormone are between 5% and 20% as compared to the hormone-dependent interaction of AR FxxLF motif with F23L/F27L-AR. Basal activities are high if these values are above 20%.

Supplemental Table III: Motifs present in subunits of SWI/SNF and Mediator/TRAP/DRIP complexes selected for interaction with AR

	Gene symbol	Aliases	Protein size (aa)	Motif starts at aa	Peptide sequence	Complex	Basal activity[b]
1	MED12	TRAP230, ARC240	2212	1024	SHLKNK**F**GE**LF**SDFCS	MED/TRAP/DRIP	
2	MED12L		2145	946	SHLRSK**F**GD**LF**SSACS	MED/TRAP/DRIP	
3	MED19		194	4	MEN**F**TA**LF**GAQAD	MED/TRAP/DRIP	Elevated
4	PB1	Polybromo 1, BAF180	1689	116	NLLTAD**F**QL**LF**NNAKS	SWI/SNF	Elevated
5	PB1	Polybromo 1, BAF180	1689	386	MDVSNP**F**YQ**LY**DTVRS	SWI/SNF	Elevated
6	PB1	Polybromo 1, BAF180	1689	728	DSMVED**F**VM**MF**NNACT	SWI/SNF	High
7	PPARBP	TRAP220, DRIP230, ARC205, MED1	1581	657	DNPAQD**F**ST**LY**GSSPL	MED/TRAP/DRIP	Elevated
8	SMARCA1	ISWI, SWI2, SNF2L	1054	368	FNSADD**F**DS**WF**DTKNC	SWI/SNF	High
9	SMARCA2[a]	BRM, SNF2a, BAF190	1586	905	FKSCST**F**EQ**WF**NAPFA	SWI/SNF	Elevated
10	SMARCA3	HIP116, HLTF, SNF2L3	1009	214	EQLKTE**F**DK**LF**EDLKE	SWI/SNF	Elevated
11	SMARCA4[a]	BRG1, SNF2b, BAF190	1647	939	FKSCST**F**EQ**WF**NAPFA	SWI/SNF	Elevated
12	SMARCD1	BAF60a, Rsc6p, CRACD1	476	194	TKQKRK**F**SS**FF**KSLVI	SWI/SNF	
13	SMARCD2	BAF60b, Rsc6p, CRACD2	456	284	INCNRY**F**RQ**IF**SCGRL	SWI/SNF	
14	THRAP3	TRAP150	955	607	NKKEQE**F**RS**IF**QHIQS	MED/TRAP/DRIP	
15	THRAP3	TRAP150	955	654	MTLHER**F**TK**YL**KRGTE	MED/TRAP/DRIP	
16	THRAP4	TRAP100, MED24, DRIP100, ARC100	989	923	AGPHTQ**F**VQ**WF**MEECV	MED/TRAP/DRIP	Elevated

[a] The FxxWF motifs in SMARCA2 and SMARCA4 are identical.

[b] Peptides have an elevated basal activity if the relative values in absence of hormone are between 5% and 20% as compared to the hormone-dependent interaction of AR FxxLF motif with F23L/F27L-AR. Basal activities are high if these values are above 20%.

CHAPTER 5

Blockade of androgen receptor function by peptides targeted to the coactivator-binding groove

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ABSTRACT

Prostate cancer growth is dependent on the androgen-androgen receptor (AR) axis. Because current androgen ablation therapies aiming at inhibition of AR function are only temporarily effective, novel approaches to repress AR function are urgently needed. Here we explored the feasibility to inhibit AR function beyond the level of hormone binding by blockade of the coactivator groove in the ligand-binding domain (LBD). To achieve this, we made use of a peptide encoding the gelsolin FxxFF motif, which has a high affinity for the coactivator groove. Effects of the gelsolin FxxFF peptide on AR functions were first determined in Hep3B cells transiently transfected with pM-peptide expression vectors. Our results demonstrated that gelsolin FxxFF efficiently interfered with AR N/C interaction and specifically inhibited the transcriptional activity of full-length AR. It did not inhibit the RSV promoter, neither did it affect transcriptional activities of constitutively active AR lacking the LBD, or full length PR and GR. As a second approach, we introduced the gelsolin FxxFF peptide coupled to the TAT cell-penetrating peptide into cells. Like the gelsolin FxxFF peptide expressed from cDNA transfected into cells, the TAT-gelsolin FxxFF peptide efficiently repressed AR N/C interaction. Moreover, the peptide inhibited full-length AR regulated gene expression and hardly affected PR and GR activity, but the effect on transcription from constitutively active promoters was variable. Our results indicate that the AR coactivator groove is a candidate target for blocking AR function in prostate cancer.

INTRODUCTION

The androgen receptor (AR) is a ligand-dependent transcription factor that is activated by the androgens testosterone and the more potent dihydrotestosterone (DHT). The androgen-AR axis is not only essential for normal male development and for maintaining function of male-specific organs, but also plays a pivotal role in the etiology of prostate cancer (1, 2). Initially, growth of prostate cancer is dependent on androgens. Therefore, patients with advanced disease are treated with androgen ablation therapy, based on suppressing AR activity indirectly by lowering the production of androgens using LHRH-analogues or directly by application of anti-androgens. Although successful initially, tumors regain the ability to grow leading to an endocrine therapy-resistant stage of the disease for which no efficient treatments are available. However, in many of these tumors a functional AR is still present (3). Mechanisms underlying therapeutic failure include AR gene amplification resulting in AR overexpression, mutations in the AR, and ligand-independent activation of the AR via other signalling pathways (4-6). In addition, it has been demonstrated that intraprostatic androgen levels after medical castration can remain sufficiently high to regulate AR-dependent gene transcription (7-9). Recently, it was reported that genes encoding members of the Ets family of transcription factors, including *ERG* and *ETV1*, are frequently fused to TMPRSS2 and other androgen-regulated genes in prostate cancers (10-12). Because of these gene fusions, expression of Ets oncogenes is now under control of AR-responsive promoters, leading to androgen-regulated aberrant overexpression of the Ets genes. Overall, these findings show that the AR remains an interesting target for inhibiting prostate cancer growth following standard endocrine therapy.

The AR belongs to the family of NRs and shares a structural and functional organization that includes an N-terminal transcription activation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (13, 14). AR transcriptional activity is regulated by interacting cofactor complexes, including histone modifying enzymes, SWI/SNF-like chromatin remodelling complexes, and Mediator complexes (15-21). Although the mode of action for the majority of cofactors remains to be elucidated, crystal structures and functional data revealed the interaction of AR with its cofactors (22-24). Upon ligand binding, the LBD undergoes major structural rearrangements, which induce the formation of a hydrophobic groove on the LBD surface (23, 24). In most NRs this groove serves as high affinity binding site for short amphipathic α-helical LxxLL sequences present in for example members of the p160 family of cofactors (25-27). Although the AR LBD is able to bind LxxLL motifs, it prefers binding of related FxxLF motifs (28-30). It has also been demonstrated that an FxxLF motif in the AR NTD is able to bind strongly to the coactivator groove (31-33). This so-called AR N/C interaction plays a role in slowing down androgen dissociation rate and in selective gene transcription (31, 34-36). Recently, we and others showed that the AR LBD not only serves as a high affinity docking site for FxxLF motifs, but is also sufficiently flexible to

accommodate other phenylalanine-rich motifs, including FxxFF, FxxMF, and FxxYF (24, 28, 37, 38). Furthermore, we demonstrated that cofactors gelsolin and PAK6 interact with AR via an FxxFF and FxxMF motif, respectively (38).

This study aims at blocking AR function beyond the level of hormone binding by targeting the interactions with the coactivator groove. We selected the gelsolin FxxFF motif to investigate blocking of AR N/C interaction and inhibiting AR transcriptional activity. The gelsolin FxxFF peptide was introduced into Hep3B cells either by transient transfection of peptide expression vectors or by coupling the synthetic peptide to the TAT cell-penetrating peptide (39, 40). Our results demonstrated that via both mechanisms the gelsolin FxxFF peptide blocks AR N/C interaction and efficiently suppressed AR transcriptional activity. Both types of peptides did not affect transcriptional activities of progesterone receptor (PR) and glucocorticoid receptor (GR), but TAT-gelsolin FxxFF was less specific in inhibition of constitutively active promoters. The results obtained in this study show that the coactivator groove is a functional target for manipulating AR activity.

EXPERIMENTAL PROCEDURES

Plasmids

Mammalian expression plasmids encoding peptides were generated by in-frame insertion of double-stranded synthetic oligonucleotides with 5'-*Bam*HI and 3'-*Eco*RI cohesive ends into the corresponding sites of pM-B/E to produce Gal_4DBD-peptide fusion proteins (31). Peptide expression constructs were sequenced to verify correct reading frame. Western blots were performed to analyze expression and size of the fusion proteins.

Mammalian vectors expressing AR NTD (pAR12.1) (41), AR NTD-DBD (pAR5) (42), AR DBD-LBD (pAR104) (42), full-length wild-type ARs pAR0 (43) and pCMVAR0 (31), and glucocorticoid receptor (GR) (44) have been described previously. The mammalian expression vector encoding progesterone receptor (PR) was provided by Dr. L. Blok, and the $(UAS)_4$TATA-Luc reporter by M. Meester (both Erasmus MC, Rotterdam, The Netherlands). The $(ARE)_2$TATA-LUC reporter construct has previously been described as $(PRE)_2$-E1b-LUC (45).

Cell-penetrating peptides

Cell-penetrating peptides were purchased from GenScript Corp. (Piscataway, NJ). Peptides were >85% purified by HPLC and verified by mass spectrometry analysis. Gelsolin FxxFF and gelsolin FxxAA peptides were first dissolved in DMSO and further diluted in water. All other peptides were dissolved in water.

Mammalian cell culture, transient transfections, and luciferase assay

Hep3B cells were cultured in αMEM (Bio-Whittaker, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) and antibiotics. For transient transfection experiments, Hep3B cells were seeded at $5x10^4$ cells per well of a 24-well plate and were allowed to grow for 24 h. Four hours prior to transfection, the medium was replaced by αMEM supplemented with 5% charcoal-stripped FCS, antibiotics, and hormone or vehicle. Twenty-four hours after addition of the transfection mixtures (described below), the cells were lysed and the luciferase activities measured as described previously (31). For mammalian one-hybrid assays, transfections were performed in the presence of Fugene 6 (Roche Diagnostics, Mannheim, Germany), 50 ng Gal_4DBD-peptide expression construct, 50 ng AR, PR, or GR expression construct, and 150 ng $(UAS)_4$TATA-LUC reporter per well.

Transfection mixtures used in the AR N/C interaction assays consisted of 50 ng pAR12.1, 50 ng pAR104, and 100 ng $(ARE)_2$TATA-LUC per well, with or without Gal_4DBD-peptide expression construct. Twenty-five ng Gal_4DBD-peptide construct was used in peptide screening, whereas increasing amounts (12.5, 25, and 50 ng) were used to determine the specific effects of the gelsolin peptide on AR N/C interaction. In the latter case, Gal_4DBD-peptide expression construct was supplemented with empty pM-B/E vector to obtain a total amount of 50 ng Gal_4DBD expression vector. In cell-penetrating peptide assays, Gal_4DBD-peptide expression constructs were replaced by the cell-penetrating peptides TAT, TAT-gelsolin FxxFF, or TAT-gelsolin FxxAA (0.1, 0.5, 1, and 5 μM).

Transfection mixtures in transcription activation assays consisted of 10 ng expression vector encoding AR, PR, GR, or AR NTD-DBD and 100 ng $(ARE)_2$TATA-LUC reporter. In indicated control assays RSV-LUC (100 ng) was used as reporter. Increasing amounts of pM-gelsolin vector (62.5, 125, 250 ng) was used, supplemented with empty pM-B/E vector to obtain a total amount of 250 ng Gal_4DBD expression vector in each assay. In cell-penetrating peptide assays, Gal_4DBD-peptide expression constructs were replaced by TAT, TAT-gelsolin FxxFF, or TAT-gelsolin FxxAA peptides (1 and 5 μM).

TR-FRET

In vitro interactions of synthetic peptides with AR LBD were determined with the Lanthascreen TR-FRET AR coactivator assay kit (Invitrogen, Carlsbad, CA). Incubation mixtures were prepared according to the manufacturer's protocol and consisted of 20 nM AR LBD, 5 μM DHT or an equal amount of DMSO, 500 nM fluorescein-labeled peptides, and 5 nM terbium-labeled anti-GST antibody in a total volume of 40 μl. Mixtures were incubated in white 384-well assay plates (Corning Inc., Corning, NY) at room temperature for 4 h while protected from light.

TR-FRET was measured using a Varioskan microplate reader (Thermo Electron, Vantaa, Finland). Assay conditions were similar as described (46). The terbium donor was excited using a 340-nm excitation filter with a 12-nm bandwidth. The wavelength of the first emission peak of terbium (490 nm) overlaps with the wavelength to excitate fluorescein. If a peptide binds to

the AR LBD, energy transfer from terbium to fluorescein takes place, resulting in an emission peak at 520 nm. Emission was assayed using a 520-nm filter with a 12-nm bandwidth. TR-FRET measurement was for 1000 ms with a 100 µs post-excitation delay and 200 µs integration time. TR-FRET was calculated on basis of the 520/490 nm signal ratios.

RESULTS

The gelsolin FxxFF motif is an AR-interacting motif that efficiently blocks AR N/C interaction

We applied FxxLF-like motif containing peptides to target the AR coactivator groove in the LBD for blocking AR function beyond the level of hormone binding. Because these peptides compete with the FxxLF motif in the AR NTD and with cofactors containing FxxLF- and LxxLL-like motifs for binding to this groove, we first selected a strong AR-interacting peptide. We determined the AR LBD interaction capacity of 18 FxxLF-like peptide motifs (Table I) in a mammalian one-hybrid assay as shown schematically in Figure 1A. These motifs, derived from known AR cofactors and from phage display screenings, were selected based on our previous results and published data (24, 29, 37, 38, 47-49). pM vectors expressing the motifs linked to Gal_4DBD were constructed and the fusion proteins were assayed for interaction with full-length wild type AR in Hep3B cells. AR recruitment to the Gal_4DBD-peptide causes transactivation of the $(UAS)_4$TATA-Luc reporter in the presence of 1 nM R1881, but not in the absence of hormone.

Most peptides interacted stronger than the AR FxxLF motif with AR (Fig. 1B). The four strongest interacting peptides, D11 FxxLF, gelsolin FxxFF, D30 FxxLF, and a novel peptide derived from the Adseverin FxxFF motif, bound with equal capacities and about 3.5-fold stronger than the AR FxxLF motif. The other peptides showed a gradual decline in the interaction capacity with weakest interactions observed with the TIF2 box I FxxLF variant motif. Western blot analysis demonstrated correct size and comparable expression levels of the Gal_4DBD-peptide fusion proteins (data not shown).

Next, we determined the capacity of the same panel of peptides to disrupt interactions of the AR NTD with the AR LBD (N/C interaction; Fig. 1C). In this assay, Hep3B cells were transiently cotransfected with expression constructs for AR NTD and AR DBD-LBD together with the $(ARE)_2$TATA luciferase reporter construct. In the presence of R1881, AR DBD-LBD binds to the reporter and efficiently recruits AR NTD thereby reconstituting transcriptional activity (Fig 1C; upper panel). Interacting peptides abrogated AR N/C interaction leading to decreased luciferase activities in this assay (Fig. 1C; lower panel).

All peptides tested, efficiently blocked AR N/C interaction (Fig. 1D). The four peptides that interacted strongest with AR also disrupted N/C interaction most efficiently, whereas weaker AR interacting peptides, including RAD9 FxxLF and TIF2 box I FxxLF, were far less efficient in

Table I: Amino acid sequences of peptides used in this study

Protein	Peptide sequence
AR	KTYRGA**F**QN**LF**QSVRE
Adseverin	GGETPI**F**KQ**FF**KDWRD
ARA54	DPGSPC**F**NR**LF**YAVDV
ARA70	RETSEK**F**KL**LF**QSYNV
Chang 4.1	QPKH**F**TE**LY**FKS
D11 FxxLF	ESGSSR**F**MQ**LF**MANDL
D11 LxxLL	ESGSSR**L**MQ**LL**MANDL
D30 FxxLF	PTHSSR**F**WE**LF**MEATP
D30 LxxLL	PTHSSR**L**WE**LL**MEATP
FLET 2	SSK**F**AA**LW**DPPKLSR
FLET 5	SSNTPR**F**KE**YF**MQSR
FLET 6	SR**F**AD**FF**RNEGLSGSR
Gelsolin	GGETPL**F**KQ**FF**KNWRD
PAK6	SLKRRL**F**RS**MF**LSTAA
RAD9	TPPPKK**F**RS**LF**FGSIL
SRC1 box I	SQTSHK**F**VQ**LF**TTTAE
SRC1 box IV	PQAQQKSL**F**QQ**LF**TE*
TIF2 box I	SKGQTK**F**LQ**LF**TTKSD

* = stop

blocking this interaction. These data demonstrate a strong correlation between the interaction capacity of a peptide (Fig. 1B) and its capacity to block AR N/C interaction.

AR binding and blocking of AR N/C interaction by the gelsolin peptide is dependent on the FxxFF motif

The gelsolin peptide was selected for further experiments. To investigate the contribution of the FxxFF motif in the gelsolin peptide for binding to AR, we determined the interaction capacity of a mutant peptide in which the FxxFF motif was substituted by FxxAA. Interaction of the gelsolin peptide with AR was dependent on an intact FxxFF motif, as the mutant peptide was unable to interact (Fig. 2A; see also (38)). In line with this observation, the gelsolin FxxFF peptide disrupted N/C interaction in a concentration dependent manner, whereas no effects were observed using the non-interacting gelsolin FxxAA peptide, even at the highest concentrations used (Fig. 2B). These results show that for blocking AR N/C interaction an intact FxxFF motif is essential.

We next determined whether the gelsolin FxxFF peptide also interacted with AR in an *in vitro* TR-FRET assay, as described previously (46). The principle of TR-FRET is schematically presented in Figure 2C and is explained in detail in the Experimental Procedures section. The results of the TR-FRET assay corresponds with the mammalian one-hybrid assay demonstrating that the gelsolin FxxFF peptide interacts hormone-dependently with AR LBD, whereas the FxxAA mutant peptide does not bind (Fig. 2D). We next investigated whether the gelsolin FxxFF peptide affected binding of fluorescein-labeled D11 FxxLF peptide in a competition

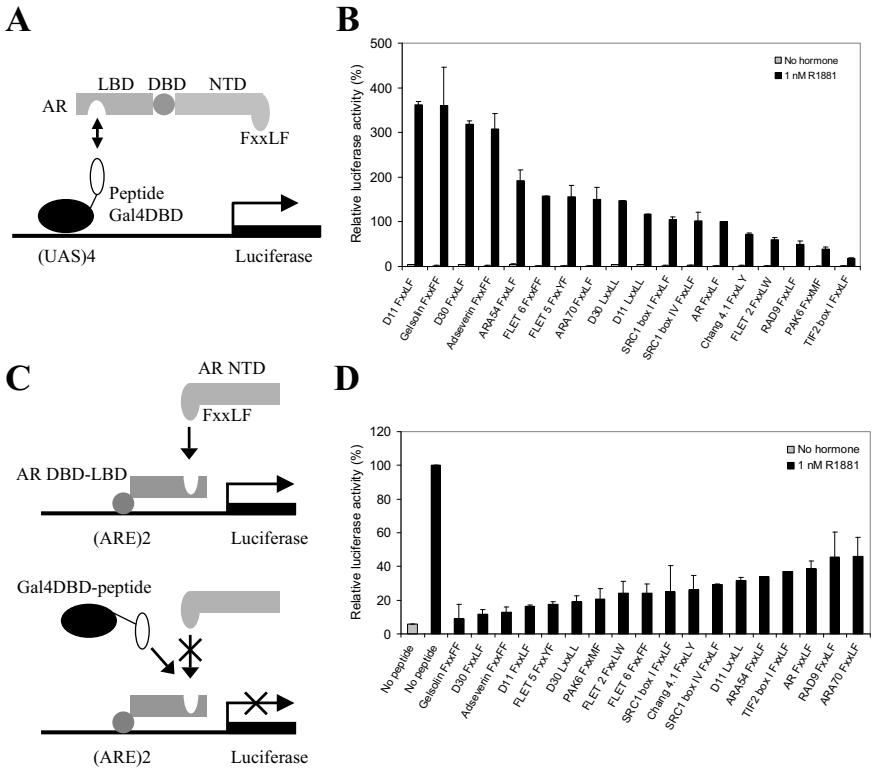

Figure 1. The gelsolin FxxFF motif strongly interacts with AR and efficiently blocks AR N/C interaction. (A) Schematic representation of the mammalian one-hybrid assay. Hep3B cells were transiently transfected with expression constructs encoding the peptides fused to Gal_4DBD, which served as bait for full-length wild type AR. In case of interaction between the peptide and the AR, the AR NTD transactivates the luciferase reporter. Interactions were determined in the absence and presence of 1 nM R1881. (B) Mammalian one-hybrid analysis of 18 selected, previously described, AR-interacting peptides. Bars represent mean relative luciferase activities in the absence (grey) or presence (black) of R1881 and are based on two independent experiments (+/- SD). Interaction of the AR FxxLF motif with AR in the presence of R1881 was set to 100%. (C) Schematic representation of the peptide competition assay for AR N/C interaction in Hep3B cells. Assay is explained in more detail in the text. (D) FxxLF-like peptide motifs efficiently disrupt AR N/C interaction. R1881-induced N/C interaction in the absence of peptides was set to 100%. The other bars represent the residual luciferase activities after co-transfection of pM constructs expressing AR-interacting peptides. Each bar represents mean relative luciferase activities in the presence of hormone. Results are based on two independent experiments (+/- SD).

assay. D11 FxxLF peptide was allowed to bind AR LBD in the absence or presence of 3- or 10-fold excess non-labeled gelsolin FxxFF or gelsolin FxxAA peptides. Figure 2E shows that excess of gelsolin FxxFF efficiently competed with the D11 FxxLF peptide for binding to the coactivator groove. The gelsolin FxxAA peptide did not affect D11 FxxLF binding to the AR LBD.

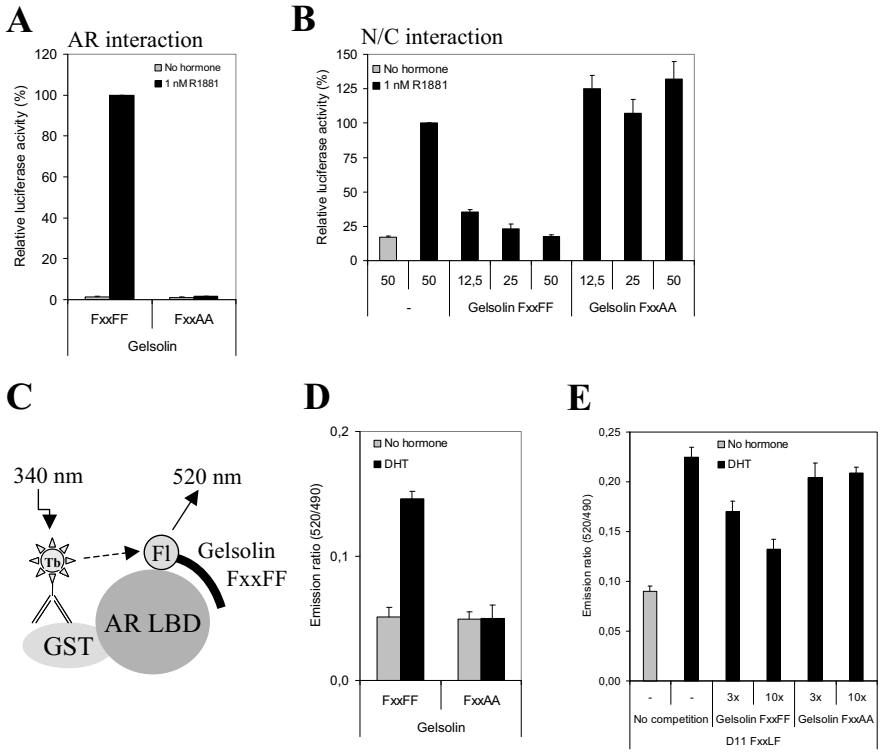

Figure 2. The gelsolin FxxFF peptide interacts with AR and blocks peptide- and protein interactions with AR LBD *in vivo* and *in vitro*. (A) Mammalian one-hybrid analysis of the gelsolin FxxFF and FxxAA mutated peptides with AR. Assays were performed as described in the legends to Figures 1A and B. Results represent the mean relative luciferase activities in the absence (grey bars) or presence (black bars) of 1 nM R1881. Results are based on two independent experiments (+/- SD). Interaction of gelsolin FxxFF with AR was set to 100%. (B) Peptide competition assay for AR N/C interaction. The assay was performed as described in the legends to Figures 1C and D, and in Experimental Procedures. Effects on AR N/C interaction were determined by transient transfection of 50 ng empty pM vector or increasing amounts of pM-peptide constructs. pM-peptide expression vector was adjusted to 50 ng using empty pM vector. Results shown are the mean luciferase activities in the absence (grey bars) or presence (black bars) of 1 nM R1881. Results are based on three independent experiments (+/- SEM). N/C interaction in the presence of 50 ng pM empty vector and in the presence of hormone was set to 100%. (C) Experimental set-up of the *in vitro* TR-FRET assay. The assay is explained in more detail in the Experimental Procedures section. (D) Representative results of a TR-FRET assay demonstrating that gelsolin FxxFF interacts with AR LBD *in vitro*. Shown are the 520/490 emission ratios in the absence (grey bars) or presence (black bars) of DHT. (E) Gelsolin FxxFF competes with the D11 FxxLF peptide for binding to the coactivator groove. Interaction of 500 nM fluorescein-labeled D11 FxxLF peptide with AR LBD was determined in the absence or presence of 3- or 10-fold competing non-labeled gelsolin FxxFF or gelsolin FxxAA peptides. Shown are the results of a representative assay.

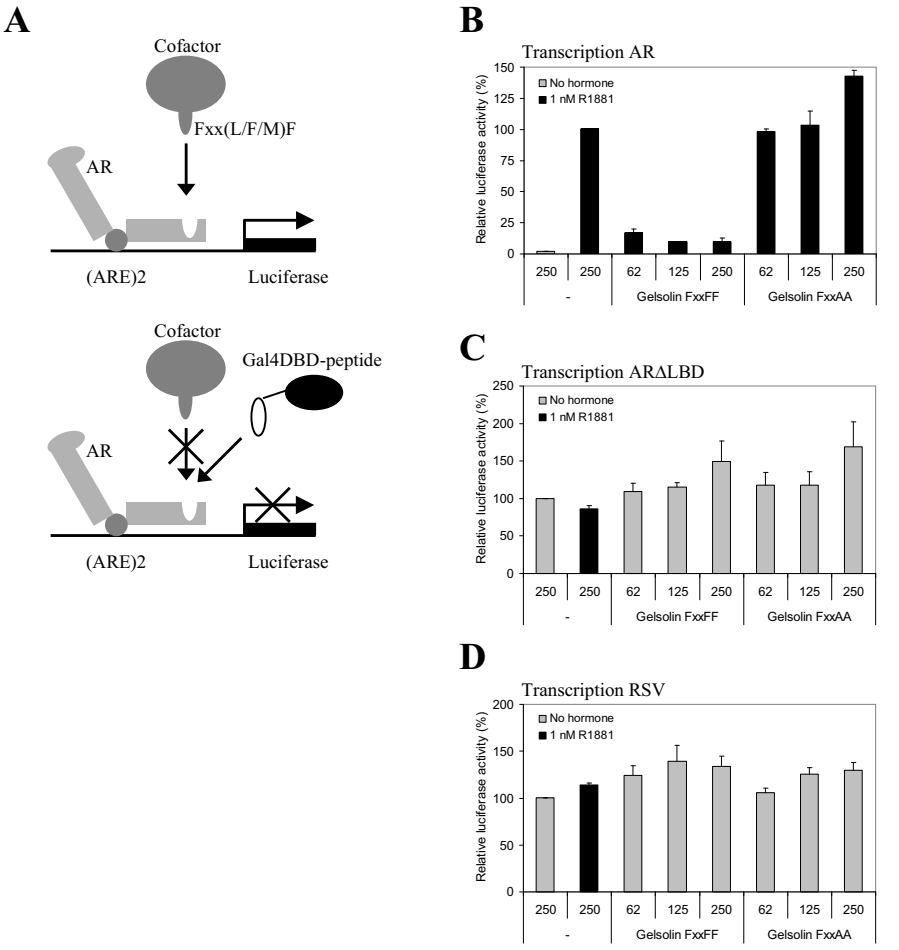

Figure 3. Gelsolin FxxFF blocks AR transcriptional activity. (A) Schematic representation of the assay to block AR transcriptional activity with peptides. Hep3B cells were transiently transfected with expression vector encoding full-length wild type AR together with $(ARE)_2$TATA-LUC reporter. In the presence of hormone, AR binds to the promoter after which cofactors are recruited via FxxLF-like motifs (upper part). In case of cotransfection of constructs encoding a peptide that binds to the coactivator groove, the peptide competes with interacting cofactors leading to repression of AR transcriptional activity (lower part). (B-D) Effect of Gal_4DBD, Gal_4DBD-gelsolin FxxFF and Gal_4DBD-gelsolin FxxAA on transcriptional activities of full-length AR (B), AR NTD-DBD (C), or on the constitutively active RSV promoter (D). Effects were determined by transient cotransfection of 250 ng empty pM vector or increasing amounts of pM-peptide constructs. pM-peptide expression vector was adjusted to 250 ng using empty pM vector. Further details are indicated in Experimental Procedures. Results shown are the mean luciferase activities of two independent experiments (+/- SD) for AR and of three independent experiments (+/- SEM) for AR NTD-DBD and RSV.

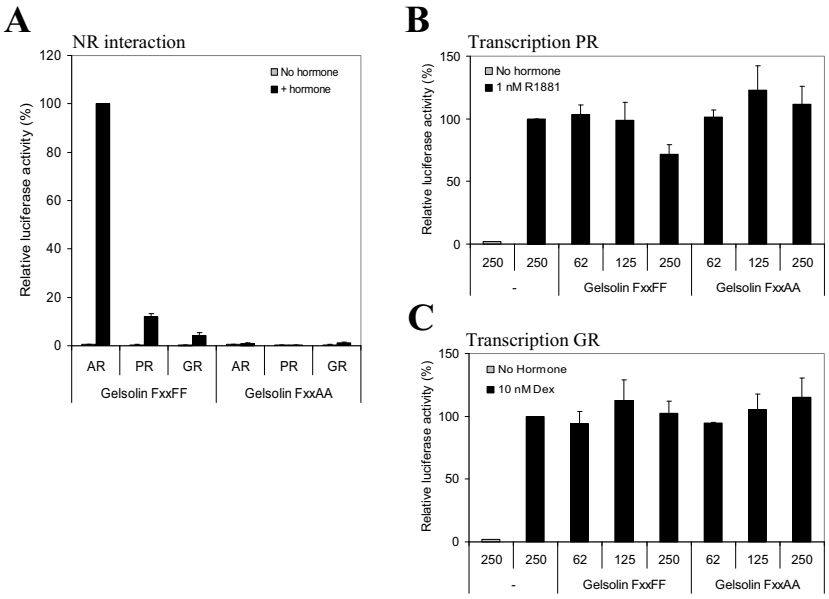

Figure 4. Gelsolin FxxFF specifically blocks transcriptional activity of AR. (A) Mammalian one-hybrid analysis to determine interaction of gelsolin FxxFF and gelsolin FxxAA peptides with AR, PR, and GR. Experimental procedures are similar to as described in the legends to Figures 1A and B. Hormones used are R1881 (1 nM) for AR and PR, and dexamethasone (10 nM) for GR. Each bar represents the mean relative luciferase activities of two independent experiments (+/- SD). (B and C) Effect of gelsolin FxxFF, and gelsolin FxxAA on transcriptional activities of PR (B) and GR (C). Assays were performed in the presence of 250 ng cotransfected empty pM vector or increasing amounts of pM-peptide expression vector. pM-expression vector was adjusted to 250 ng using empty pM vector. Further details are indicated in the Experimental Procedures section. Results shown are the mean relative luciferase activities of three independent experiments (+/- SEM), in which transcriptional activities of PR and GR in the presence of empty pM vector were set to 100%.

The gelsolin FxxFF peptide blocks AR-regulated gene expression

Because the gelsolin FxxFF peptide efficiently blocked peptide and protein interactions with the AR LBD, we investigated whether this peptide could affect gene expression regulated by full length AR as well (schematically presented in Fig. 3A). R1881-induced expression of the $(ARE)_2$TATA-LUC reporter was efficiently suppressed by increasing concentrations of the gelsolin FxxFF peptide (Fig. 3B), although higher amounts of plasmid were needed as compared to the AR N/C interaction assay. Even at the highest concentrations used, no inhibitory effects on AR-regulated gene expression were observed with the gelsolin FxxAA control peptide. Similar effects were observed using MMTV-LUC as reporter (data not shown).

Importantly, blocking of AR regulated gene expression by gelsolin FxxFF occurred via the LBD because no inhibitory effects were observed on the constitutive transcriptional activity

of AR lacking the LBD (Fig. 3C). The gelsolin FxxFF peptide did not affect the constitutively active RSV promoter, ruling out non-specific effects (Fig. 3D).

The gelsolin FxxFF peptide specifically blocks AR-regulated gene expression

Because the LBDs of PR and GR are structurally highly similar to AR LBD, we determined whether binding of the gelsolin FxxFF peptide was AR specific. The results of the mammalian one-hybrid assay demonstrated that, in contrast to strong interactions with AR, gelsolin FxxFF weakly interacted with PR and GR (Fig. 4A). Further analysis demonstrated that the gelsolin FxxFF peptide weakly affected PR activity only at the highest concentrations used, whereas no effects were observed on GR (Figs. 4B and C). The gelsolin FxxAA peptide did not affect activities of PR and GR, which is consistent with its inability to interact with both receptors.

In conclusion, the results demonstrate that the gelsolin FxxFF peptide coupled to Gal_4DBD efficiently blocks AR N/C interaction and specifically inhibits AR activity via the LBD.

The TAT-gelsolin FxxFF peptide interacts with AR LBD in vitro and efficiently blocks AR N/C interaction in vivo

In order to block AR-mediated expression of endogenous genes, peptides need to be efficiently introduced in almost all cells. Because transfection efficiencies are usually not of sufficient efficacy, we investigated a different approach to obtain peptides in all cells by applying synthetic gelsolin FxxFF peptide coupled to the TAT cell-penetrating peptide.

To verify that synthetic TAT-gelsolin FxxFF (Fig. 5A) is able to interact with AR LBD we used the TR-FRET assay as described in Figure 2C. In contrast to the TAT and TAT-gelsolin FxxAA mutant control peptides, TAT-gelsolin FxxFF showed a hormone-dependent recruitment by the AR LBD demonstrating that the TAT-gelsolin FxxFF peptide adopts a correct conformation and that this peptide binds AR LBD *in vitro* (Fig. 5B).

To investigate whether the TAT-gelsolin FxxFF peptide also functions in cultured cells, we determined its effect on the recruitment of AR NTD by AR LBD (Fig. 5C). TAT-gelsolin FxxFF completely abrogated AR N/C interaction at a peptide concentration of 0.5 μM, whereas TAT and TAT-gelsolin FxxAA had minimal effects. These results demonstrate that the synthetic TAT-gelsolin FxxFF peptide efficiently blocks protein interactions with AR LBD in cell cultures.

TAT-gelsolin FxxFF blocks AR-mediated reporter gene expression

Because TAT-gelsolin FxxFF interacts with AR LBD *in vitro* and competes with AR NTD to bind AR LBD *in vivo*, we argued that this peptide may serve as peptide antagonist to block AR-regulated reporter gene expression as well. Hep3B cells were transiently transfected with AR expression construct and $(ARE)_2$TATA-LUC reporter, and the peptides were added. As is shown in Fig. 6A, TAT-gelsolin FxxFF indeed strongly inhibited AR-regulated gene expression in a dose-dependent manner. Although the repression was less effective than for N/C interaction, still about 70% inhibition was observed using 5 μM peptide. No effects were observed for the

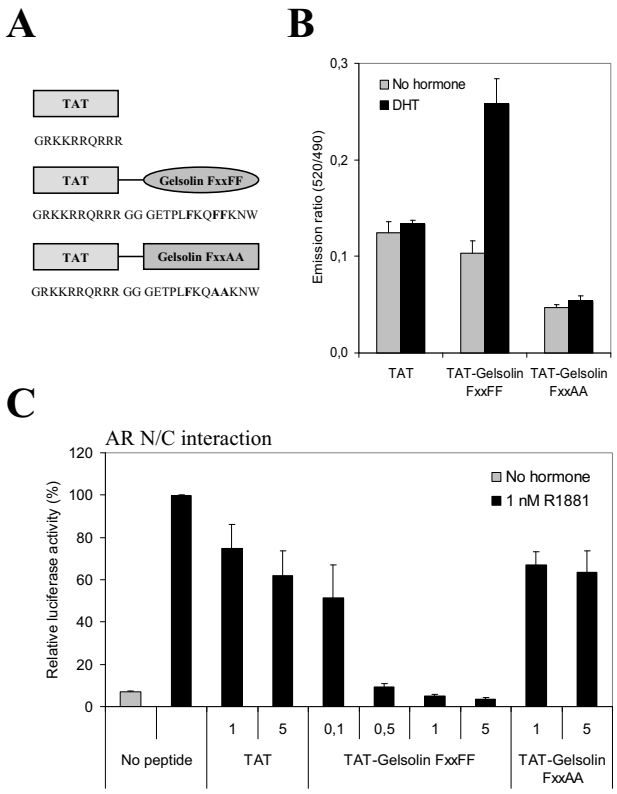

Figure 5. TAT-gelsolin FxxFF interacts with AR LBD *in vitro* and efficiently blocks AR N/C interaction *in vivo*. (A) Amino acid sequences of synthetic peptides used in this study. (B) Representative results of a TR-FRET assay demonstrating that TAT-gelsolin FxxFF interacts with AR LBD. Shown are the 520/490 emission ratios in the absence (grey bars) or presence (black bars) of DHT. (C) TAT-gelsolin FxxFF efficiently disrupts AR N/C interaction. Hep3B cells were transiently cotransfected with expression constructs encoding AR NTD and AR DBD-LBD together with $(ARE)_2$TATA-Luc reporter construct. AR N/C interaction was measured in the absence or presence of increasing concentrations of cell-penetrating peptides (μM). Bars represent the mean relative luciferase activities of three independent experiments (+/- SEM). Hormone induced N/C interaction in the absence of peptides was set to 100%.

two control peptides. Similar results were obtained in CHO cells with the same reporter or with MMTV-LUC as reporter in Hep3B cells (data not shown).

Effects of TAT-gelsolin FxxFF were specific for AR, as no effects were observed on activities mediated by PR and GR (Figs. 6B and C). Effects of TAT-gelsolin FxxFF on AR activity occurred via the LBD, because the constitutively active AR lacking the LBD was not affected by the peptide (Fig. 6D). However, whereas the expressed gelsolin peptide specifically blocked AR-regulated gene expression, synthetic TAT-gelsolin FxxFF was less specific as it also efficiently

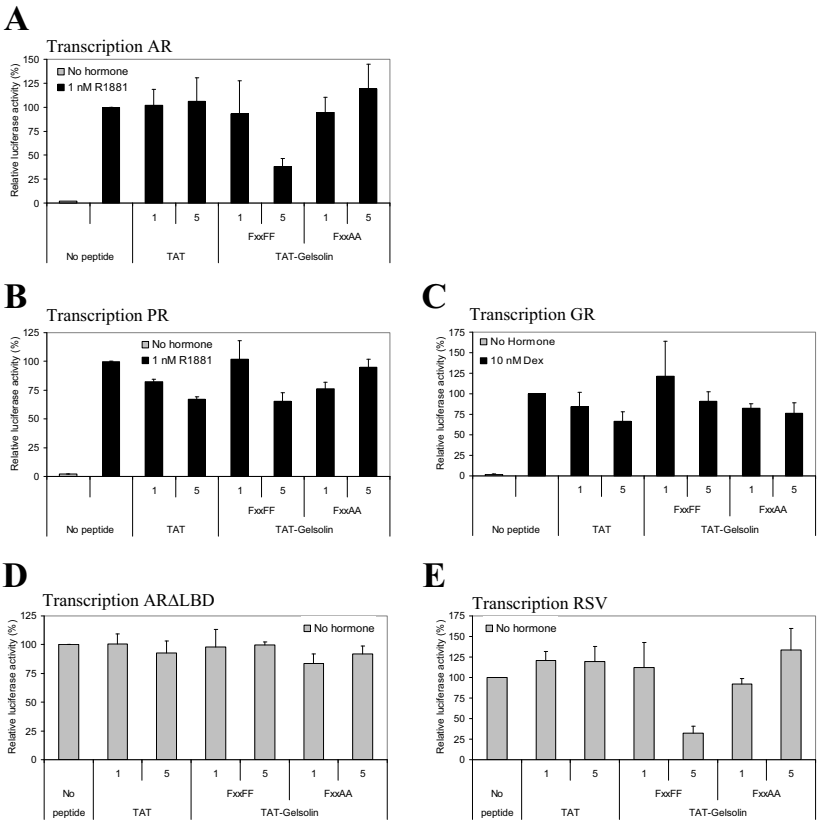

Figure 6. TAT-gelsolin FxxFF represses transcriptional activity of AR, but is less specific. Effect of the cell-penetrating peptides TAT, TAT-gelsolin FxxFF, and TAT-gelsolin FxxAA on transcriptional activities of AR (A), PR (B), GR (C), AR NTD-DBD (D), and on the constitutively active RSV promoter (E). Hep3B cells were transiently cotransfected as described in the Experimental Procedures section. Cells were incubated in the absence or presence of increasing concentrations of cell-penetrating peptides (μM). Bars represent the mean relative luciferase activities of four independent experiments (+/- SEM) for AR, AR NTD-DBD, and RSV or of three experiments (+/-SEM) for PR and GR. No peptide controls were set to 100%.

blocked transcription from the constitutively active RSV promoter (Fig. 6E). We also tested a peptide in which TAT was fused to the FxxLF motif of ARA54, but the TAT-ARA54 FxxLF peptide behaved similarly as the TAT-gelsolin FxxFF motif (data not shown).

DISCUSSION

This study aimed at the identification and characterization of peptides that block AR function beyond the level of hormone binding by targeting the coactivator binding groove in the AR

LBD. Our results demonstrated that a peptide withh the gelsolin FxxFF motif did not only efficiently block AR N/C interaction, but also AR-mediated reporter gene expression. This was true for both the peptide expressed from transfected pM vector and as well as a synthetic peptide coupled to the TAT cell-penetrating peptide.

The first part of this study focused on the selection of the most suitable peptide to block AR function. Because the peptide has to compete with cofactors for binding to the coactivator groove and with AR N/C interaction, we selected the peptide that interacted strongest with AR and was most efficient in blocking the recruitment of the AR NTD to AR LBD. We compared several FxxLF-like peptide motifs present in known AR cofactors as well as peptides identified in phage display screenings using AR LBD as bait (24, 29, 37, 38, 47-49). In general, we found a positive correlation between the interaction of the peptides and their capacity to block AR N/C interaction. The gelsolin FxxFF peptide scored very high in both assays and was selected for further experiments. We also tested peptides that were previously identified in a phage display screening using full-length AR as bait (28). Although most of these peptides were reported to interact with AR already in the absence of hormone, we found clear hormone-inducible interactions with low background values (data not shown). These discrepancies may be due to the usage of an additional VP16 activation domain fused to the AR in the previously reported experiments (28). However, none of these peptides displayed stronger interactions with AR or were more efficient in blocking AR N/C interaction than the gelsolin FxxFF peptide (data not shown).

Our results demonstrated that the gelsolin FxxFF peptide strongly repressed AR transcriptional activity without affecting transcription mediated by PR, GR, and the constitutively active AR NTD-DBD fragment. It is generally believed that AR transcriptional activity is largely mediated via the NTD, because in gene expression assays an AR fragment comprising the NTD and DBD is constitutively strongly active. In contrast, AR LBD is weakly transcriptionally active, suggesting that the AR LBD is a less likely target to block AR function (50). Consistent with this observation, p160 cofactors have been shown to interact with AR NTD. Although p160 cofactors may interact with AR LBD as well via the LxxLL motifs, these interactions are not essential (51, 52). In addition, the AR has a unique N/C interaction that involves binding of the FxxLF motif in the NTD to the coactivator groove in the LBD (32, 33, 41). N/C interaction may compete with endogenous cofactors for binding to the same LBD surface. This contrasts with other NRs, in which the LBD is considered to be the most important transcription activation domain. Several studies demonstrated that LBDs of most NRs have autonomous transcription activation capacity, suggesting that the LBD is capable of recruiting most cofactors necessary to activate transcription (53, 54). It is, therefore, not surprising that the coactivator groove has been successfully targeted by LxxLL-motif containing peptides to repress the function of various NRs (30, 47, 55-60).

Our results demonstrated that the role of the AR LBD may be more important than previously thought. Recently, we published that AR N/C interaction preferentially takes place in the

mobile AR fraction, probably to prevent unwanted cofactor binding (61). Upon DNA binding N/C interaction is relieved and the coactivator groove becomes accessible for FxxLF-motif containing cofactors. The data presented in this study are in agreement with the findings that the coactivator groove and the LBD play an important role in AR-regulated gene expression and show that this groove also in AR serves as a target (28, 37, 62, 63).

We showed that the gelsolin peptide inhibited AR activity, without affecting activities mediated by the highly related PR and GR. We previously demonstrated that AR prefers binding of phenylalanine-rich motifs over LxxLL motifs and that phenylalanine-rich motifs prefer to bind AR (29, 31, 38). Therefore, and as shown in this study, usage of FxxLF-like motifs instead of LxxLL will increase specificity for blocking gene expression mediated by AR.

We demonstrated that both gelsolin FxxFF peptide expressed from transfected vectors and synthetic gelsolin FxxFF coupled to TAT had similar effects, with exception of constitutively active promoters. Whereas Gal_4DBD-gelsolin was completely specific for AR, TAT-gelsolin FxxFF was less specific. It also inhibited not only the RSV promoter, but also the promoters of MSV and TK (data not shown).

Several additional synthetic peptides were tested in order to try to increase AR specificity (data not shown). In two of these, gelsolin FxxFF was fused to two other cell-penetrating peptides, i.e. penetratin and an arginine-stretch (R9) (64, 65). Both peptides did not have an effect, even on AR transcriptional activity, suggesting that internalisation of these peptides was less efficient or that gelsolin had a less optimal conformation in these specific fusions. In addition, we also tested a peptide in which TAT was coupled to the FxxLF motif of ARA54. However, this peptide also repressed RSV activity. For unclear reasons, the effects on constitutively active promoters appear to be due to the combination of TAT and the FxxLF(-like) motif.

In the majority of prostate tumors an active AR is still present, indicating that the AR remains a therapeutic target. Development of peptides or compounds targeted to the AR coactivator groove may overcome therapeutic failure that usually arises during conventional endocrine therapies, such as hormone antagonists. The results obtained in this study are instrumental and of importance in the design of such peptides and compounds in order to specifically repress the transcriptional activity of AR.

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REFERENCES

1. Feldman BJ, Feldman D 2001 The development of androgen-independent prostate cancer. Nat Rev Cancer 1:34-45
2. Trapman J, Brinkmann AO 1996 The androgen receptor in prostate cancer. Pathol Res Pract 192:752-760
3. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, Trapman J 1991 Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int J Cancer 48:189-193
4. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP 1995 In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 9:401-406
5. Balk SP 2002 Androgen receptor as a target in androgen-independent prostate cancer. Urology 60:132-138
6. Taplin ME 2007 Drug insight: role of the androgen receptor in the development and progression of prostate cancer. Nat Clin Pract Oncol 4:236-244
7. Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL 2005 Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. Clin Cancer Res 11:4653-4657
8. Mohler JL, Gregory CW, Ford OH, 3rd, Kim D, Weaver CM, Petrusz P, Wilson EM, French FS 2004 The androgen axis in recurrent prostate cancer. Clin Cancer Res 10:440-448
9. Mostaghel EA, Page ST, Lin DW, Fazli L, Coleman IM, True LD, Knudsen B, Hess DL, Nelson CC, Matsumoto AM, Bremner WJ, Gleave ME, Nelson PS 2007 Intraprostatic androgens and androgen-regulated gene expression persist after testosterone suppression: therapeutic implications for castration-resistant prostate cancer. Cancer Res 67:5033-5041
10. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM 2005 Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644-648
11. Hermans KG, van Marion R, van Dekken H, Jenster G, van Weerden WM, Trapman J 2006 TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. Cancer Res 66:10658-10663
12. Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, Yu J, Wang L, Montie JE, Rubin MA, Pienta KJ, Roulston D, Shah RB, Varambally S, Mehra R, Chinnaiyan AM 2007 Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature 448:595-599
13. Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT 2007 Nuclear receptor structure: implications for function. Annu Rev Physiol 69:201-220
14. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-839
15. Kang Z, Janne OA, Palvimo JJ 2004 Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. Mol Endocrinol 18:2633-2648
16. Louie MC, Yang HQ, Ma AH, Xu W, Zou JX, Kung HJ, Chen HW 2003 Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. Proc Natl Acad Sci U S A 100:2226-2230
17. Marshall TW, Link KA, Petre-Draviam CE, Knudsen KE 2003 Differential requirement of SWI/SNF for androgen receptor activity. J Biol Chem 278:30605-30613
18. Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. Mol Cell 9:601-610

19. Wang Q, Carroll JS, Brown M 2005 Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 19:631-642
20. Huang ZQ, Li J, Sachs LM, Cole PA, Wong J 2003 A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. Embo J 22:2146-2155
21. Vijayvargia R, May MS, Fondell JD 2007 A coregulatory role for the mediator complex in prostate cancer cell proliferation and gene expression. Cancer Res 67:4034-4041
22. Estebanez-Perpina E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, Buehrer BM, Webb P, Fletterick RJ, Guy RK 2005 The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280:8060-8068
23. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM 2004 Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425-438
24. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ 2004 Recognition and Accommodation at the Androgen Receptor Coactivator Binding Interface. PLoS Biol 2:E274
25. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, Stallcup MR 1998 Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 12:302-313
26. Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733-736
27. Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ, Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 12:3343-3356
28. Chang CY, Abdo J, Hartney T, McDonnell DP 2005 Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol Endocrinol 19:2478-2490
29. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, Trapman J 2006 Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping. Mol Endocrinol 20:1742-1755
30. Hall JM, Chang CY, McDonnell DP 2000 Development of peptide antagonists that target estrogen receptor beta-coactivator interactions. Mol Endocrinol 14:2010-2023
31. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J 2004 Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132-2150
32. He B, Kemppainen JA, Wilson EM 2000 FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986-22994
33. Steketee K, Berrevoets CA, Dubbink HJ, Doesburg P, Hersmus R, Brinkmann AO, Trapman J 2002 Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor. Eur J Biochem 269:5780-5791
34. Askew EB, Gampe RT, Jr., Stanley TB, Faggart JL, Wilson EM 2007 Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. J Biol Chem 282:25801-25816
35. Callewaert L, Verrijdt G, Christiaens V, Haelens A, Claessens F 2003 Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements. J Biol Chem 278:8212-8218
36. He B, Lee LW, Minges JT, Wilson EM 2002 Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. J Biol Chem 277:25631-25639
37. Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C 2003 The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691-23698

38. van de Wijngaart DJ, van Royen ME, Hersmus R, Pike AC, Houtsmuller AB, Jenster G, Trapman J, Dubbink HJ 2006 Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain. J Biol Chem 281:19407-19416
39. Vives E, Brodin P, Lebleu B 1997 A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272:16010-16017
40. Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB 2000 The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Natl Acad Sci U S A 97:13003-13008
41. Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J 1997 Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Biochemistry 36:1052-1064
42. Jenster G, van der Korput HA, Trapman J, Brinkmann AO 1995 Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 270:7341-7346
43. Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris C, Klaassen P, van der Korput JA, Voorhorst MM, van Laar JH, Mulder E, Trapman J 1989 The human androgen receptor: domain structure, genomic organization and regulation of expression. J Steroid Biochem 34:307-310
44. Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J 1996 Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 271:6379-6388
45. Jenster G, Spencer TE, Burcin MM, Tsai SY, Tsai MJ, O'Malley BW 1997 Steroid receptor induction of gene transcription: a two-step model. Proc Natl Acad Sci U S A 94:7879-7884
46. Ozers MS, Marks BD, Gowda K, Kupcho KR, Ervin KM, De Rosier T, Qadir N, Eliason HC, Riddle SM, Shekhani MS 2007 The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay. Biochemistry 46:683-695
47. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19:8226-8239
48. He B, Minges JT, Lee LW, Wilson EM 2002 The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226-10235
49. Wang L, Hsu CL, Ni J, Wang PH, Yeh S, Keng P, Chang C 2004 Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol 24:2202-2213
50. Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO 1991 Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol 5:1396-1404
51. Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B 1999 The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 19:6085-6097
52. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG 1999 The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 19:8383-8392
53. Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR 1996 GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci U S A 93:4948-4952
54. Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H 1996 TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. Embo J 15:3667-3675

55. Gaillard S, Dwyer MA, McDonnell DP 2007 Definition of the molecular basis for estrogen receptor-related receptor-alpha-cofactor interactions. Mol Endocrinol 21:62-76
56. Kurebayashi S, Nakajima T, Kim SC, Chang CY, McDonnell DP, Renaud JP, Jetten AM 2004 Selective LXXLL peptides antagonize transcriptional activation by the retinoid-related orphan receptor RORgamma. Biochem Biophys Res Commun 315:919-927
57. Mettu NB, Stanley TB, Dwyer MA, Jansen MS, Allen JE, Hall JM, McDonnell DP 2007 The nuclear receptor-coactivator interaction surface as a target for peptide antagonists of the peroxisome proliferator-activated receptors. Mol Endocrinol 21:2361-2377
58. Northrop JP, Nguyen D, Piplani S, Olivan SE, Kwan ST, Go NF, Hart CP, Schatz PJ 2000 Selection of estrogen receptor beta- and thyroid hormone receptor beta-specific coactivator-mimetic peptides using recombinant peptide libraries. Mol Endocrinol 14:605-622
59. Pathrose P, Barmina O, Chang CY, McDonnell DP, Shevde NK, Pike JW 2002 Inhibition of 1,25-dihydroxyvitamin D3-dependent transcription by synthetic LXXLL peptide antagonists that target the activation domains of the vitamin D and retinoid X receptors. J Bone Miner Res 17:2196-2205
60. Pike JW, Pathrose P, Barmina O, Chang CY, McDonnell DP, Yamamoto H, Shevde NK 2003 Synthetic LXXLL peptide antagonize 1,25-dihydroxyvitamin D3-dependent transcription. J Cell Biochem 88:252-258
61. van Royen ME, Cunha SM, Brink MC, Mattern KA, Nigg AL, Dubbink HJ, Verschure PJ, Trapman J, Houtsmuller AB 2007 Compartmentalization of androgen receptor protein-protein interactions in living cells. J Cell Biol 177:63-72
62. Hsu CL, Chen YL, Ting HJ, Lin WJ, Yang Z, Zhang Y, Wang L, Wu CT, Chang HC, Yeh S, Pimplikar SW, Chang C 2005 Androgen receptor (AR) NH2- and COOH-terminal interactions result in the differential influences on the AR-mediated transactivation and cell growth. Mol Endocrinol 19:350-361
63. Minamiguchi K, Kawada M, Ohba S, Takamoto K, Ishizuka M 2004 Ectopic expression of the amino-terminal peptide of androgen receptor leads to androgen receptor dysfunction and inhibition of androgen receptor-mediated prostate cancer growth. Mol Cell Endocrinol 214:175-187
64. Derossi D, Joliot AH, Chassaing G, Prochiantz A 1994 The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269:10444-10450
65. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB 2000 Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res 56:318-325

CHAPTER 6

General discussion

In this thesis, several aspects of hormone-binding and protein interactions with the coactivator groove in the LBD are described. First, the molecular mechanisms by which an AR prostate cancer mutant, AR L701H, and related mutants are activated by non-androgenic ligands were investigated (Chapter 2). In the context of a broad study of LxxLL and FxxLF motifs, screening of L+4 in the FxxLF motif of the AR NTD shows that other residues may be present at this position, including F and M, while maintaining strong interaction capacities with the AR LBD (Chapter 3). The AR-interacting proteins gelsolin and PAK6 appeared to be dependent on these novel motifs for their interaction with the AR LBD. Screening of FxxLF-like motifs resulted in the identification of BAF60a, a subunit of the SWI/SNF chromatin remodelling complex, as a novel AR cofactor (Chapter 4). Furthermore, it is shown in this thesis that a peptide containing an FxxLF-like motif can be efficiently used to abrogate protein interactions with the AR LBD and to specifically inhibit the transcriptional activity of the AR (Chapter 5). This general discussion focuses on two important aspects of these studies. First, the interaction of FxxLF-like motifs present in peptides and cofactors with the AR coactivator groove is discussed in more detail. Further it is discussed whether interactions of the AR LBD with peptide motifs may function as alternative target to block androgen-dependent prostate cancer growth. Where appropriate, important directions for future research are indicated.

1. PEPTIDE INTERACTIONS WITH THE AR COACTIVATOR GROOVE

Random mutagenesis studies of the AR FxxLF motif, screenings of random and focused peptide libraries, and structural analyses provided detailed insights in the structure of the AR coactivator groove and how it is bound by FxxLF-like motifs (1-7). These studies revealed that although the AR coactivator groove is sufficiently flexible to accommodate other large residues at positions +1 and +5, F residues are clearly preferred for strong AR LBD interactions (6, 7). These studies were extended by analysis of the +4 residue (Chapter 3). A systematic mutational screening of L+4 in the AR FxxLF motif showed that this residue can be substituted by an F (FxxFF) or M (FxxMF) without losing AR interaction capacity. FxxFF and FxxMF variants of the FxxLF motifs of ARA54 and ARA70 were also compatible with strong AR LBD binding (Chapter 3), as were the FxxLF and FxxMF variants of the gelsolin FxxFF motif, and the FxxLF and FxxFF variants of the PAK6 FxxMF motif (data not shown). This shows that in AR-binding motifs the +4 residue is exchangeable between L, F, or M while maintaining AR interaction capacity. In another study we have found that also FxxWF motifs may interact strongly with the AR LBD (Chapter 4), whereas others identified FxxYF (3, 4). In our mutational screening of L+4 (Chapter 3), the FxxWF and FxxYF variants of the AR FxxLF motif weakly interacted with the AR LBD, suggesting that in the context of this peptide motif W+4 and Y+4 adopt a less optimal conformation for AR LBD binding. All data demonstrate that large hydrophobic residues are preferred at position +4 of AR-interacting motifs, indicating that

only these residues are able to form sufficient hydrophobic contacts with the +4 binding site in the coactivator groove to allow stable peptide binding. Thus, interaction of a peptide with the AR LBD is driven by phenylalanine residues at positions +1 and +5, whereas the residue at position +4 seems less restricted.

Crystal structures of several FxxLF, FxxFF, and FxxYF motifs bound to the AR coactivator groove have been solved (1-3). These structures show that the FxxYF motif binds the AR LBD in a similar way as the FxxLF motifs. However, the FxxFF peptide has shifted in the coactivator groove toward the K720 residue in the one known AR LBD/FxxFF complex. Further, the crystal structure of the complex showed that F+4 has a different orientation than an L or Y at this position. The most likely explanation for these changes is that the peptide shift to K720 is caused by the face-down orientation of F+4 and not the other way around. Direct comparison of the crystal structures of the AR LBD complexed with FxxLF peptides, like AR FxxLF and its FxxFF variant, would shed more light on this question. So far, crystal structures of the AR LBD in complex with FxxMF or FxxWF motifs are not available. Because of the variability in the orientation of the +4 residue and the highly flexible side chain of methionine, the precise positioning of M and W to the coactivator groove cannot be accurately predicted by structural modelling.

Out of all other NRs tested so far, only PR LBD is able to bind a subset of FxxLF motifs although LxxLL motifs are preferred (4, 6, 8). This suggests that FxxLF motifs create a level of specificity for interactions with the AR LBD. However, here it is shown that the interaction of the ARA70 FxxLF motif with PR LBD is abrogated if L+4 is substituted by an F or M residue, whereas interactions with AR LBD remain unaffected (Chapter 3). Crystal structures of the PR LBD in complex with a peptide have so far not been published. Sequence comparison reveals that the +4 binding site in the coactivator groove of PR LBD is composed of the same residues as the AR LBD, except for a leucine (L727) which is a smaller valine (V713) in AR. The larger side chain of L727 probably precludes binding of peptide motifs containing bulky F and M residues at position +4. If this is true, FxxFF and FxxMF motifs may add an additional level of specificity for the AR compared to FxxLF motifs.

Phage display screenings of random peptide sequences with AR LBD and full-length AR only retrieved FxxLF-like motifs (3-5). This suggests that the coactivator groove is the major compact high-affinity protein interaction site in the AR. Although it cannot be excluded that peptides interact at other locations in the AR, they probably bind with too low affinities to be identified by the phage display screenings. Another option is that the interaction surfaces are larger than can be covered by the peptides or that a combination of multiple low-affinity binding sites is needed for stable binding, as may be true for the AR NTD (4, 9). Therefore, additional peptide screenings will probably not yield novel types of motifs that bind with high affinity to the coactivator groove or anywhere else in the AR.

It has recently been shown that the AR T877A mutant shows differential peptide preferences depending on the bound ligand (10, 11). Future studies may be aimed at more detailed

investigation of the effects of AR mutations and/or different ligands on the structure of the coactivator groove and on subsequent peptide interactions.

2. PROTEIN INTERACTIONS WITH THE AR COACTIVATOR GROOVE

So far, (fragments of) nearly 200 proteins have been claimed to interact with the AR (12). However, for only a small subset of proteins it is known that they interact with the AR via an LxxLL (p160 cofactors and MED1/TRAP220) or FxxLF (ARA54, ARA70, Rad9) motif (6, 8, 13-15). For the remaining proteins the mode of interaction with AR is not or not fully understood. In this thesis it is shown that two of these proteins, PAK6 and gelsolin, are dependent on FxxMF and FxxFF motifs, respectively, for their interaction with the AR (Chapter 3). It was demonstrated that the interaction of PAK6 with AR could be established with (almost) full-length protein. This indicates that PAK6 is a *bona fide* AR partner. The specific function of the interaction remains to be established. The relevance of AR-gelsolin interaction is less clear. In the experiments described in Chapter 3 a large fragment of gelsolin was used. Follow-up experiments with even longer fragments, however, could not confirm the interaction.

A screening of *in silico* selected FxxLF-like motifs was performed to identify additional physiologically relevant AR binding partners (Chapter 4). In contrast to more conventional screening methods such as the yeast two-hybrid assay of cDNA libraries, this approach would identify only those proteins that interact with the cofactor groove in the AR LBD. To limit the number of peptides to be analysed, additional selection criteria were used, including conservation between species, tissue of expression, and subcellular localization. From the selected motifs only a small percentage (~10%) showed interaction with the AR LBD. The one motif tested as full-length protein did not interact with the AR. Although not all proteins have been analysed, these findings suggest that random *in silico* screening is not an ideal start for identification of AR interacting proteins.

A second screening, focused on FxxLF-like motifs in proteins present in complexes essential in the transcription process, such as chromatin remodelling complexes and Mediator, was more successful. Because of the limited number of proteins present in these complexes we could include other types of motifs in the searches. This screening resulted in the identification of BAF60a as a novel AR cofactor (Chapter 4). AR binding of BAF60a, a component of the SWI/SNF chromatin remodelling complex, is dependent on hormone and requires an intact FxxFF motif. Downregulation of BAF60a with siRNA differentially affects expression of AR target genes (Chapter 4). Expression of TMPRSS2 is strongly inhibited, whereas expression of other AR target genes, such as SGK and SARG, is hardly affected. This indicates that transcription initiation complexes on androgen-regulated genes can have different cofactor composition. Preliminary microarray analysis confirms the important role of BAF60a in the expression of TMPRSS2, as TMPRSS2 is one of the genes with strongest downregulation by BAF60a siRNA

(data not shown). Furthermore, the microarray data indicated that many AR target genes affected by BAF60a encode proteins that play a role in lipid metabolism or function as solute carriers in the cell membrane. Interestingly, BAF60a has recently been shown to affect expression of similar genes regulated by PPAR (16). BAF60a seems the first AR cofactor that plays a role in the selective expression of a subset of AR target genes with a specific function.

3. THE AR AS THERAPEUTIC TARGET IN PROSTATE CANCER

As discussed earlier, in prostate cancer the AR-controlled balance between cell proliferation, survival, and differentiation is disturbed, leading to the growth of an androgen-dependent tumour (17, 18). Treatments of metastasized prostate tumours are based on androgen ablation and/or anti-androgens. Although initially effective, within 2-3 years the tumour progresses to a hormone-refractory state for which effective treatments are currently not available. However, in the majority of these tumours the AR signalling pathway still plays a critical role, indicating that not only in primary tumours, but also in hormone-refractory tumours the AR remains a target for blocking prostate cancer growth, as has been described in the General Introduction.

3.1 MECHANISMS OF BLOCKING AR FUNCTION

Because progression of anti-androgen responsive prostate tumours to an anti-androgen refractory state is a common phenomenon, there is an urgent need for the development of improved anti-androgens or novel types of drugs that block AR function via other mechanisms. Alternative approaches to inhibit AR-dependent prostate cancer growth are discussed in more detail below.

The first approach is the development of improved anti-androgens. Anti-androgens, such as hydroxyflutamide and bicalutamide, compete with androgens for binding to the AR ligand-binding pocket. If bound by an anti-androgen, the AR is still capable of entering the nucleus and to (transiently) bind DNA, but is unable to recruit cofactors because of an inactive conformation (10, 19-22). Approximately 10% of the hormone-refractory prostate tumours escape anti-androgen therapy because of mutations in the AR. The AR T877A mutation has been identified frequently in patients who received flutamide, whereas the AR W741C mutation has been found in prostate tumours of patients treated with bicalutamide (23-25). The AR T877A and W741C mutations have also been identified in prostate cancer cell lines after long-term treatment with hydroxyflutamide or bicalutamide, respectively (26, 27). Secondary or tertiairy anti-androgens should be developed for treatment of AR positive patients resistant against flutamide or bicalutamide. Improved hydroxyflutamide or bicalutamide might be developed based on the crystal structures of T877A and W741L bound by the respective anti-androgens (28, 29).

A recent screening of small molecules identified compound RD162, that functions as an anti-androgen via a novel mechanism. Unlike the current anti-androgens, which prevent cofactor recruitment, RD162 impairs AR nuclear translocation by a so far unknown mechanism (30). Elucidation of this mechanism by functional and structural analyses may provide important clues for the development of novel types of anti-androgens.

A novel approach of blocking AR function is to specifically target crucial steps in the AR transcription activation pathway other than hormone binding. Binding of the AR to DNA has been successfully inhibited in LNCaP cells by using ARE decoy molecules (31, 32). These decoys, consisting of double-stranded oligonucleotides based on the ARE sequence of the PSA promoter, competitively inhibit AR transcriptional activity by blocking the DNA-binding site in the DBD. Because the effects of these decoys were limited due to low transfection efficiencies, further optimisations are needed to improve their therapeutic potential. Another mechanism indirectly affecting AR binding to DNA is to target the D-box dimerization interface in the AR DBD. Interestingly, recent experiments suggest that dimerization via the D-box is critical for stable AR binding to selective AREs, but it seems less important for binding to non-selective AREs (33). In addition, ChIP-chip data indicate that the AR may bind as monomer to single ARE half sites, although the functionality of these interactions remains to be investigated (34-36). Overall, targeting the AR D-box dimerization interface possibly affects transcription of AR target genes that are under control of the selective AREs. Because for the majority of AR target genes it is unknown how the expression is regulated by AR, it remains unclear whether it would be advantageous or disadvantageous to abrogate AR-dependent prostate cancer growth by targeting the D-box. Identification of these genes will be necessary. Abrogating other important protein interactions of the AR, such as AR N/C interaction and cofactor recruitment, may also serve as interesting mechanism to block AR transcriptional activity. The potential of targeting these AR-protein interactions will be discussed in more detail in section 3.2.

A next approach to impair AR function is to block its expression. Targeted inhibition of AR using ribozyme, antisense, and siRNA approaches have been demonstrated to reduce AR-dependent target gene expression, cell proliferation, and survival in hormone-refractory prostate cancer cell lines and xenografts (37-42). Thus, approaches involving AR knock-down may have therapeutic potential in prostate cancer.

An alternative approach is not to attack the function of AR itself, but to inhibit the expression of AR target genes and/or their protein products that play a critical role in the prostate cancer process. Good candidates are the members of the Ets family that are overexpressed specifically in the majority of prostate tumours (43, 44). However, because, like AR, Ets factors are transcription factors, targeting will not be easy. Another option is to target important Ets target genes. Gene expression profiling of ERG-positive prostate tumours revealed epigenetic reprogramming, including upregulation of the histone deacetylase 1 (HDAC1) gene and downregulation of its target genes (45). The first studies in TMPRSS2-ERG positive

prostate cancer cells showed that HDAC inhibitor treatment reduces cell growth and induces apoptosis (46).

3.2 ABROGATING AR PROTEIN INTERACTIONS

The best-studied mechanism of blocking AR function besides competition of hormone binding to the ligand-binding pocket is the abrogation of the necessary protein interactions. D-box interactions have been described above. Important other AR protein interactions include inter –or intramolecular N/C interaction and the binding of cofactors (47). In contrast to other NRs, the NTD in the AR appears to be an important transcription activation domain, suggesting that key AR cofactors are recruited by the NTD (48, 49). The lack of data indicates that the most important cofactors that bind the AR NTD remain to be identified. The mode of interaction of most proteins that bind the NTD is not clear. The lack of a stable AR NTD structure and observations that indicate that protein interactions with the NTD may require a relatively large surface area hamper the use of small peptides or compounds to target a specific cofactor binding site (4, 9). In one study, the complete AR NTD has been used as decoy molecule to competitively bind the interacting proteins that are required for the activation of the endogenous full-length AR (50). This AR NTD decoy reduces AR transcriptional activity, tumour incidence and growth, and delays progression to the hormone-refractory stage if stably expressed in an LNCaP xenograft model. Moreover, intra-tumour injection of lentiviruses expressing the AR NTD strongly inhibited growth of established xenografts. The mechanism by which the AR NTD inhibits AR function is not fully understood. Besides functioning as decoy molecule, the AR NTD may also bind the AR LBD thereby impairing AR N/C interaction and cofactor recruitment to the coactivator groove. To apply the AR NTD as a therapeutic target, it is of high importance to identify the binding proteins that play an important role AR transcriptional activity. This might allow targeting these protein interactions more specifically using small protein fragments or low-molecular weight compounds. Potential targets include the interaction of p160 cofactors with the TAU-5 region in AR and binding of MAGE-11 to the AR region that overlaps the FxxLF motif (14, 51, 52).

In the present study a different approach was followed by targeting the protein interactions that act via the coactivator groove in the AR LBD. The compact interface allows the usage of relatively small protein fragments. Moreover, the structure of the groove differs among the NR LBDs, which will aid in the development of drugs with high specificity. In most NRs, the LBD is the most important transcription activation domain, in which the coactivator groove plays an essential role. LxxLL-motif containing peptides targeted to the coactivator groove have been successfully used to compete for cofactor interactions and to inhibit the transcriptional activity of various NRs (53-60). The role of the LBD in transcriptional activity of the AR is less clear. An AR fragment encompassing the DBD and LBD shows weak transcriptional activity. Furthermore, the AR LBD binds LxxLL motifs present in the p160 cofactors with less affinity than LBDs of other NRs (7, 61-63). However, recent experiments showed that AR N/C

interaction, involving the FxxLF motif in the AR NTD, is relieved upon DNA binding and will be available for interacting cofactors (64), including cofactors that bind via motifs with lower affinity, such as p160 cofactors. This indicates that the AR coactivator groove does play a role in AR transcriptional activity, as also shown in this study for binding of BAF60a via this groove. Our data, demonstrating that AR transcriptional activity can be efficiently and specifically repressed by FxxLF-like peptides confirms and extends these observations.

The effects of peptides on NR transcriptional activities have been largely determined on transiently transfected reporters using vectors expressing the peptides linked to Gal_4DBD. However, transient transfections are not efficient enough to investigate the effects of such peptides on the expression of endogenous target genes or on cell growth. Therefore, different methods to deliver peptides into cells were investigated. First, the effects of peptides delivered into cells via cell-penetrating peptides (CPPs) on AR N/C interaction and AR transcriptional activity was investigated (Chapter 5). CPPs or protein transduction domains (PTDs) are highly basic motifs consisting of approximately 10 amino acid residues that are claimed to enter most cell types in a fast and non-toxic way if added to the culture medium (reviewed in (65-68)). Peptides and heterologous proteins that do not cross cell membranes have been efficiently transported into cultured cells if coupled to CPPs. The mechanism by which CPPs enter cells is poorly understood. CPPs have also been used *in vivo* to deliver β-galactosidase into mouse tissues (69, 70). β-Galactosidase was detected in most tissues already 15 minutes after intraperitoneal or intravenous injection of the CPP-coupled protein. Although far less efficient, the fusion protein was also present in brain, suggesting that CPPs are even capable of crossing the blood-brain-barrier. These properties would make CPPs interesting carriers for peptide or protein delivery, not only *in vitro*, but also *in vivo*.

Although it was found that the gelsolin FxxFF peptide coupled to TAT could enter cells, it turned out that the peptide only exerted its effects efficiently if added to the cells after pre-incubation with the transfection mixtures used to introduce the luciferase reporter and the AR expression vectors (Chapter 5). This suggests that the TAT-gelsolin peptide forms a complex with the transfection mixtures, possibly via the positively charged TAT and the negatively charged DNA. The data showed that the application of CPPs is not as straightforward as indicated by literature data. Clearly, further studies are needed to optimize the use of CPPs.

We observed differential effects between the gelsolin FxxFF peptide coupled to the Gal_4DBD and expressed from a vector, and peptide introduced into cells via the TAT CPP (Chapter 5). Whereas Gal_4DBD-gelsolin FxxFF efficiently and specifically represses AR N/C interaction and AR transcriptional activity, TAT-gelsolin FxxFF is less specific since it also efficiently inhibits several constitutively active promoters. The mechanisms behind these discrepancies are currently not known. Preliminary data show that these effects are neither due to the cell line used nor to toxicity of the peptide. The effects observed are also not due to the gelsolin FxxFF motif per se, because a peptide in which TAT is coupled to the FxxLF motif of ARA54 also represses transcription from the RSV promoter. Replacement of TAT by other cell-penetrating

peptides, including penetratin and R9 (a stretch of 9 arginine residues) was not beneficial (71, 72). Both peptides neither affected AR N/C interaction nor AR transcriptional activity, suggesting that internalization of these peptides is even less efficient. In further transfection studies it turned out that the Gal_4DBD part of FxxLF expression constructs is of importance for efficient function of the peptide. The Gal_4DBD might function as a scaffold or alternatively stabilizes the peptide to prevent premature degradation.

So far, the observations that the TAT-gelsolin FxxFF peptide does not efficiently translocate into cells and that it has non-specific effects, hampers the application of this approach to block transcriptional activities of endogenous AR target genes and to study an effect on cell growth. Therefore, a start was made with the application of peptide expression by lentivirus delivery. Recently, it has been demonstrated that a Gal_4DBD-LxxLL peptide was capable of inhibiting transcription of endogenous PPAR target genes if delivered by lentiviruses (57). Preliminary data show that the gelsolin-FxxFF fusion protein is expressed and interacts with endogenous AR in LNCaP cells. However, so far repression of AR transcriptional activity was not found, suggesting that the expression level of the peptide was not high enough. Protein expression may be increased by using stronger promoters.

Another strategy to repress endogenous AR activity via the coactivator groove, is by developing small molecules. Such compounds may have several advantages compared to peptides. They are smaller and can be relatively easily modified to improve affinity, selectivity, solubility, and bioavailability. Helix mimetics resembling the α-helical LxxLL motif, which target the coactivator groove of ERα, have been developed (73). These compounds bound ERα with similar affinities as the second LxxLL motif of SRC1 and competed with LxxLL peptides for binding to the same LBD surface *in vitro*. In another study, β-aminoketones have been identified that covalently bind the coactivator groove of TR (74, 75). These compounds do not only inhibit TR-LxxLL peptide interaction *in vitro*, but also suppress TR transcriptional activity. This demonstrates the applicability of small molecules that target the coactivator groove for blocking NR function. Screenings to identify compounds that target the coactivator groove of the AR are currently under way.

In conclusion, it is clear from the data collected so far that the coactivator groove in the AR LBD is an attractive target for blocking of AR function. However, the ideal blocking molecule and the most efficient method to deliver such a molecule into the target cell remains a challenge.

REFERENCES

1. Estebanez-Perpina E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, Buehrer BM, Webb P, Fletterick RJ, Guy RK 2005 The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280:8060-8068
2. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, Stewart EL, Kalman RI, Minges JT, Wilson EM 2004 Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol Cell 16:425-438
3. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ 2004 Recognition and Accommodation at the Androgen Receptor Coactivator Binding Interface. PLoS Biol 2:E274
4. Chang CY, Abdo J, Hartney T, McDonnell DP 2005 Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display. Mol Endocrinol 19:2478-2490
5. Hsu CL, Chen YL, Yeh S, Ting HJ, Hu YC, Lin H, Wang X, Chang C 2003 The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs. J Biol Chem 278:23691-23698
6. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, Trapman J 2006 Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping. Mol Endocrinol 20:1742-1755
7. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, Ziel-van der Made AC, Brinkmann AO, Pike AC, Trapman J 2004 Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 18:2132-2150
8. He B, Minges JT, Lee LW, Wilson EM 2002 The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 277:10226-10235
9. Lavery DN, McEwan IJ 2006 The human androgen receptor AF1 transactivation domain: interactions with transcription factor IIF and molten-globule-like structural characteristics. Biochem Soc Trans 34:1054-1057
10. Brooke GN, Parker MG, Bevan CL 2008 Mechanisms of androgen receptor activation in advanced prostate cancer: differential co-activator recruitment and gene expression. Oncogene 27:2941-2950
11. Ozers MS, Marks BD, Gowda K, Kupcho KR, Ervin KM, De Rosier T, Qadir N, Eliason HC, Riddle SM, Shekhani MS 2007 The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay. Biochemistry 46:683-695
12. Heemers HV, Tindall DJ 2007 Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 28:778-808
13. Coulthard VH, Matsuda S, Heery DM 2003 An extended LXXLL motif sequence determines the nuclear receptor binding specificity of TRAP220. J Biol Chem 278:10942-10951
14. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG 1999 The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 19:8383-8392
15. Wang L, Hsu CL, Ni J, Wang PH, Yeh S, Keng P, Chang C 2004 Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol 24:2202-2213
16. Li S, Liu C, Li N, Hao T, Han T, Hill DE, Vidal M, Lin JD 2008 Genome-wide coactivation analysis of PGC-1alpha identifies BAF60a as a regulator of hepatic lipid metabolism. Cell Metab 8:105-117
17. Trapman J 2001 Molecular mechanisms of prostate cancer. Eur J Cancer 37 Suppl 7:S119-125
18. Dehm SM, Tindall DJ 2006 Molecular regulation of androgen action in prostate cancer. J Cell Biochem 99:333-344
19. Kang Z, Janne OA, Palvimo JJ 2004 Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor. Mol Endocrinol 18:2633-2648

20. Kang Z, Pirskanen A, Janne OA, Palvimo JJ 2002 Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. J Biol Chem 277:48366-48371
21. Shang Y, Myers M, Brown M 2002 Formation of the androgen receptor transcription complex. Mol Cell 9:601-610
22. Farla P, Hersmus R, Trapman J, Houtsmuller AB 2005 Antiandrogens prevent stable DNA-binding of the androgen receptor. J Cell Sci 118:4187-4198
23. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP 1999 Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 59:2511-2515
24. Haapala K, Hyytinen ER, Roiha M, Laurila M, Rantala I, Helin HJ, Koivisto PA 2001 Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide. Lab Invest 81:1647-1651
25. Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EJ, Cancer and Leukemia Group BS 2003 Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. J Clin Oncol 21:2673-2678
26. Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, Miyamoto M 2003 Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. Cancer Res 63:149-153
27. Marques RB, Erkens-Schulze S, de Ridder CM, Hermans KG, Waltering K, Visakorpi T, Trapman J, Romijn JC, van Weerden WM, Jenster G 2005 Androgen receptor modifications in prostate cancer cells upon long-termandrogen ablation and antiandrogen treatment. Int J Cancer 117:221-229
28. Bohl CE, Miller DD, Chen J, Bell CE, Dalton JT 2005 Structural basis for accommodation of nonsteroidal ligands in the androgen receptor. J Biol Chem 280:37747-37754
29. Bohl CE, Gao W, Miller DD, Bell CE, Dalton JT 2005 Structural basis for antagonism and resistance of bicalutamide in prostate cancer. Proc Natl Acad Sci U S A 102:6201-6206
30. Clegg N, Tran C, Ouk S, Wongvipat J, Watson P, Welsbie D, Chen C, Jung M, Sawyers CL 2008 Development of androgen receptor antagonists with a novel mechanism of action. Keystone Symposium, Whistler, 2008
31. Kuratsukuri K, Sugimura K, Harimoto K, Kawashima H, Kishimoto T 1999 "Decoy" of androgen-responsive element induces apoptosis in LNCaP cells. Prostate 41:121-126
32. Zhang P, Zhang J, Young CY, Kao PC, Chen W, Jiang A, Zhang L, Guo Q 2005 Decoy androgen-responsive element DNA can inhibit androgen receptor transactivation of the PSA promoter gene. Ann Clin Lab Sci 35:278-284
33. van Royen ME, Houtsmuller AB, Trapman J 2009 A two-step model for androgen receptor dimerization in living cells. Submitted
34. Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR 2007 Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev 21:2005-2017
35. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, Mills IG 2007 New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. EMBO Rep 8:871-878
36. Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M 2007 A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 27:380-392
37. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ 2002 Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 62:1008-1013
38. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE 2005 Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. J Steroid Biochem Mol Biol 96:251-258

39. Eder IE, Haag P, Basik M, Mousses S, Bektic J, Bartsch G, Klocker H 2003 Gene expression changes following androgen receptor elimination in LNCaP prostate cancer cells. Mol Carcinog 37:181-191
40. Liao X, Tang S, Thrasher JB, Griebling TL, Li B 2005 Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. Mol Cancer Ther 4:505-515
41. Compagno D, Merle C, Morin A, Gilbert C, Mathieu JR, Bozec A, Mauduit C, Benahmed M, Cabon F 2007 SIRNA-directed in vivo silencing of androgen receptor inhibits the growth of castration-resistant prostate carcinomas. PLoS ONE 2:e1006
42. Li TH, Zhao H, Peng Y, Beliakoff J, Brooks JD, Sun Z 2007 A promoting role of androgen receptor in androgen-sensitive and -insensitive prostate cancer cells. Nucleic Acids Res 35:2767-2776
43. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM 2005 Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310:644-648
44. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM 2008 Recurrent gene fusions in prostate cancer. Nat Rev Cancer 8:497-511
45. Iljin K, Wolf M, Edgren H, Gupta S, Kilpinen S, Skotheim RI, Peltola M, Smit F, Verhaegh G, Schalken J, Nees M, Kallioniemi O 2006 TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming. Cancer Res 66:10242-10246
46. Bjorkman M, Iljin K, Halonen P, Sara H, Kaivanto E, Nees M, Kallioniemi OP 2008 Defining the molecular action of HDAC inhibitors and synergism with androgen deprivation in ERG-positive prostate cancer. Int J Cancer 123:2774-2781
47. Trapman J, Dubbink HJ 2007 The role of cofactors in sex steroid action. Best Pract Res Clin Endocrinol Metab 21:403-414
48. Jenster G, van der Korput HA, Trapman J, Brinkmann AO 1995 Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 270:7341-7346
49. Jenster G, van der Korput JA, Trapman J, Brinkmann AO 1992 Functional domains of the human androgen receptor. J Steroid Biochem Mol Biol 41:671-675
50. Quayle SN, Mawji NR, Wang J, Sadar MD 2007 Androgen receptor decoy molecules block the growth of prostate cancer. Proc Natl Acad Sci U S A 104:1331-1336
51. Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B 1999 The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 19:6085-6097
52. He B, Bai S, Hnat AT, Kalman RI, Minges JT, Patterson C, Wilson EM 2004 An androgen receptor NH2-terminal conserved motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP). J Biol Chem 279:30643-30653
53. Chang C, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19:8226-8239
54. Gaillard S, Dwyer MA, McDonnell DP 2007 Definition of the molecular basis for estrogen receptor-related receptor-alpha-cofactor interactions. Mol Endocrinol 21:62-76
55. Hall JM, Chang CY, McDonnell DP 2000 Development of peptide antagonists that target estrogen receptor beta-coactivator interactions. Mol Endocrinol 14:2010-2023
56. Kurebayashi S, Nakajima T, Kim SC, Chang CY, McDonnell DP, Renaud JP, Jetten AM 2004 Selective LXXLL peptides antagonize transcriptional activation by the retinoid-related orphan receptor RORgamma. Biochem Biophys Res Commun 315:919-927
57. Mettu NB, Stanley TB, Dwyer MA, Jansen MS, Allen JE, Hall JM, McDonnell DP 2007 The nuclear receptor-coactivator interaction surface as a target for peptide antagonists of the peroxisome proliferator-activated receptors. Mol Endocrinol 21:2361-2377

58. Northrop JP, Nguyen D, Piplani S, Olivan SE, Kwan ST, Go NF, Hart CP, Schatz PJ 2000 Selection of estrogen receptor beta- and thyroid hormone receptor beta-specific coactivator-mimetic peptides using recombinant peptide libraries. Mol Endocrinol 14:605-622
59. Pathrose P, Barmina O, Chang CY, McDonnell DP, Shevde NK, Pike JW 2002 Inhibition of 1,25-dihydroxyvitamin D3-dependent transcription by synthetic LXXLL peptide antagonists that target the activation domains of the vitamin D and retinoid X receptors. J Bone Miner Res 17:2196-2205
60. Pike JW, Pathrose P, Barmina O, Chang CY, McDonnell DP, Yamamoto H, Shevde NK 2003 Synthetic LXXLL peptide antagonize 1,25-dihydroxyvitamin D3-dependent transcription. J Cell Biochem 88:252-258
61. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM 1995 Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 9:208-218
62. He B, Bowen NT, Minges JT, Wilson EM 2001 Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 276:42293-42301
63. He B, Kemppainen JA, Wilson EM 2000 FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986-22994
64. van Royen ME, Cunha SM, Brink MC, Mattern KA, Nigg AL, Dubbink HJ, Verschure PJ, Trapman J, Houtsmuller AB 2007 Compartmentalization of androgen receptor protein-protein interactions in living cells. J Cell Biol 177:63-72
65. Chauhan A, Tikoo A, Kapur AK, Singh M 2007 The taming of the cell penetrating domain of the HIV Tat: myths and realities. J Control Release 117:148-162
66. Gump JM, Dowdy SF 2007 TAT transduction: the molecular mechanism and therapeutic prospects. Trends Mol Med 13:443-448
67. Vives E, Schmidt J, Pelegrin A 2008 Cell-penetrating and cell-targeting peptides in drug delivery. Biochim Biophys Acta 1786:126-138
68. Wadia JS, Dowdy SF 2005 Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. Adv Drug Deliv Rev 57:579-596
69. Cai SR, Xu G, Becker-Hapak M, Ma M, Dowdy SF, McLeod HL 2006 The kinetics and tissue distribution of protein transduction in mice. Eur J Pharm Sci 27:311-319
70. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF 1999 In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285:1569-1572
71. Derossi D, Joliot AH, Chassaing G, Prochiantz A 1994 The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269:10444-10450
72. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB 2000 Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res 56:318-325
73. Becerril J, Hamilton AD 2007 Helix mimetics as inhibitors of the interaction of the estrogen receptor with coactivator peptides. Angew Chem Int Ed Engl 46:4471-4473
74. Arnold LA, Estebanez-Perpina E, Togashi M, Jouravel N, Shelat A, McReynolds AC, Mar E, Nguyen P, Baxter JD, Fletterick RJ, Webb P, Guy RK 2005 Discovery of small molecule inhibitors of the interaction of the thyroid hormone receptor with transcriptional coregulators. J Biol Chem 280:43048-43055
75. Estebanez-Perpina E, Arnold LA, Jouravel N, Togashi M, Blethrow J, Mar E, Nguyen P, Phillips KJ, Baxter JD, Webb P, Guy RK, Fletterick RJ 2007 Structural insight into the mode of action of a direct inhibitor of coregulator binding to the thyroid hormone receptor. Mol Endocrinol 21:2919-2928

Summary

Samenvatting

SUMMARY

The androgen receptor (AR) is a ligand-dependent transcription factor, which is activated by the androgens testosterone and dihydrotestosterone (DHT). The androgen-AR pathway is essential for normal male development and plays a critical role in maintaining the functions of the male-specific organs, such as the prostate. A disregulated androgen-AR pathway has been implicated in several diseases, including prostate cancer. AR transcriptional activity is regulated by interacting proteins, so-called cofactors. The best-studied protein interaction surface in the AR is the coactivator groove in the ligand-binding domain (LBD) to which cofactors can bind via FxxLF motifs. In this thesis several properties of the AR LBD are investigated, including ligand binding and interactions with peptides and proteins. This knowledge is applied to investigate whether the abrogation of protein interactions with the coactivator groove may serve as an alternative target to inhibit AR transcriptional activity.

Chapter 1 gives an overview of the current knowledge on AR structure and function. The three functional domains of the AR (the N-terminal domain (NTD), the DNA-binding domain (DBD), and the LBD) are introduced and their role in AR-regulated transcription are described. The binding of ligand in the ligand-binding pocket and the interaction of FxxLF-like motifs with the AR coactivator groove are described in more detail.

Mutations in the AR LBD provide a mechanism for prostate tumours to escape from androgen ablation therapies. Mutant ARs may be activated by non-androgenic ligands and/or anti-androgens. In **Chapter 2** the mechanism by which the ligand-binding pocket mutant AR L701H is activated by the endogenous hormone cortisol is described. Systematic structure-function analysis of AR amino acid residue 701 shows that in addition to L701H, also L701Q can be activated by cortisol. Another AR mutant, L701M, is strongly activated by progesterone. Using a panel of structurally related steroids it is shown that the hydroxyl group at position 17α is essential for the cortisol response of AR L701H and AR L701Q. Molecular 3D-modelling indicates that a hydrogen-bond network involving the steroidal 17α-hydroxyl group, H701, and the backbone of S778, which is conserved in L701Q, underlies the cortisol response of the AR L701H mutant.

In **Chapter 3** the identification of two novel AR-interacting FxxLF-like motifs is described. Systematic mutational analysis of the leucine residue at position +4 (L+4) of the AR FxxLF motif shows that this residue can be substituted by phenylalanine (F) or methionine (M) without losing AR interaction capacity. Also FxxFF and FxxMF variants of the FxxLF motifs in the cofactors ARA54 and ARA70 retain strong AR LBD binding. Like the corresponding FxxLF motifs, interaction of the FxxFF and FxxMF variants of the AR and ARA54 motifs are AR specific, whereas the variants of the less AR-selective ARA70 motif increase AR specificity. It is demonstrated that the interaction of the cofactors gelsolin and PAK6 with the AR is dependent on an FxxFF and an FxxMF motif, respectively.

Chapter 4 describes the identification of BAF60a as a novel AR cofactor after *in silico* selection and functional screening of FxxLF-like motifs present in potentially AR-interacting proteins. Candidate interacting motifs were selected from a proteome-wide screening and from a supervised screening focusing on components of protein complexes involved in transcriptional regulation. The FxxFF motif present in SMARCD1/BAF60a, a subunit of the SWI/SNF chromatin remodelling complex, not only interacts with AR as peptide but also drives the interaction of the full-length protein. BAF60a depletion in LNCaP cells by siRNA shows differential effects on the expression of endogenous AR target genes. AR-driven expression of TMPRSS2 is almost completely blocked, whereas expression of other AR target genes, such as SGK and SARG, is hardly affected. This demonstrates that BAF60a plays a critical role in the expression of a subset of AR target genes.

Chapter 5 describes the feasibility to inhibit AR transcriptional activity beyond the level of hormone binding by targeting the coactivator groove. A peptide containing the FxxFF motif of gelsolin, which is the strongest AR-interacting motif, is delivered into cells either by transfection of vectors expressing the peptide or by the TAT cell-penetrating peptide. Via both approaches the gelsolin FxxFF peptide inhibits AR N/C interaction and blocks transcriptional activity of AR, but not of PR and GR. In contrast to expressed gelsolin, TAT-gelsolin FxxFF has variable effects on constitutively active promoters. These results show that the AR coactivator groove is a candidate target for blocking AR function in prostate cancer.

In **Chapter 6** the results obtained in Chapters 2-5 are discussed in more detail. The General Discussion focuses on the interaction of FxxLF-like motifs with the AR coactivator groove and addresses the question whether these interactions may function as alternative target to block androgen-dependent prostate cancer growth.

SAMENVATTING

De androgeenreceptor (AR) is een ligand-afhankelijke transcriptiefactor, die geactiveerd wordt door de androgenen testosteron en dihydrotestosteron (DHT). Androgenen en de AR zijn essentieel voor de normale ontwikkeling van de man en spelen een belangrijke rol bij het instandhouden van de functies van de mannelijke geslachtsorganen, waaronder de prostaat. Het verstoren van het functioneren van de androgeen-AR as kan leiden tot verschillende ziektes, zoals prostaatkanker. De transcriptieactiviteit van de AR wordt gereguleerd door interacterende eiwitten, de zogenaamde cofactoren. Het best beschreven gebied voor interacties met eiwitten in de AR is de coactivator groef in het ligand-bindend domein (LBD) waaraan eiwitten kunnen binden via FxxLF motieven. In dit proefschrift zijn verschillende eigenschappen van het AR LBD onderzocht, waaronder het binden van het ligand en interacties met peptiden en eiwitten. De vergaarde kennis is vervolgens gebruikt om te onderzoeken of het verbreken van eiwit-interacties met de coactivator groef kan dienen als een alternatieve target om AR transcriptieactiviteit te remmen.

Hoofdstuk 1 geeft een overzicht van de huidige kennis van de structuur en functie van de AR. De drie functionele domeinen van de AR (het N-terminale domein (NTD), het DNA-bindend domein (DBD), en het LBD) worden geintroduceerd en hun rol in de AR-gereguleerde transcriptie wordt beschreven. Het binden van het ligand en de interactie van FxxLF-achtige motieven met de coactivator groef worden gedetailleerd beschreven.

Mutaties in het AR LBD vormen een mechanisme voor prostaattumoren om zich te onttrekken aan therapieën die gericht zijn op het tegengaan van de werking van androgenen. Gemuteerde ARs kunnen geactiveerd worden door niet-androgene liganden en/of zelfs anti-androgenen. In **Hoofdstuk 2** wordt het mechanisme beschreven waardoor een AR met een mutatie in de ligand-binding pocket, AR L701H, geactiveerd kan worden door het endogene hormoon cortisol. Systematische structuur-functie analyse van aminozuur 701 in de AR toont aan dat naast L701H ook de L701Q mutant geactiveerd kan worden door cortisol. Een andere AR mutant, L701M, wordt sterk geactiveerd door progesteron. Door gebruik te maken van een set van sterk gelijkende steroïden is aangetoond dat de hydroxyl-groep op positie 17α een essentiële rol speelt bij de respons op cortisol van AR L701H en AR L701Q. Met behulp van 3D-modelleren kan de cortisol respons van AR L701H verklaard worden door de aanwezigheid van een netwerk van waterstofbruggen tussen de 17α-hydroxyl-groep van het steroïde, H701 en de "backbone" van aminozuur S778.

In **Hoofdstuk 3** wordt de identificatie van twee nieuwe AR-interacterende FxxLF-achtige motieven beschreven. Systematische analyse van leucine op positie +4 (L+4) toont aan dat dit aminozuur vervangen kan worden door een fenylalanine (F) en methionine (M) zonder dat de interactie met de AR verloren gaat. Ook de FxxFF en FxxMF varianten van de FxxLF motieven in ARA54 en ARA70 behouden hun sterke binding met het AR LBD. Net als de oorspronkelijke FxxLF motieven interacteren de FxxFF en FxxMF varianten van de AR en ARA54 motieven

specifiek met de AR. De varianten van het ARA70 motief verhogen de specificiteit voor de AR. Er wordt verder aangetoond dat de interactie van de cofactoren gelsolin en PAK6 met de AR afhankelijk is van respectievelijk een FxxFF en een FxxMF motief.

Hoofdstuk 4 beschrijft de identificatie van BAF60a als een nieuwe AR cofactor na het *in silico* selecteren en functioneel screenen van FxxLF-achtige motieven die aanwezig zijn in potentiële AR-interacterende eiwitten. Mogelijke interacterende motieven werden geselecteerd na een proteoom-brede screening en na een gerichte screening van eiwitten die een onderdeel vormen van grote complexen die betrokken zijn bij regulering van transcriptie. Het FxxFF motief in SMARCD1/BAF60a, een onderdeel van het SWI/SNF chromatine remodelling complex, vertoont niet alleen interactie met de AR als peptide, maar is ook essentieel voor de interactie van het volledige eiwit. Verlagen van BAF60a in LNCaP cellen met behulp van siRNA leidde tot differentiële effecten op de expressie van endogene AR targetgenen. De AR-gereguleerde expressie van TMPRSS2 is bijna volledig geblokkeerd, terwijl er nauwelijks effecten zijn op de expressie van andere AR targetgenen, zoals SGK en SARG. Dit toont aan dat BAF60a een cruciale rol speelt in de expressie van een gedeelte van de AR-gereguleerde genen.

Hoofdstuk 5 beschrijft de mogelijkheid om de transcriptieactiviteit van de AR te remmen door de coactivator groef als target te gebruiken. Een peptide met het FxxFF motief van gelsolin, dat het sterkst AR-interacterende motief is, werd in cellen gebracht door middel van transfectie van vectoren die het peptide tot expressie brengen of door te koppelen aan het TAT cel-penetrerende peptide. Via beide benaderingen is het gelsolin FxxFF peptide in staat om zowel AR N/C interactie als AR transcriptie-activiteit te blokkeren, maar niet die van de PR en GR. In tegenstelling tot het intracellulair geproduceerde gelsolin, vertoont TAT-gelsolin FxxFF variabele effecten op constitutief actieve promotoren. De resultaten tonen aan dat de coactivator groef in de AR een potentiële target is voor het blokkeren van de AR in prostaatkanker.

In **Hoofdstuk 6** worden de resultaten die verkregen zijn in Hoofdstukken 2 tot en met 5 in meer detail bediscussieerd. De algemene discussie richt zich vooral op de interactie van FxxLF-achtige motieven met het AR LBD en bestudeert de vraag of deze interacties als alternatieve target kunnen dienen om androgeen-afhankelijke groei van prostaatkanker te remmen.

CURRICULUM VITAE

Dennis van de Wijngaart was born on the 25th of May 1977 in Rotterdam. He attended secondary school at the Erasmiaans Gymnasium in Rotterdam, where he graduated in 1997. In the same year he started the study Biology at the University of Utrecht. His first graduation project, entitled "Coupling between release and biosynthesis of adipokinetic hormones in the corpus cardiacum in the African migratory locust, *Locusta migratoria*", was performed at the Department of Biochemical Physiology at the University of Utrecht, under the supervision of Dr. L.F. Harthoorn and Prof. D.J. van der Horst. His second graduation project, entitled "Structure-function analysis of the human androgen receptor mutated at positions 701, 877, and 880", was performed at the Department of Pathology (Josephine Nefkens Institute) at the Erasmus MC in Rotterdam, under the supervision of Dr. H.J. Dubbink and Prof. J. Trapman. Specializing in Molecular Cell Biology he graduated in Biology in November 2001. In December 2001 he started his PhD project at the Departments of Urology and Pathology (Josephine Nefkens Institute) at the Erasmus MC in Rotterdam, under the supervision of Dr. G.W. Jenster and Prof. J. Trapman. The results of this project are described in this thesis. As of March 2007, Dennis is working as a post-doc in de group of Prof. J. Trapman. In his current project he studies the mechanism of expression of Ets fusion genes in prostate cancer, focussed on the role of endogenous retroviral sequences. This project is supported by the VanderEs-stichting.

LIST OF PUBLICATIONS

Van de Wijngaart DJ, Dubbink HJ, Molier M, Jenster G, and Trapman J. Blockade of androgen receptor function by peptides targeted to the coactivator-binding groove.
Submitted for publication.

Van de Wijngaart DJ, Molier M, Lusher SJ, Hersmus R, Jenster G, Trapman J, and Dubbink HJ. Differential ligand-responsiveness of androgen receptor L701 mutants.
Submitted for publication.

Van de Wijngaart DJ, Dubbink HJ, Molier M, Trapman J, and Jenster G. Functional screening of FxxLF-like peptide motifs identifies SMARCD1/BAF60a as an androgen receptor cofactor that selectively modulates TMPRSS2 expression.
Submitted for publication.

Van Royen ME, **Van de Wijngaart DJ**, Cunha SM, Trapman J, and Houtsmuller AB. A FRET-based assay to study ligand-induced androgen receptor activation.
Manuscript in preparation.

Wong HY, Hoogerbrugge JW, Pang KL, Van Leeuwen M, Van Royen ME, Molier M, Berrevoets CA, Dooijes D, Dubbink HJ, **Van de Wijngaart DJ**, Wolffenbuttel KP, Trapman J, Kleijer WJ, Drop SLS, Grootegoed JA, and Brinkmann AO. A novel mutation F826L in the human androgen receptor results in increased NH2-/COOH-terminal domain interaction and TIF2 co-activation.
Mol Cell Endocrinol. 2008; 292(1-2):69-78.

Hermans KG, Van der Korput HA, Van Marion R, **Van de Wijngaart DJ**, Ziel-Van der Made A, Dits NF, Boormans JL, Van der Kwast TH, Van Dekken H, Bangma CH, Korsten H, Kraaij R, Jenster G, and Trapman J. Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer.
Cancer Res. 2008; 68(18):7541-7549

Van de Wijngaart DJ, Van Royen ME, Hersmus R, Pike ACW, Houtsmuller AB, Jenster G, Trapman J, and Dubbink HJ. Novel FxxFF and FxxMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain.
J Biol Chem. 2006; 281(28):19407-19416.

Harthoorn LF, Oudejans RC, Diederen JH, **Van de Wijngaart DJ**, and Van der Horst DJ. Absence of coupling between release and biosynthesis of peptide hormones in insect neuroendocrine cells.
Eur J Cell Biol. 2001; 80(7); 451-457

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