Dynamics of the Androgen Receptor in Living Cells

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Introduction

Androgens, such as testosterone or the more potent 5α-dihydrotestosterone, are essential in development and maintenance of the male phenotype. Androgens exert their actions by binding to the androgen receptor (AR), an intracellular receptor, which functions as a transcription factor to regulate expression of target genes. The human AR is a 100-110 kDa protein encoded by an eight exon gene (Brinkmann et al., 1989) located on chromosome Xq11-12 (Migeon et al., 1981; Brown et al., 1989).

1.1. The nuclear receptor family

The AR is a member of the family of nuclear receptors (NRs). This family can be divided into 3 subclasses (Wahli and Martinez, 1991; Mangelsdorf et al., 1995; McKenna and O'Malley, 2002). The first subgroup consists of the steroid receptors: AR, glucocorticoid receptor (GR), estrogen receptor (ER), progesterone receptor (PR) and mineralocorticoid receptor (MR). After hormone binding these receptors bind as homodimers to response elements in promoter regions of their target genes. The second subclass consists of receptors that form heterodimers and may also display binding to response elements in the absence of ligand. Those receptors include thyroid hormone receptor (TR), retinoic acid receptor (RAR), retinoic X receptor (RXR) and vitamin D receptor (VDR). The third subclass of NRs is formed by the orphan receptors for which no natural ligands have yet been identified or function without ligand.

1.2. Modular structure of the androgen receptor

The steroid receptors (SRs) all have a similar modular structure, consisting of an amino-terminal domain (NTD), a DNA-binding domain (DBD) and a carboxyl-terminal ligand-binding domain (LBD), which binds the hormone. The

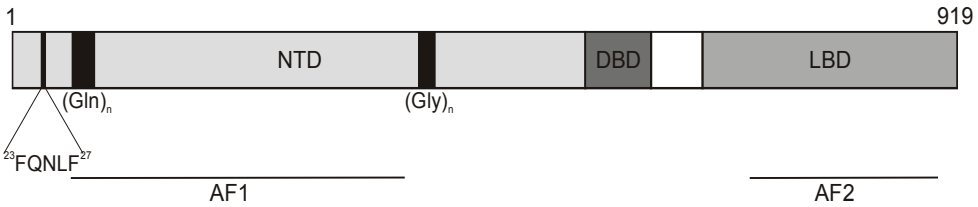

Figure 1.1. The domain structure of the androgen receptor
NTD: amino-terminal domain, DBD: DNA-binding domain, LBD: ligand binding domain, AF: Activation function. See text for details.

domain structure of the AR is depicted in Fig.1.1. The modular structure is also reflected in the genomic organization of the AR gene. The NTD, which has a highly variable structure, is encoded by a single exon. The DBD in the middle of the protein is highly conserved and forms two zinc clusters, which can bind to specific DNA-sequences and is encoded by two small exons (2 and 3). At the C-terminal side the DBD is linked via a flexible hinge region to the LBD. The genomic information for the LBD, which binds androgens that activate the receptor, is encoded by exons 4-8 (Brinkmann et al., 1989).

1.2.1. Amino-terminal domain

The NTD of NRs is highly variable and flexible and contains an interaction domain, which has a transactivation function (AF-1). The AR-NTD contains a highly conserved region (aa. 234-247 in the human AR), not present in other NRs, with the exception of a partial sequence in the GR of human and Xenopus (Betney and McEwan, 2003; He et al., 2004a; Han et al., 2005) which forms a binding interface for several coactivators and general transcription factors (McEwan, 2004). It has been suggested that interactions with components of the general transcription machinery with this region induce a more structured conformation of the NTD (McEwan, 2004), which in turn would allow binding of other cofactors. Mutations in this region (A229T and E231G) in mouse AR (corresponding to A234T and E236G, respectively in human AR) have been associated with higher transcriptional activity in absence of hormone. Moreover the E231G mutation showed increased responsiveness to coactivators ARA70 and ARA160 (Han et al., 2001). The carboxyl-terminus of the hsp70-interacting protein (CHIP) was found to interact with the conserved region in the AR-NTD and to inhibit AR transcriptional activation (He et al., 2004a). Furthermore, the AR-NTD can interact in a ligand-dependent manner with the AR-LBD (Langley et al., 1995; Doesburg et al., 1997) via an LXXLL-like (L: leucine, X: any amino acid) motif ^{23}FQNLF^{27} (Berrevoets et al., 1998; He et al., 2000). LXXLL-motifs are present in most NR coactivators and interact with activation function-2 (AF-2) of agonist liganded NRs (Heery et al., 1997). Correspondingly, the ^{23}FQNLF^{27}-motif (He et al., 2000) can interact in a ligand dependent way with the cofactor binding groove in the AR-LBD, although the physiological role of this interaction is still unclear. The FXXLF-motif mediated interaction with the AR-LBD is highly specific for AR (Steketee et al., 2002; Dubbink et al., 2004). Similar FXXLF-motifs are present in the specific AR coactivators ARA54, 55 and 70 (He et al., 2002) and the repressor Rad9

(Wang et al., 2004). Furthermore, the AR coding sequence for the NTD contains two polymorphic repeats $(CAG)_n$, which codes for a glutamine (Gln or Q) repeat of on average 20 copies, and a $(GGC)_n$ repeat, which codes for a glycine (Gly or G) repeat of on average 16 copies. Extremely short or long length of the repeats has been associated with diseases such as prostate cancer and Kennedy's disease, respectively (see 1.3).

1.2.2. DNA-binding domain

The AR DBD contains two zinc-fingers that can bind to sequences in promoter/enhancer regions of androgen-regulated genes. The AR recognizes consensus sequences consisting of inverted hexamer repeats of AGAACA with a spacing of three nucleotides (IR3 repeats, 5'-**AGAACA**nnn**TGTTCT**-3'**)** (Roche et al., 1992). This consensus DNA-binding site is similar for GR, MR and PR, although *in vivo* the receptors have completely different biological functions. Sequences more related to direct repeats with a three nucleotide spacer (5'-**AGAACA**nnn**AGAACA**-3'**)**, ADR3 repeats, as present in the specific androgen regulated gene probasin (Claessens et al., 1996), determine specificity for the AR (Claessens et al., 2001). On these sequences, AR dimers were expected to bind in a head-to-tail configuration similar to the situation observed with the VDR (Shaffer and Gewirth, 2002). However, crystal structures revealed that AR dimers were bound in a head-to-head configuration

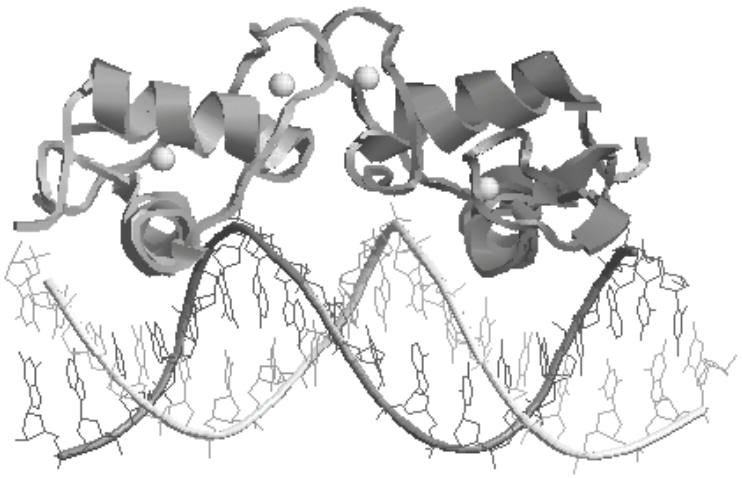

Figure 1.2. Model of the crystal structure of the AR-DBD bound to DNA as published by Shaffer et al. (Shaffer et al., 2004).

(Fig. 1.2) as expected for an inverted repeat configuration (Shaffer et al., 2004). This configuration is similar to the one observed with crystal structures of ER (Schwabe et al., 1993) and GR (Luisi et al., 1991) on IR3 repeats. Therefore, although it is energetically less favorable AR binds to the ADR3 in an antiparallel orientation, with one AR binding to a high-affinity half-site, whereas the other AR in the dimer binds to a lower-affinity half-site, implying that DNA target recognition by the AR is strongly determined by the dimerization behavior (Shaffer et al., 2004). This indicates that dimer interactions strongly influence specificity of binding to response elements.

1.2.3. Ligand-binding domain

A flexible hinge region that contains highly positively charged amino acids with bipartite nucleoplasmin-like nuclear localization signal (NLS) motifs links the DBD to the LBD (Jenster et al., 1993). The structure of the AR-LBD, like that of other NRs consists of 10-12 helices. AR contains no helix 2, whereas helices 4/5 and helices 10/11 are contiguous (Matias et al., 2000; Sack et al., 2001). In the absence of ligand helix 12 extends out of the LBD structure. When the hormone binds in the ligand-binding pocket, helix 12 functions as a lid and thereby fixes the hormone in the ligand-binding pocket. This also results in the formation of a hydrophobic coactivator-binding groove on the LBD-surface

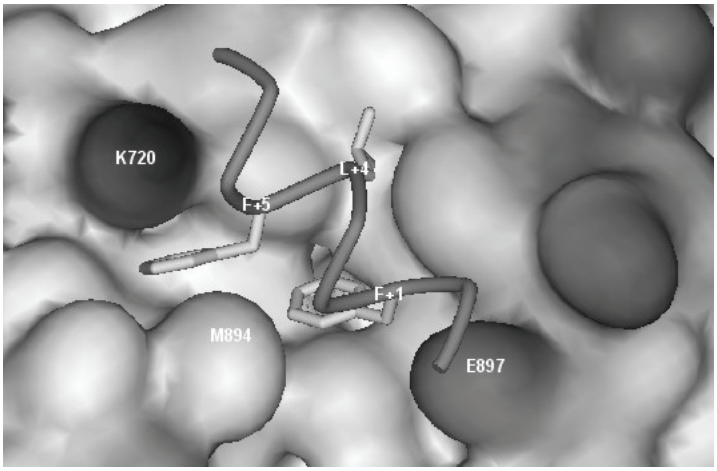

Figure 1.3. Representation of the binding of the FXXLF motif in the AR-NTD to the surface of the coactivator-binding groove in the AR-LBD in presence of R1881 as published by He et al. (He et al., 2004b). Charged amino acids, mainly the lysine on position 720 and glutamic acid on position 897 of the AR, form a charged clamp. Phenylalanines at positions 1 and 5 of the peptide fit into a hydrophobic pocket bordered by the positively and negatively charged amino acids (Dubbink et al., 2004; Hur et al., 2004; Estébanez-Perpiñá et al., 2005).

bordered by positively and negatively charged amino acids. This groove functions as an interaction interface for LXXLL- and FXXLF-motif containing cofactors (Fig. 1.3 and Dubbink et al., 2004; He et al., 2004b; Hur et al., 2004; Estébanez-Perpiñá et al., 2005) as well as the FXXLF motif in the AR-NTD (see above). This hydrophobic interaction interface forms the basis for the ligand dependent activation function 2 (AF-2) (Danielian et al., 1992; Berrevoets et al., 1998).

1.3. Diseases associated with androgen receptor dysfunction

Besides the role in development of the male phenotype, the AR has also been shown to be involved in disease. The three major diseases in which the AR is involved are: Androgen insensitivity syndrome (AIS) (1.3.1), spinal bulbar muscular atrophy (SBMA) or Kennedy's disease (1.3.2) and prostate cancer (1.3.3).

1.3.1. Androgen insensitivity syndrome

Androgen insensitivity syndrome (AIS) is a rare hereditary disease caused by inactivating mutations in the AR, resulting in inability of the receptor to bind its ligand or to bind to DNA (Griffin et al., 1982). Alternatively, it can be caused by defects in coactivators (Adachi et al., 2000) or a defect in the enzyme 5α-reductase which converts testosterone to more potent 5α-dihydrotestosterone. Patients with complete AIS clinically display as XY-individuals with female external genitalia and breast development, a blind ending vagina, absence of uterus and female adnexa (Fallopian tubes and ovaries), abdominal or inguinal testes and absence of pubic and axial hair. Partial AIS is clinically referred to as Reifenstein syndrome and displays as a XY-individual with male pseudohermaphroditism characterized by hypospadias, hypogonadism and gynecomastia (Griffin et al., 1982).

1.3.2. Spinal bulbar muscular atrophy/Kennedy's disease

Spinal bulbar muscular atrophy (SBMA) or Kennedy's disease is characterized by adult-onset spinobulbar motor neuronopathy associated with mild AIS (Kennedy et al., 1968). It was shown to correlate with an abnormal expansion of the CAG repeats in exon 1 of the AR, resulting in increased length of the Gln-repeat (La Spada et al., 1991; Doyu et al., 1992). Cells expressing ARs with expanded Gln-repeats were less viable. Addition of testosterone increased cell

viability and resulted in formation of cytoplasmic aggregates, although this did not seem to correlate with cell survival (Simeoni et al., 2000). However, the details on the role of the AR in the disease are still unknown (Walcott and Merry, 2002).

1.3.3. Prostate cancer

Prostate cancer cells, like normal cells of the prostate are dependent on androgens for their survival. Therefore metastasized prostate cancer is treated by androgen-ablation or antiandrogen therapy. Although initially most prostate cancers respond to this therapy, in essentially all patients the cancer relapses. Most prostate cancers treated by endocrine therapy still show expression of the AR, indicating that relapse is not due to selection of AR^- cells (van der Kwast et al., 1991). Relapse of prostate cancers might be a result of overexpression of the AR or coactivators, mutation of the AR or alternative signaling pathways that bypass androgen signaling (reviewed by Culig et al., 2000; Feldman and Feldman, 2001; Trapman, 2001). Furthermore, it has been suggested that men who have an AR with a short length of the CAG repeat are prostate cancer prone (Klotz et al., 2005 and references therein). However, meta-analysis of several studies revealed that shorter repeats are only modestly associated with prostate cancer risk and the absolute difference in number of repeats between cases and controls was less than one repeat (Zeegers et al., 2004).

Most mutations identified in prostate cancer patients (see http://www.mcgill.ca/androgendb) reside in the AR-LBD (Linja and Visakorpi, 2004; Taplin and Balk, 2004), most likely due to selection by the therapeutic regime. For most mutations in the AR-LBD the effect or pathogenicity has not been proven (Gottlieb et al., 2004), the mutations which have been demonstrated to result in altered ligand specificity are shown in Table 1.1.

Mutations in the AR might explain some of the prostate cancer recurrences seen after anti-androgen therapy. However mutations are not very frequent in prostate cancers (Ruizeveld de Winter et al., 1994; Culig et al., 2000). In endocrine therapy resistant prostate cancer expression of AR may be increased (Visakorpi et al., 1995; Koivisto et al., 1997; Linja et al., 2001). In fact, an increase in AR mRNA expression, although modest, was the only factor consistently associated with the development of endocrine therapy resistance (Chen et al., 2004). In addition, the expression of coactivators, such as SRC-1 or TIF2, may be increased, allowing the AR to help in survival of cancer cells at low remaining levels of (dihydro)testosterone, or adrenal androgens (Gregory et

Table 1.1. Mutations in the AR-LBD found in prostate cancers that result in broadened ligand specificity

Mutation	Activated by[1]	Reference[2]
L701H	Glucocorticoids	Zhao et al., 2000
V715M	Adrenal steroids, DHT metabolites, progesterone and hydroxyflutamide	Culig et al., 1993
R726L	Estradiol	Elo et al., 1995
V730M	DHT metabolites, hydroxyflutamide	Peterziel et al., 1995
W741C	Bicalutamide	Hara et al., 2003
H874Y	Estradiol, DHEA, progesterone and hydroxyflutamide	Tan et al., 1997
T877A	Estradiol, progesterone, hydroxyflutamide, nilutamide	Veldscholte et al., 1990
T877S	Estradiol, progesterone, hydroxyflutamide	Taplin et al., 1995

[1] All these mutants, except L701H, are also activated by androgens

[2] Reference where altered ligand specificity was first described

al., 2001). Finally, there might be alternative ways of activation and/or stabilization of the AR: the HER2/ERBB3 signaling pathway has been shown to stabilize AR protein (Mellinghoff et al., 2004), whereas the MAPK pathway has been shown to be able to activate AR transcription (Culig et al., 1994). Several other signaling pathways have been shown to influence AR function. However the clinical relevance of these findings remains to be determined.

Chapter

2

Regulation of transcription activation by nuclear receptors

2.1. Androgen action in the cytoplasm

In cell cultures depleted of hormones, ARs are mainly localized in the cytoplasm (Jenster et al., 1993; Georget et al., 1997). This provides a primary step in regulation of androgen-regulated gene expression. The androgen testosterone can enter the cell by passive diffusion due to its lipophilic nature (Fig. 2.1, step 1). In prostate cells 5α-reductase converts testosterone into the more potent androgen 5α-dihydrotestosterone. Androgens then bind chaperone associated AR complexes in the cytoplasm of a target cell (Fig. 2.1, step 2).

Chaperone-associated SR complexes assemble in a stepwise fashion in a mature SR complex that can bind hormones with high affinity (reviewed in Pratt and Toft, 1997; Pratt and Toft, 2003). Functionally mature SR complexes consist of a dimer of heat shock protein (hsp) 90, p23 and an immunophilin (*e.g.* FKBP52) (Pratt and Toft, 1997). Binding of hormone to the receptor results in import of receptors to the nucleus. Previously it was thought that molecular chaperones upon hormone binding immediately release from the complex. However, more recently it has been reported that chaperones are also required for translocation of SRs from the cytoplasm to the nucleus. In this process the cytoskeleton might be involved (Galigniana et al., 1998; Silverstein et al., 1999; Ozanne et al., 2000; Whitaker et al., 2004). Inhibition of hsp90 activity by geldanamycin has been shown to inhibit nuclear import of AR (Georget et al., 2002) and GR (Galigniana et al., 1998). However, geldanamycin no longer inhibited GR nuclear import after disruption of the cytoskeleton. Therefore, it was proposed that GR under physiological conditions requires molecular chaperones to overcome the limited diffusion due to the cytoskeletal network (Galigniana et al., 1998). Cytoplasmic dynein, a component of microtubules (Walker and Sheetz, 1993), was co-immunoabsorbed with GR and its co-chaperone FKBP52 (Silverstein et al., 1999), which can bind to GR directly (Silverstein et al., 1999) as well as via hsp90 (Young et al., 1998).

Recently, GR retrograde movement from cytoplasm to the nucleus was shown to be dynein dependent (Harrell et al., 2004). Furthermore, the AR was shown to interact with the actin-binding protein filamin and AR remained cytoplasmic in filamin deficient cells. Reintroduction of filamin restored nuclear translocation after androgen induction (Ozanne et al., 2000). After translocation to the nucleus SRs lose the associated chaperone proteins (Fig. 2.1, step 3) and bind as dimers via their DBD to response elements in promoters of target genes (Fig. 2.1, step 4).

2.2. Gene activation by steroid receptors

In the nucleus SRs bind as homodimers to cognate response elements in promoter and enhancer regions of their target genes. In addition to the SR other gene specific coregulators are required to activate transcription of target genes. In eukaryotes DNA is packed into chromatin, which prevents some sequence specific transcription factors and the basal RNA-polymerase II (RNAP2) transcription machinery from accessing promoters, thereby inhibiting transcription of genes.

The fundamental component of chromatin is the nucleosome core particle, which consists of 146 bp of DNA wound in two superhelical turns around a histone octamer consisting of 2 copies of four core histones (H2A, H2B, H3 and H4) (Kornberg and Lorch, 1999). The central turn of DNA is induced by a tetramer of $(H3)_2(H4)_2$, whereas two H2A-H2B dimers lie on both sides of this tetramer, each associated with half a turn of DNA (Klug et al., 1980; Arents et al., 1991; Kornberg and Lorch, 1999). For other gene specific transcription factors to be able to access their binding sites on DNA, SRs are thought to recruit factors that modify the chromatin structure and make the binding sites accessible (Fig. 2.1, step 5). This can be performed by sliding or displacement of nucleosomes by ATP-dependent chromatin remodeling factors (Fig. 2.1, step 5a) or loosening of interactions of DNA with the nucleosomal structure by modification of histone tails (Fig.2.1, step 5b) (Hebbar and Archer, 2003).

In the following part, the different types of chromatin remodeling complexes (2.2.1) and histone modifying factors (2.2.2) will be introduced. Subsequently the combinatorial action of the factors will be discussed in Chapter 3, with a focus on transcription activation in yeast (3.1), transcription activation by NRs *in vitro* (3.2) and an extensive focus on transcription activation by NRs in mammalian cells (3.3).

2.2.1. ATP dependent chromatin remodeling

ATP-dependent chromatin remodeling complexes can dislocate or displace nucleosomes and thereby facilitate access for other transcription factors (Fig.2.1, step 5a). The ATP-dependent chromatin remodeling complexes involved in activation of transcription can be divided into three subclasses based on their core ATPases Swi2/Snf2 (2.2.1.1), ISWI (2.2.1.2) and Mi-2/NuRD (2.2.1.3) (Vignali et al., 2000; Becker and Hörz, 2002; Aoyagi et al., 2005). Those three enzymes are engines of the remodeling complexes, since they possess *in vitro* remodeling activity in the absence of associated subunits. The

remodeling activity can be quantitatively and qualitatively modulated by interacting subunits. The subunits may be involved in regulation or targeting of remodeling activity, or the integration of nucleosome remodeling into a physiological context (Becker and Hörz, 2002).

Most experiments involving ATP-dependent chromatin remodeling and SRs have been performed using the mouse mammary tumor virus (MMTV)-promoter, which can be activated by liganded AR, GR, PR and MR. Apart from binding sites for these SRs, the MMTV proximal promoter contains binding sites for nuclear factor (NF1), octamer transcription factors (OTFs) and TATA-binding protein (TBP) (Richard-Foy and Hager, 1987; Hebbar and Archer, 2003). In addition to binding of a SR, binding of these factors is required for maximal transcription activation from the MMTV-promoter. In absence of hormone, access of NF1 to its binding sites in MMTV-templates, which have stably integrated into cells and have assembled into chromatin, is blocked (Archer et al., 1992). In contrast NF1 is constitutively bound on "naked" MMTV-DNA, lacking nucleosomal structures. Therefore MMTV-promoter activation occurs via a bimodal mechanism: First, chromatin modifiers in collaboration with SRs modify chromatin structure. In the second step SRs and NF1 bind, and cooperate in activation of transcription. In absence of ATP GR was shown to bind to multiple sites on an MMTV-long terminal repeat (LTR) DNA polynucleosomal array and prevented access of restriction enzymes to restriction sites (Fletcher et al., 2000). When ATP was added in the presence of HeLa nuclear extract or purified SWI/SNF components, GR induced nucleosome remodeling, most likely due to SWI/SNF ATP-dependent chromatin remodeling, and GR was simultaneously lost from the template as indicated by an increase of restriction enzyme access to GR binding sites (Fletcher et al., 2000; Fletcher et al., 2002).

2.2.1.1. SWI/SNF

The Swi/Snf family of chromatin remodelers was first discovered in yeast genetic screens for factors responsible for mating type switch (swi) or sucrose non-fermenting (snf) mutations. Biochemical characterization of these factors led to the identification of a SWI/SNF complex consisting of at least 10 subunits (Côté et al., 1994; Vignali et al., 2000), which was able to increase the accessibility of nucleosomal DNA in an ATP dependent fashion.

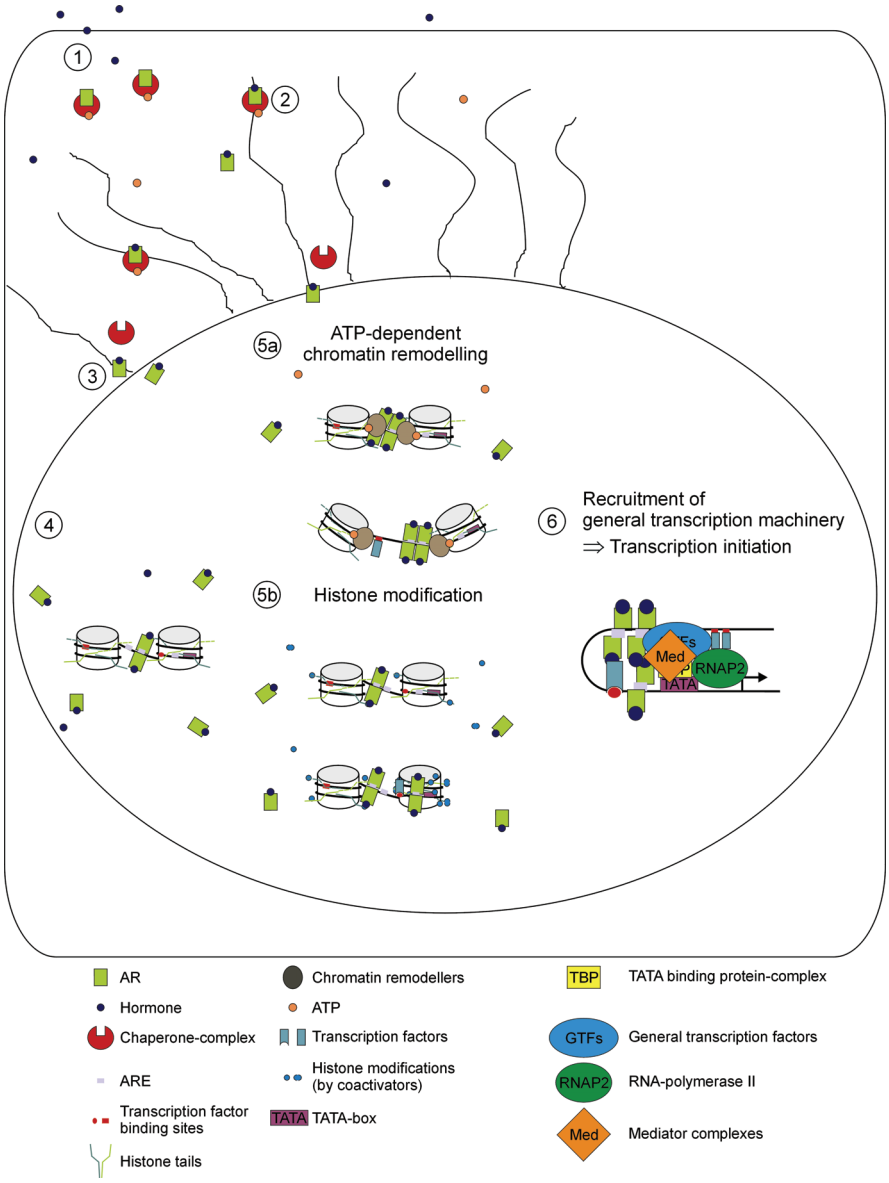

Figure 2.1. Mechanism of transcription activation by the androgen receptor
(1) Androgens enter the target cell by passive diffusion through the cell membrane. (2) In the cytoplasm androgens bind to ARs, which are in complex with chaperones. (3) Liganded ARs are retrogradely transported to the nucleus via interactions of co-chaperones with the cytoskeleton. (4) In the nucleus ARs bind as dimers to androgen response elements (AREs) in promoter/enhancer regions of androgen regulated genes. (5) Chromatin structure of androgen-regulated genes is altered by ATP-dependent chromatin remodeling (a) and histone modifications (b). These alterations of chromatin structure facilitate the access of others transcription factors. (6) Eventually a transcription initiation complex is formed which includes ordered recruitment of general transcription factors (GTFs) and RNA-polymerase II and involves actions of mediator complexes.

The ATPase Swi2/Snf2 was found to be the motor of this nucleosome-remodeling complex. Although the proteins in the SWI/SNF complex were originally discovered and characterized as transcriptional activators, they seem to be involved in repression of about the same number of genes (Holstege et al., 1998; Sudarsanam and Winston, 2000).

A distinct complex with the capacity to remodel the structure of chromatin (RSC) was isolated from *S. Cerevisiae* on the basis of homology to the SWI/SNF complex. RSC is at least 10 fold more abundant than SWI/SNF complexes and essential for mitotic growth (Cairns et al., 1996).

Like in yeast, in human cells at least two SWI/SNF related complexes can be distinguished, the brahma related gene 1 (BRG1) (also named BAF or hSwi/Snf-A)- complex and human brahma (hBRM) (also named PBAF or hSwi/Snf-B)-complex. The other proteins found in these complexes have been termed hBRM- and BRG-associated factors (BAFs). Identification of BAF250, which is related to Swi1, in BRG1 but not hBRM complexes suggested that the BRG1 complex is closer related to the SWI/SNF complex and complex B is more related to RSC (Nie et al., 2000). hBRM and BRG1 have been shown to coactivate transcription by the GR (Muchardt and Yaniv, 1993) and ERα (Ichinose et al., 1997). The core promoters of androgen regulated genes prostate specific antigen (PSA) and probasin were demonstrated to require hBRM activity for their transcriptional activity. Addition of PSA-enhancer (Cleutjens et al., 1997) to the core PSA-promoter bypassed SWI/SNF-requirement, but did not relief SWI/SNF requirement of the core probasin-promoter (Marshall et al., 2003). BAF57 can directly bind to the AR and is recruited to endogenous AR targets upon ligand activation and increases AR transcriptional activity via activation of hBRM (Link et al., 2005). Moreover, BAF57 and hBRM were shown to be required for the proliferation of AR-dependent prostatic adenocarcinoma cells. AR interacting protein (ARIP4) coactivates AR transcription on androgen regulated promoters and was shown to be a member of the SWI/SNF family (Rouleau et al., 2002).

2.2.1.2. ISWI

The ATPase imitation switch (ISWI) was first identified in *D. Melanogaster* because of its similarity to ATPase domain of brahma. The ISWI protein is 70% identical to the human hSNFL2 protein (Elfring et al., 1994). ISWI is a core component of NURF (Tsukiyama et al., 1995), CHRAC (Varga-Weisz et al., 1997) and ACF (Ito et al., 1997) complexes. The isolated ATPase ISWI is able

to remodel and rearrange nucleosomes and assemble chromatin (Corona et al., 1999). ISWI complexes induce sliding of nucleosomes by movement of intact histone octamers to neighboring DNA segments (Längst et al., 1999) rather than by disassembly and reassembly of the histone octamer on a different site as was observed for the RSC-complex (Lorch et al., 1999). Synergistic transcription activation by PR and NF1 of an MMTV promoter assembled in minichromosomes was observed in the absence of SWI/SNF and was enhanced by recombinant ISWI, suggesting ISWI induce a chromatin remodeling event that facilitates NF1 binding (Di Croce et al., 1999).

2.2.1.3. Mi-2/NuRD complex

Mi-2 was first identified as a dermatomyositis-specific autoantigen (Seelig et al., 1995) and was later shown to reside in the so-called nucleosome remodeling and deacetylation complex (NuRD, NURD or NRD) complex (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998b). Mi-2 is a member of the CHD-protein family, like all other CHD-proteins it contains a pair of chromodomains (CHD), in addition Mi-2α (CHD3) and Mi-2β (CHD4) contain two plant homeodomain (PHD) zinc clusters (Woodage et al., 1997). Similar to ISWI complexes, the NuRD complex ATPase activity is only stimulated by nucleosomes (Guschin et al., 2000). However, in contrast to ISWI and SWI/SNF complexes the NuRD complex does not require histone tails for interactions with nucleosome core particles (Brehm et al., 2000). In addition to the ATP dependent nucleosomal remodeling activity the NuRD complex contains histone deacetylase activity (Knoepfler and Eisenman, 1999; Guschin et al., 2000) (see also 2.2.2.2).

2.2.2. Histone modification

In addition to ATP-dependent chromatin remodeling, histones can also be modified by acetylation, methylation, phosphorylation (Loury and Sassone-Corsi, 2003), ubiquitination (Sun and Allis, 2002) and ADP-ribosylation (de Murcia et al., 1988) (Fig.2.1, step 5b). Already in the 1960s it was proposed that modification of histones by acetylation and methylation was associated with increased transcriptional activity (Allfrey et al., 1964). Recently it has been suggested that a certain pattern of histone modifications, referred to as the "histone code" (Strahl and Allis, 2000), is recognized by certain proteins, including transcription factors and coactivators. For example, heterochromatin protein 1 (HP1) specifically binds to methylated lysine 9 (K9) in histone 3

created by histone methyltransferases (HMTs) and thereby induces gene silencing by changing supra-nucleosomal chromatin structure (Bannister et al., 2001; Lachner et al., 2001).

2.2.2.1. Acetylation

Acetylation of histones occurs on the amino terminal tails of histones at lysine residues. Acetylation of lysines neutralizes the positive charge of the histone tails and thereby decreases their affinity for DNA (Hong et al., 1993). This results in altered conformation of nucleosomes (Norton et al., 1989), making the binding sites for transcriptional regulatory proteins more accessible (Lee et al., 1993; Vettese-Dadey et al., 1996). A large number of histone acetyl transferases (HATs) and deacetylases (HDACs) have been identified. Several subgroups of histone acetylases can be distinguished (Struhl, 1998; Glass and Rosenfeld, 2000): (1) Gcn5 (Brownell et al., 1996) (2) $TAF_{II}130/250$ (Mizzen et al., 1996) (3) p160 coactivators (Leo and Chen, 2000; Xu and Li, 2003) (4) p300/CBP (Ogryzko et al., 1996) and (5) p300/CBP-associated factor (P/CAF) (Yang et al., 1996). P160 coactivators, p300/CBP and P/CAF are only found in multicellular organisms, whereas Gcn5 and $TAF_{II}130/250$ also exist in yeast (Ogryzko et al., 1996; Struhl, 1998; Glass and Rosenfeld, 2000).

p160-coactivators

Proteins with a molecular mass of ~160 kDa were among the first factors identified, that were found to interact with AF-2 of NRs in a hormone-dependent way (Cavaillès et al., 1994; Halachmi et al., 1994). Three related p160-coactivators can be distinguished (Leo and Chen, 2000; Xu and Li, 2003): Steroid receptor coactivator (SRC)-1, -2 and -3 (or NR coactivator NCoA-1, -2 and -3), which have been assigned several names, depending on the context in which they were discovered (Table 2.1). The carboxyl-terminal regions of SRC-1 and -3, but not SRC-2, contain HAT activity (Chen et al., 1997; Spencer et al., 1997). However, this activity is much weaker than the HAT activity of p300/CBP.

All SRC members contain a highly conserved basic helix-loop-helix (bHLH) PAS domain, which mediates protein-protein interactions. However, the importance of the bHLH/PAS domain is unclear, since it was shown not to be required for SRC-1 coactivator activity in transcriptional activation studies (Oñate et al., 1995). The conserved central region of the SRC proteins contains three LXXLL (L for leucine and X for any amino acid)-motifs, referred to as NR boxes I, II and III, which interact with ligand-bound NRs (Chen et al., 1997; Heery et al., 1997; Torchia et al., 1997; Voegel et al., 1998). The LXXLL-motif forms an amphiphatic α-helix that can interact with a hydrophobic cleft, formed after binding of an agonistic ligand to the LBD of a NR (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998). SRC proteins contain two activation

Table 2.1. Alternative names of SRC (or NCoA)-complexes

	Abbreviation	Full name	Reference
SRC-1		Steroid receptor coactivator-1	Oñate et al., 1995
SRC-2	TIF2	Transcription intermediary factor 2	Voegel et al., 1996
	GRIP1*	Glucocorticoid receptor interacting protein	Hong et al., 1997
SRC-3	ACTR	Activator of thyroid and retinoic acid receptor	Chen et al., 1997
	RAC3	Receptor associated coactivator 3	Li et al., 1997
	AIB-1	Amplified in breast cancer-1	Anzick et al., 1997
	p/CIP*	p300/CBP-interacting protein	Torchia et al., 1997

* Mouse homologue

domains: AD1 and AD2. The AD1 domain contains three additional LXXLL-like motifs (NR boxes IV, V and VI), which are binding sites for p300 and CBP (Voegel et al., 1996; Li et al., 1997; Oñate et al., 1998), however the AD1 domain does not interact with NRs. Mutation of one or more NR boxes in AD1 results in impaired interaction with p300/CBP and reduced coactivator function (McInerney et al., 1998; Voegel et al., 1998). SRC-1 and -2 have been demonstrated to bind and coactivate the AF-1 domain of AR (Alen et al., 1999; Bevan et al., 1999; Ma et al., 1999). This activation occurs through AD1 rather than by interaction of NR boxes I to III. Mutation of NR boxes I, II and III did not impair transcriptional activation by full-length AR, but only influenced the relatively weak transcriptional activation of the isolated AR AF2-domain (Alen

et al., 1999; Bevan et al., 1999). At the C-terminus SRC proteins contain an AD2 domain. This domain forms an interaction surface for histone methyltransferases (HMTs), such as CARM1 and PRMT1 (Chen et al., 1999; Ma et al., 1999; Koh et al., 2001) (see 2.2.2.3). In summary, the role of p160-coactivators seems to be recruitment of other HATs and HMTs.

p300/CBP

p300 and cyclic AMP response element binding protein (CREB)-binding protein (CBP) are two closely related proteins. p300 was first identified as an adenovirus E1A oncoprotein-associated factor (Eckner et al., 1994), whereas CBP was discovered as a protein binding to CREB (Kwok et al., 1994). The acetyl transferase activity of p300 and CBP was initially demonstrated using histones. However, *in vitro* CPB and p300 not only acetylate free histones but also histones assembled into nucleosomal complexes, suggesting that the nucleosomes can be similarly modified *in vivo* (Ogryzko et al., 1996). p300/CBP was found to be tightly associated with RNAP2, suggesting that p300/CBP is a more general component of the RNAP2 transcription machinery (Ogryzko et al., 1996; Struhl, 1998; Glass and Rosenfeld, 2000).

In addition to acetylating histones, p300 in combination with P/CAF (see below) can acetylate the AR at lysines 632 and 633, present in a highly conserved lysine-rich motif carboxyl-terminal to the DBD (Fu et al., 2000). Similarly, CBP was shown to be able to acetylate the NR hepatocyte nuclear factor-4 (HNF-4), which increased HNF-4 DNA-binding and its affinity for CBP itself, and was required for target gene activation (Soutoglou et al., 2000). Therefore p300/CBP acetylation not only affects transcription by acetylation of histones, but also the activity of non-histone proteins, including NRs.

P/CAF

p300/CBP-associated factor (P/CAF) is structurally similar to the Gcn5 enzyme from yeast (Brownell et al., 1996), but distinct from the human Gcn5 homologue (Candau et al., 1996). Like Gcn5, P/CAF contains protein-protein interaction motifs. In addition P/CAF contains an amino-terminal extension that appears to mediate additional protein-protein interactions. P/CAF was initially identified as a factor that interacts with the C/H3 domains of p300 and CBP (Yang et al., 1996) It was shown that P/CAF can also interact with the p160-coactivators SRC-1 (Spencer et al., 1997) and ACTR (Chen et al., 1997).

2.2.2.2. Deacetylation

Histone deacetylation, the counterpart of histone acetylation is performed by histone deacetylases. Histone deacetylation is correlated with reduced transcriptional activity (Pazin and Kadonaga, 1997; Hu and Lazar, 2000). HDACs can be divided into two subclasses, class I (HDAC 1-3 and 8), which are related to yeast RPD3 and class II (HDACs 4-7), which are related to yeast HDA1 (Rundlett et al., 1996). HDACs 1 and 2 are components of two repressor complexes, the mSin3 complex and the NuRD complex. Although these two complexes contain a number of proteins in common, the mSin3 complex contains the protein mSin3A and Sin3A associated proteins (SAPs), whereas the NuRD complex contains CHD3/CHD4 and MTA1/MTA2. The NuRD-complex in addition to deacetylase activity contains nucleosome remodeling activity (see 2.2.1.3) (Ayer, 1999).

In absence of ligand thyroid and retinoid receptors bind NR corepressors like silencing mediator of retinoid and thyroid receptors (SMRT) (Chen and Evans, 1995)and NR-corepressor (N-CoR) (Horlein et al., 1995). N-CoR and SMRT have also been shown to associate with ER (Jackson et al., 1997; Smith et al., 1997; Zhang et al., 1998a; Shang et al., 2000; Liu and Bagchi, 2004) and AR (Shang et al., 2002; Berrevoets et al., 2004), which results in repression of transcription activation. N-CoR and SMRT, contain "corner"-box (CoRNR)-motifs, which are similar to LXXLL motifs present in coactivators. Sequences flanking the CoRNR-motif were shown to determine the specificity (Hu and Lazar, 1999). Earlier work indicated that N-CoR and SMRT recruit mSin3 complexes to deacetylate histones (Heinzel et al., 1997; Nagy et al., 1997), although Sin3/HDAC1-independent transcriptional repression by N-CoR via class II HDACs has also been reported (Huang et al., 2000; Liu and Bagchi, 2004). However, neither N-CoR nor SMRT were purified with the mSin3 complex (Zhang et al., 1997).

Purification of SMRT and N-CoR complexes resulted in a third 1.5-2 MDa complex with HDAC-activity, containing core proteins HDAC3 and transducin-β-like 1 (TBL1) (Guenther et al., 2000; Li et al., 2000). Moreover, recombinant HDAC3 requires binding of N-CoR or SMRT as a cofactor for its HDAC activity (Guenther et al., 2001). Sin3 and NuRD complexes showed constitutive association with chromatin, whereas liganded TR specifically recruited SMRT and N-CoR/HDAC3 complexes. Therefore two mechanisms of HDAC activity were suggested (Li et al., 2002). Furthermore, the different repressor complexes have differential histone tail specificity, mSin3 complexes were shown to

deacetylate both histones H3 and H4, whereas N-CoR/SMRT could only deacetylate histone H3 (Vermeulen et al., 2004).

2.2.2.3. Methylation

Coactivator-associated arginine (R) methyltransferase 1 (CARM1) (Chen et al., 1999) and protein arginine methyltransferase 1 (PRMT1) (Lin et al., 1996) can coactivate NR transcription in presence of p160-coativators and ligand (Chen et al., 1999; Koh et al., 2001). CARM1 and PRMT1 bind to the AD2 domain of SRC-1, -2 and -3 (Chen et al., 1999; Koh et al., 2001) and can synergistically coactivate transcription by NRs (Koh et al., 2001). Although the proteins are quite different in size they share extensive homology in the central portion of the coding region (Chen et al., 1999). CARM1 preferentially methylates histone H3, whereas PRMT1 preferentially methylates histone H4. Both proteins were shown to be able to methylate individually purified H2A, but not H2A in unfractionated histone preparations (Chen et al., 1999). Proteins containing arginines in glycine rich regions, which include the RNA-interacting proteins heterogeneous ribonuclein particle (hnRNP) A and B, RNA, fibrillarin and nucleolin, are also good substrates for PRMT1 (Najbauer et al., 1993; Lin et al., 1996). Therefore, in addition to histone methylation, methylation of non-histone proteins might also play a role in transcriptional coactivation by PRMT1 and CARM1.

2.2.3. Mediator complexes

Complexes related to the yeast mediator complex have been found to stimulate transcriptional activity of NRs in a ligand dependent way. The thyroid receptor associated proteins (TRAP)-complex was shown to enhance transcription by T_3-liganded thyroid hormone receptor (TR) (Fondell et al., 1996). Later it was shown that the human mediator-equivalent SRB/MED containing cofactor complex (SMCC) (Gu et al., 1999) was identical to TRAP (Ito et al., 1999). Other mediator (MED)-complexes (Bourbon et al., 2004) have been denoted vitamin-D-receptor interacting protein (DRIP) or activator-recruited cofactor (ARC) (Näär et al., 1999; Rachez et al., 1999). The protein MED1 (also named TRAP220/DRIP205, PPAR-binding protein (PBP) or RB18A (Drane et al., 1997; Zhu et al., 1997; Yuan et al., 1998)) was identified as the subunit that binds to the AF-2 domain of ligand activated NRs (Kang et al., 2002a) and was shown to be required for ER-mediated transcription and growth of estrogen-dependent breast cancer cells (Zhang et al., 2005).

Like p160-coactivators and p300/CBP, mediator complexes interact with AF2 of NRs via LXXLL-motif domains (Yuan et al., 1998). The mediator complex can interact with NRs and other transcriptional activators as well as with general transcription factors and RNAP2. Mediator may facilitate RNAP2 recruitment and promote formation of the preinitiation complex (PIC), whereas it may also modulate the function of RNAP2 in the PIC (reviewed in (Malik and Roeder, 2005)). Electron microscopic 3D image-reconstruction revealed that mediator makes multiple contacts with RNAP2 subunits (Davis et al., 2002). The cyclin and kinase subunits of mediator, cyclin C and cdk8, phosphorylate the CTD of RNAP2 (Liao et al., 1995). CyclinC/cdk8 can also phosphorylate cyclin H, a subunit of TFIIH, which results in repression of the ability of TFIIH to activate transcription and its RNAP2 CTD-kinase activity (Akoulitchev et al., 2000).

2.2.4. Transcription activation by RNA-polymerase II

After opening of chromatin structure by chromatin remodeling and histone modifying coactivators recruited by SRs, other transcription factors and the general transcription machinery can bind to the promoter. This eventually results in launch of RNAP2 transcription of target genes (Fig.2.1, step 6). This involves sequential ordered recruitment of general transcription factors (TFs) to the promoters of genes (Roeder, 1996). First, TFIID, which consists of TATA binding protein (TBP) and several TBP-associated factors (TAF_{II}s) bind to the TATA-box in the promoter. TFIIA stabilizes the interactions of TBP with the TATA-box and TAF_{II}s with DNA. Subsequently TFIIB binds through direct interactions with TBP and DNA. Like TFIIA, TFIIB stabilizes TBP-TATA interactions. TFIIB and TFIIA can bind simultaneously to a TBP-TATA complex. TFIIA and TFIIB show no overlapping contacts with TBP or DNA and no direct contacts with each other (Nikolov and Burley, 1997). TFIIB is involved in start-site selection by RNAP2 (Leuther et al., 1996). TFIIB recruits a pre-assembled TFIIF-RNAP2 complex through interaction with TFIIF. This destabilizes non-specific RNAP2 DNA interactions. Subsequently TFIIE binds through direct interactions with RNAP2 and potentially TFIIF and TBP. TFIIE is suggested to be associated with promoter melting. Binding of TFIIE recruits the helicase TFIIH.

TFIIH has been reported to stabilize TFIIE binding and a site important for RNAP2 C-terminal (CTD) phosphorylation by TFIIH has been mapped on the large subunit of TFIIE. TFIIH has also been suggested to be involved in

promoter melting and in addition it has been shown that cdk7 kinase in the CAK subunit of TFIIH can phosphorylate the CTD of RNAP2 (Feaver et al., 1994; Roy et al., 1994), thereby promoting the transition from initiation to elongation (Dahmus, 1996). In addition it has been shown that phosphorylation by cdk7 can phosphorylate a serine residue in AF1s of ERα (Chen et al., 2000) and RARα (Rochette-Egly et al., 1997), resulting in stimulation of ERα and RARα activity.

Chapter

3

Dynamics of transcription activation

Steroid receptors associate with multiple factors and complexes, including ATP-dependent chromatin remodeling factors, histone modifiers and general transcription factors. For example the AR has been found to interact with Swi/Snf chromatin remodeling factors (Rouleau et al., 2002; Marshall et al., 2003), histone modifiers (Bevan et al., 1999; Ma et al., 1999) as well as with mediator complexes (Wang et al., 2002) and components of the general transcription factors TFIIF (McEwan and Gustafsson, 1997; Reid et al., 2002) and TFIIH (Lee et al., 2000). Are all these components influencing transcription simultaneously or do they influence transcription in temporally different intervals, perhaps sequentially? If so are the activities of certain coactivator proteins required for others to perform their actions?

3.1. Recruitment of chromatin remodeling and modifying complexes in yeast

The recruitment and timing of recruitment of the different factors involved in transcription activation in eukaryotes has been most extensively studied in yeast. Acetylation of the *HO* gene by Gcn5 required primary ATP-dependent chromatin-remodeling action by SWI/SNF complexes (Krebs et al., 1999). Furthermore, it was shown that for methylation of lysine 4 (K4) in H3 primary ubiquitination of a lysine residue (K123) in H2B was required (Sun and Allis, 2002), indicating that complexes with different activities are necessary to dislodge nucleosomes. These observations in yeast suggest a sequential and ordered recruitment of coactivators to promoters. Whether histone acetylation occurs before nucleosome remodeling by ATP-dependent chromatin remodeling factors or the other way around depends on the gene studied (Cosma, 2002).

3.2. Dynamics of *in vitro* transcription activation by steroid receptors

Regulation of transcription by SRs in mammalian cells likely occurs in a similar spatial and temporal order as observed in yeast. Both p160-coactivator- and mediator-complexes have been shown to interact with AF-2 in the LBD of NRs, suggesting they cannot act simultaneously (Treuter et al., 1999). Moreover, HAT containing p300/CBP-SRC complexes and mediator synergistically influenced transcriptional activation from chromatin templates by ERα (Acevedo and Kraus, 2003), indicating that they do not compete but rather

cooperate in transcription activation. Accordingly, activation of transcription by AR and TR required both p300/CBP and SWI/SNF activities. Histone acetylation by p300/CBP facilitated binding of SWI/SNF and mediator complexes (Huang et al., 2003). Transcriptional activation by RAR/RXR was shown to require both ATP-dependent hSWI/SNF chromatin remodeling as well as p300/SRC-coactivators with HAT-activity. The corresponding chromatin remodeling events were shown to occur in temporally separated, but interdependent steps (Dilworth et al., 2000). Although RAR/RXR dimers could bind to their cognate (DR5) response elements, disruption of nucleosomal structure by ATP-dependent hISWI/SNF chromatin remodeling complexes was necessary to induce "tight" ligand independent binding of RAR/RXR dimers, a process which did not require histone acetylation by p300/TIF2 coactivators. As a second step in the process leading to transcriptional initiation by RAR/RXR dimers ligand-dependent recruitment/targeting of coactivators with HAT activity was observed. For this second step to be efficient, it had to be preceded by the ATP-dependent ligand-independent "tight" NR binding step (Dilworth et al., 2000). These observations suggest that action ATP-dependent chromatin remodelers is required before histone modifiers such as p300/CBP can act.

3.3. Dynamics of steroid receptors and coactivators in the nucleus of mammalian cells

Although *in vitro* studies provide important biochemical, biophysical and structural information on the working mechanism of DNA-interacting proteins such as SRs. They are inefficient artificial systems, in which the factors are usually more abundant than they would be in the living cell, moreover certain structures can only be detected in living cells.

There are a number of techniques to study the dynamics of proteins in the cell nucleus that have recently revolutionized our insight in transcription activation in living cells. First, the discovery and cloning of green fluorescent protein (GFP), which allows the study of protein dynamics and interactions in the living cell by several fluorescence-based techniques (3.3.1). One of them is fluorescence recovery after photobleaching (FRAP) (3.3.2), which can be used to study the dynamics of fluorescently labeled molecules (including proteins) in cellular compartments or exchanges with subcellular/nuclear structures in a single cell (3.3.3).

Another technique is chromatin immunoprecipitation (ChIP), which can be

used to study recruitment of factors to specific promoter/enhancer regions and represents an overall response in the total cell population (3.3.4).

3.3.1. Green fluorescent protein

The isolation of the gene coding for GFP, a protein of the jellyfish *Aeqorea Victoria* enabled biologists to study localization and dynamics of proteins in living cells by genetically linking GFP to their protein of interest (Tsien, 1998; Lippincott-Schwartz and Patterson, 2003). The crystal structure of GFP revealed that it has a barrel like-structure (Fig.3.2, (Ormö et al., 1996)), which is very favorable since it is therefore unlikely to interfere with function of most tagged proteins. However the functionality of the GFP-tagged protein always needs to be checked to ensure that GFP itself does not interfere with the function of the protein. GFP-technology enables biologists to study the localization of proteins, including SRs and their cofactors, in the living cell (Georget et al., 1999; Hager, 1999). It has to be noted however that high expression levels that are usually obtained when cells are transiently transfected might result in mislocalization of the protein. A way to circumvent this problem is to generate stably transfected cell lines, which usually results in more physiological expression levels of proteins.

Figure 3.1. Ribbon model representation of the crystal structure of *Aeqorea Victoria* green fluorescent protein (GFP).

Genetic modifications in the GFP gene have resulted in GFP proteins with more stable and brighter fluorescence, specifically the replacement of serine 65 by

threonine (S65T), which is present in the commonly used GFP-derived enhanced fluorescent proteins (Clontech, Palo Alto, CA). In addition several mutants have been made that resulted in different emission wavelengths (Palm and Wlodawer, 1999), such as cyan and yellow fluorescent protein (CFP and YFP), allowing the study of multiple labeled proteins and their potential colocalization.

Although colocalization is sometimes considered as an indirect indication that proteins are in the same complex or structure, the different color variants can also be used to study interaction of proteins directly using fluorescence energy transfer (FRET). When the two fluorescent proteins (e.g. CFP and YFP) are in very close proximity (<80 Å or 8 nm, also referred to as Förster distance (Förster, 1948)) *i.e.* when there is an interaction between the two, the donor (e.g. CFP) is excited and non-radiative fluorescence resonance energy transfer to the donor (e.g. YFP) can occur when the emission and excitation spectra of the donor and acceptor overlap. The energy transfer results in emission of light at the emission wavelength of the donor (e.g. YFP). Using FRET interaction of SRC-1 as well as MED1 with ER and RAR was demonstrated (Llopis et al., 2000). In addition to the interaction between two individual proteins, FRET can also be used to study conformational changes within proteins *i.e.* intramolecular interactions. GFP-tagged proteins cannot only be applied to study subcellular localization of proteins but can also be used to study dynamics of proteins in the living cell using FRAP or fluorescence correlation spectroscopy (FCS)(Elson et al., 1976).

3.3.2. Fluorescence recovery after photobleaching (FRAP)

If fluorescent molecules or proteins such as GFP are exposed to light they emit fluorescent light of a longer wavelength. However too much light destroys the fluorophore and the fluorescent molecule looses its fluorescent properties, a principle referred to as photobleaching. Although in general in fluorescent imaging photobleaching is considered disadvantageous, because continuous monitoring of the specimen results in loss of the fluorescent signal, this principle forms the basis of FRAP.

FRAP was first described in the 1970's, as a technique to measure lateral diffusion dynamics of membrane proteins and lipids using fluorescent probes (Axelrod et al., 1976; Elson et al., 1976). Recently, development of confocal laser scanning microscopes equipped with acousto-optic transmission filters (AOTF) and increasingly sensitive detectors has revolutionized application of

FRAP by enabling the bleaching of a small volume with high laser intensity and scanning of only a small region at low light intensities, reducing the bleaching in the rest of the sample.

In a typical FRAP experiment, fluorescence in a region (e.g. a square, circle or strip) of a subcellular compartment, such as the cell nucleus or structures therein, is (partly) bleached out by bleaching with high intensity and subsequently the fluorescence recovery in the region is monitored using low laser intensities at which ideally no bleaching occurs ((Houtsmuller and Vermeulen, 2001; Houtsmuller, 2005), Fig. 3.2). From a FRAP experiment several kinetic parameters can be derived (Fig. 3.2), such as (effective) diffusion, immobile fraction and mean residence time of individual proteins in the immobile fraction. By combining these parameters with known biological properties of the protein under investigation and the use of mutant proteins, chemical inhibitors, activating stimuli (e.g. radiation or ligand) and/or changing other experimental conditions such as temperature, dynamic properties of protein (complexes) can be deduced.

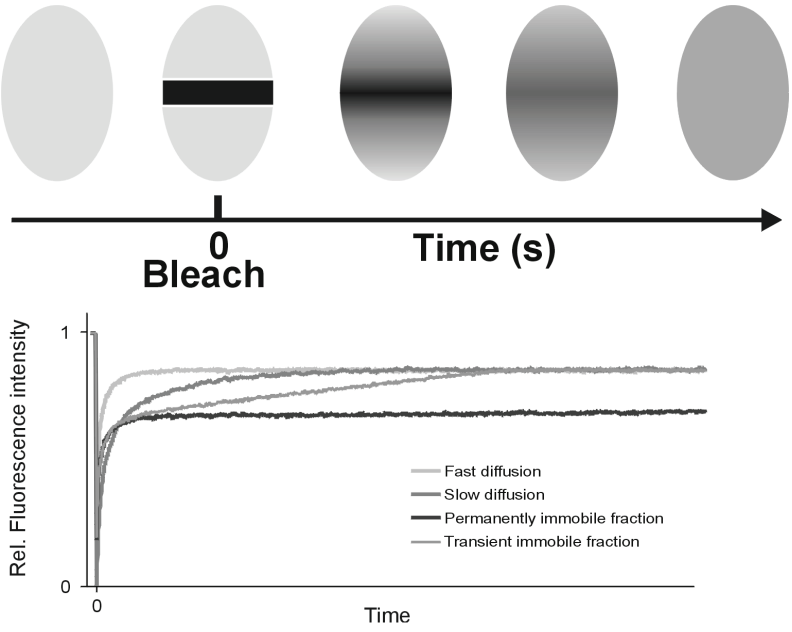

Figure 3.2. Fluorescence recovery after photobleaching
Fluorescence in an area of a subcellular structure, for example the cell nucleus is bleached with high laser intensity. If molecules/proteins are mobile, the bleached (dark) molecules will exchange with fluorescent (light) molecules from outside the bleached region. This fluorescence recovery can be monitored by low laser power at which minimal bleaching occurs. From this fluorescence recovery kinetic information can be obtained.

A variant of the FRAP technique is fluorescence loss in photobleaching (FLIP), where an area is bleached and decrease of fluorescence due to redistribution of proteins from the bleached area is measured (Houtsmuller and Vermeulen, 2001). FLIP can also be combined with FRAP, which allows easier distinction of small differences in diffusion (Hoogstraten et al., 2002; van den Boom et al., 2004). A combination of those FRAP methods can also be used to distinguish between long-term binding events and slow diffusion (see Chapters 4 and 5).

If fluorescent proteins show slower effective diffusion under a certain condition than in another condition, this slower effective diffusion is indicative of the protein being in a larger complex. Alternatively slower recovery of fluorescence can be due to interactions or restrictions with/by immobile structures. By studying proteins at different temperatures it is possible to distinguish between diffusion or binding events, since temperature change will hardly affect diffusion, but will have a large impact on biochemical reactions (Hoogstraten et al., 2002; van den Boom et al., 2004). It can also be that a fraction of the protein is immobilized due to permanent binding to/in immobile structures, which will be observed as less final recovery of fluorescence. In this case the fraction of the protein that is immobilized can be directly deduced from the curves.

3.3.3. Application of FRAP

FRAP has been used to study dynamics of exchange between the cytoplasmic and nuclear compartment of GFP-tagged proteins. One of the early GFP-based FRAP studies focused on the dynamics of proteasomes in living cells. GFP-tagging of one of its protein subunits revealed that proteasomes can freely diffuse in the cell during metaphase. However, when the cellular envelope is formed, only slow unidirectional import to the nucleus occurs (Reits et al., 1997). Within the cellular or nuclear compartment proteasomes showed rapid diffusion, which suggested that proteins that have to be degraded do not need to be transported to the proteasome and will encounter proteasomes by random collision.

3.3.3.1. Dynamics of nuclear proteins studied by FRAP

FRAP is also an ideal method to study the mobility of nuclear proteins that are involved in processes such as DNA-repair (Houtsmuller et al., 1999; Essers et

al., 2002; Hoogstraten et al., 2002; Rademakers et al., 2003), replication (Essers et al., 2002; Mattern et al., 2004) and transcription in the living cell.

FRAP experiments have revealed surprising dynamics of several cellular proteins. Houtsmuller et al. used photobleaching to study the dynamics of the DNA-repair protein excision repair cross complementation group 1/xeroderma pigmentosum (XP) group F (ERCC1/XPF) in nuclei of living cells (Houtsmuller et al., 1999). They showed that ERCC1/ERCC1 is much more dynamic than would be expected if it would be in large pre-assembled holo-complexes, suggesting that nucleotide excission repair (NER) complexes assemble on the spot on damaged lesions. This is supported by later observations that another NER-protein, XP group A (XPA) moves rapidly through the nucleoplasm with a diffusion rate different from those of other NER factors tested (Rademakers et al., 2003). TFIIH, a protein complex involved in RNAP1 and 2 transcription as NER was shown to switch between transcription and repair, with different association times. The rapid exchange of TFIIH suggested that it moves according to a model of diffusion and random collisions (Hoogstraten et al., 2002). Furthermore HP1, a protein that binds methylated lysine 9 (K9) in histone tails of H3 and is associated with heterochromateous DNA (Bannister et al., 2001; Lachner et al., 2001) was shown to dynamically exchange, suggesting that heterochromatin is not as static as previously thought (Cheutin et al., 2003).

3.3.3.2. Dynamics of steroid receptors studied by FRAP

FRAP has also been used to study the dynamics of SRs in nuclei of living cells. FRAP in nuclei of GFP (variant) tagged ERα expressing cells revealed that in absence of hormone fluorescence recovered within seconds, whereas the E2 liganded ERα showed about 5 times slower fluorescence recovery ($t_{1/2}$ of ~5s) (Stenoien et al., 2001b; Reid et al., 2003). Proteasome inhibition by MG132 resulted in almost no recovery of fluorescence after photobleaching (Stenoien et al., 2001b; Reid et al., 2003), whereas transcription inhibition by α-amanitin resulted in slower recovery with $t_{1/2}$ of ~12s (Reid et al., 2003). Similarly GR was shown to exhibit slower dynamics in presence of activating ligands, moreover the decrease in mobility was found to be related to ligand affinity (Schaaf and Cidlowski, 2003). Molecular chaperones, such as hsp90 and p23 were suggested to play a role as nuclear mobility factors of GR and PR (Elbi et al., 2004). Additionally to the mobility of SRs in the total nucleus, mobility has also been studied on artificial structures consisting of promoter repeats, where

dynamics can be tracked as a result of accumulation of receptors at the repeat. Previously SR binding to promoters of target genes was considered a static event, where once the receptor had bound the promoter it would be immobile until disassembly of the transcription initiation complex. This view has changed dramatically by the above-mentioned dynamics of liganded SRs, but especially by a report in which a cell line containing an array of ~200 MMTV-promoters was used. GFP-tagged glucocorticoid receptors accumulated at the site of integration of the MMTV-promoter repeat and using FRAP and FLIP, it was shown that glucocorticoid receptors exchange rapidly with target promoters (McNally et al., 2000). The GR and the p160-coactivator GRIP1 were shown to display similar fluorescence recovery kinetics ($t_{1/2}$ of ~5 seconds) on this promoter array (Becker et al., 2002). In contrast the large subunit (RPB1) of RNAP2 required 13 min for complete fluorescence recovery. Longer immobilization of RNAP2 was also observed with FRAP on total nuclei ($t_{1/2}$ of ~20 min) and in accompanying ^{3}H-uridine labeling of nascent transcripts ($t_{1/2}$ of ~14 min) (Kimura et al., 2002), indicating that this longer immobilization of RNAP2 is most likely not MMTV-promoter specific and occurs at all RNAP2 activated genes. Remarkably, inhibition of molecular chaperone hsp90 resulted in faster exchange of GR at the promoter repeat (Stavreva et al., 2004), in contrast with the results of experiments on GR mobility in total nuclei (Elbi et al., 2004). Dynamic exchange was also suggested by experiments in which recruitment of ATP-dependent chromatin remodelers by GR resulted in simultaneous loss of GR from GREs, indicated by increased restriction enzyme access (Fletcher et al., 2002). These observations suggested that GR, GRIP-1 and SWI/SNF are in a dynamic equilibrium with the promoter ("hit-and-run" model) and must return to the template many times during the course of RNAP2-driven transcription activation (Becker et al., 2002; Fletcher et al., 2002). Similar dynamic exchange of a yellow fluorescent protein (YFP)-SRC-1 with a cyan fluorescent protein *lac*-repressor ER chimera (CFP-LacER) bound to a *lac*-operator-array was observed in presence of E2 (Stenoien et al., 2001a).

3.3.4. Chromatin immunoprecipitation (ChIP)

ChIP is a method that can be used to study the binding of proteins to specific promoter/enhancer regions in a population of synchronized cells (Fig. 3.3) and represents an overall response in the total cell population.

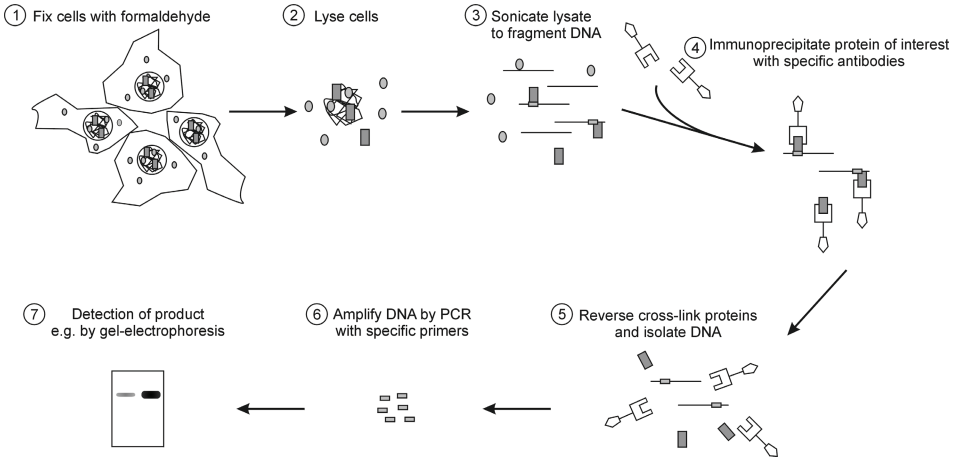

Figure 3.3 Experimental setup of a typical chromatin immunoprecipitation (ChIP) experiment
In ChIP experiments cells are fixed, usually by chemical crosslinking with formaldehyde (1). Subsequently cells are lysed (2) and the lysate is sonicated to fragment the DNA (3), followed by immunoprecipitation with specific antibodies recognizing the protein of interest (4). Thereafter, proteins and DNA are reverse crosslinked (5) and DNA sequences (e.g. promoter/enhancer regions), which were immunoprecipitated with the protein of interest, are amplified by PCR with sequence specific primers (6). Finally, the amplified DNA is either separated by electrophoresis on an agarose gel or assayed by more quantitative methods (7).

3.3.4.1. Dynamics of steroid receptors studied by ChIP

Using ChIP the binding to specific promoter regions in a population of synchronized cells can be studied. ChIP studies on the estrogen regulated Cathepsin D-promoter indicate that estradiol (E2) liganded ERα associates with the promoter in a cyclic fashion with cycles of approximately 45 minutes. In the first cycle, starting 15 minutes after addition of E2, in addition to ERα, p300, SRC-3 (AIB1), MED1 (PBP) and RNAP2 were detected on the promoter. Association of CBP and P/CAF lagged somewhat behind (Shang et al., 2000). This was in agreement with a previous observation that p300 interacts specifically with the non-phosphorylated, initiation-competent form of RNA polymerase II, whereas pCAF interacts with the elongation-competent, phosphorylated form (Cho et al., 1998). p300 was only recruited in the first cycle and not in subsequent cycles (Shang et al., 2000), consistent with *in vitro*

transcription data suggesting that while p300 plays a role in transcription initiation by ERα, it does not participate in reinitiation (Kraus and Kadonaga, 1998). These findings indicate that histone acetylation and chromatin remodeling are a step-wise process in which each cofactor exerts a distinct and non-redundant role and each of these three HAT proteins exhibit different substrate specificity, as also suggested by *in vitro* studies (Schiltz et al., 1999).

A similar cyclic pattern of gene activation was observed in ChIP experiments on the estrogen regulated pS2 gene promoter in MCF-7 cells. In absence of hormone ERα cycled on the pS2 promoter with a periodicity of 20 minutes, whereas in presence of E2 cycles where prolonged (45 minutes) (Reid et al., 2003). RNAP2 was not detected on the promoter in absence of hormone, consistent with the absence of transcriptional activity. In presence of E2 RNAP2 also cycled on pS2-promoters with a periodicity similar to ERα, however recruitment and activation lagged behind ERα association by approximately 10

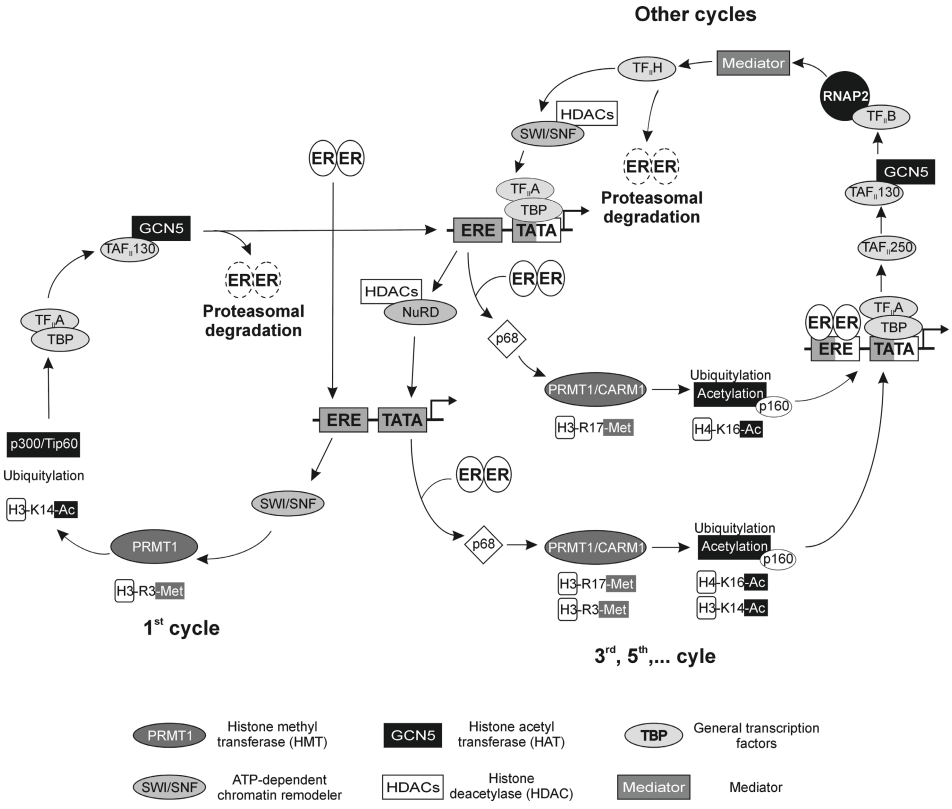

Figure 3.4 Model of association of ER and coactivator and repressor complexes
ER attracts different coactivator and repressor complexes in sequential transcriptional cycles on the estrogen regulated pS2-promoter. Adapted from (Métivier et al., 2003)

minutes. In presence of E2 and the proteasome inhibitor MG132 cycling on the promoter was considerably slower (2 hours) and RNAP2 never associated with the promoter (Reid et al., 2003). Subsequent studies revealed that ERα as well as cofactor proteins showed ordered and cyclic recruitment to the promoter (Fig. 3.4 and Métivier et al., 2003).

A mechanism of a "transcriptional clock" was suggested, in which three different types of cycles could be distinguished (Fig.3.4). In the initial transcriptionally unproductive cycle (RNAP2 is not detected on the promoter), SWI/SNF chromatin remodelers were recruited to the promoter, followed by recruitment of PRMT1 and HATs (Tip60 and p300). Next, the components of the basal transcription machinery are attracted. Following action of GCN5 and $TAF_{II}130$, ERα was targeted to the proteasome. This initial cycle was followed by a transcriptionally productive cycle, where p68 RNA helicase, which may serve as an adapter protein to associate with AF-2 coactivators (Endoh et al., 1999), was recruited first. Subsequently combinatorial sequestering of HMTs (PRMT1 and CARM1) was observed, followed by p160-coactivators and later other HATs resulting in a large complex on the promoter that presumably directed further modification of histones. The engagement of p160-coactivators prior to other HATs was also observed by others (Shang et al., 2000; Liu et al., 2001) and confirms their essential role as scaffold proteins in the construction of complexes involved in NR-mediated transactivation. Subsequently $TAF_{II}250$, $TAF_{II}130$ and mediator-complexes were recruited to the promoter. After transcription elongation, ERα was again targeted to the proteasome, and HDACs and SWI-SNF complexes remodeled the nucleosomal organization of the pS2 promoter, presumably allowing the subsequent cycles to proceed. At the end of the second productive cycle, NuRD was specifically recruited to the promoter, which might result in removal of remaining TFIIA/TBP on the TATA box of the promoter. A new initiation cycle is then needed to remodel nucleosomal structure of the pS2 promoter and to allow subsequent cycles to occur.

Some factors could functionally substitute for each other, *e.g.* P/CAF or Tip60 could functionally substitute each other in activation of the transcriptional cycle. In contrast, when TBP and TFIIA were recruited to the pS2 promoter a SWI/SNF-complex containing BRG1 was specifically recruited and BRG1 could not be substituted by hBRM in these complexes (Métivier et al., 2003). A similar specific requirement was also demonstrated for transcription activation by the AR on promoters of androgen regulated genes, although here there was a

preference for hBRM (Marshall et al., 2003).

A cyclic association pattern was also shown for the AR and the coactivators GRIP1 and CBP as well as RNAP2 on the androgen regulated PSA- and kalikrein 2 (KLK2)-promoters (Kang et al., 2002b; Kang et al., 2004). In presence of the pure antiandrogen bicalutamide, AR associated with the promoter regions and corepressor complexes, but RNAP2 and HATs were not detected on the promoter (Kang et al., 2002b; Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004). Therefore, it is likely that similar mechanisms involving cycling on promoter regions and sequential recruitment of complexes with different activities are also involved in regulation of gene expression by androgens via the AR.

Gene repression of SR regulated genes also seems to involve sequential recruitment of complexes, since ERα bound to antagonist OH-tamoxifen was shown to sequentially recruit different repressor complexes with HDAC-activity, with N-CoR-HDAC3 complexes being detected before NuRD-complexes (Liu and Bagchi, 2004). These observations are in support of a model of transcription initiation resulting from sequential and stochastic recruitment allowing combinatorial regulation of transcription (Vermeulen and Houtsmuller, 2002) by specific protein complexes from a large panel of potentially redundant factors and confirm previous observations of FRAP studies.

3.4. Outline of this thesis

Biochemical studies have revealed the AR interacts with many factors to coordinate transcription activation. However, nothing was known on the dynamics of the AR in living cells. Therefore, in this thesis we studied the dynamics of the AR tagged with GFP in living cells using confocal microscopy and FRAP.

In Chapter 4 the functionality of GFP-AR fusion constructs is shown. By introducing linkers consisting of glycine and alanine repeats, a GFP-fusion protein with minimal effects on activity was obtained. We investigated the dynamics and localization of this GFP-tagged “wild-type” AR, a non-DNA-binding mutant containing a mutation in the DBD (A573D) and an AR lacking the LBD that is constitutively active in activating transcription. Two different FRAP-methods are introduced, which in combination with computer modeling of the FRAP experiments allow to distinguish between slower diffusion or a transiently immobile fraction.

In Chapter 5 the effect of antiandrogens, bicalutamide and OH-flutamide that are clinically used to treat patients with metastasized prostate cancer were studied. Using the two different FRAP-methods and computer modeling of the FRAP-experiments we studied the working mechanism of antiandrogens. Furthermore, mutants that have been found in patients treated with bicalutamide or OH-flutamide (W741C and T877A) and were proven to confer resistance to these drugs were studied.

The molecular chaperone hsp90 has been associated with maturation of SRs, including the AR, in the cytoplasm of cells. Recently, there have been indications that hsp90 may also be involved in SR mobility in the nucleus. In Chapter 6 we investigated the effects of inhibition of molecular chaperone hsp90 on cytoplasmic and nuclear mobility of the androgen. Several experiments in which we tried to unravel the nature of the hsp90 inhibition of AR activity are described.

Import of SRs has been suggested to occur via the cytoskeleton. Therefore, in Chapter 7 we studied the involvement of the microtubule (MT) cytoskeleton in the import of ARs from the cytoplasm to the nucleus after hormone induction. To this end, GFP-tagged AR import was studied in cells with nocodazole disrupted MTs and mouse embryonic fibroblast (MEF) cells deficient for one or more proteins associated with plus ends of microtubules (+TIPs).

In Chapter 8 the findings of the studies in this thesis are put into perspective and directions for future research are suggested.

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Chapter

4

The androgen receptor ligand-binding domain stabilizes DNA binding in living cells

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4.1. Abstract

The androgen receptor (AR) is a member of the steroid receptor family, a group of transcription factors that activate steroid regulated genes. Live cell studies of several steroid receptors have shown that the mobility of the liganded receptor is strongly reduced compared to the unliganded receptor. To investigate the nature of this reduced mobility, we generated Hep3B cells stably expressing green fluorescent protein (GFP)-AR at physiological levels. Computer-aided analysis of photobleaching experiments showed that in the presence of ligand on average one out of five ARs is immobilised, each individual AR being immobile for 1 to 2 minutes. This immobilization depended on DNA binding since GFP-ARs mutated in the DNA binding domain (DBD) were not immobilised. Interestingly, a truncated AR lacking the ligand binding domain (LBD) displayed substantially shorter immobilizations, in the order of seconds, although its transcriptional activation function was stronger. Our data suggest the LBD has a role in maintaining the stability of AR-DNA complexes.

Keywords: Steroid receptors – Fluorescence recovery after photobleaching – Transcription factors – DNA-binding proteins – Cell nucleus structures

4.2. Introduction

The androgen receptor (AR) is a member of the steroid receptor family of ligand-activated nuclear receptors (Wahli and Martinez, 1991). Steroid receptors regulate the transcription of target genes by binding to hormone response elements (HREs). HRE bound steroid receptors act as 'founder' molecules that recruit transcription coregulators, including factors that have histone acetyl transferase and chromatin remodelling properties (Beato et al., 1995; McKenna and O'Malley, 2002). As a next step general transcription factors are recruited to the transcription preinitiation complex allowing RNA polymerase II (RNAP2) to initiate transcription (Beato et al., 1995; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002) .

Like all other steroid receptors, the AR has a modular structure (Brinkmann et al., 1989) consisting of a carboxyl-terminal ligand binding domain (LBD), a DNA binding domain (DBD) and an amino-terminal transactivating domain (NTD). The transactivating function and subcellular distribution of the AR is regulated by the LBD, which harbours a ligand-binding pocket to which androgens can bind. The DBD enables the AR to specifically bind to androgen response elements (AREs) in the promoters of target genes (Roche et al., 1992; Claessens et al., 2001).

The subcellular distribution of steroid receptors has been the subject of a number of microscopic investigations. In the absence of ligand, AR is part of a large multi-protein complex, including heat shock proteins, that resides predominantly in the cytoplasm (Smith and Toft, 1993). Ligand binding induces release from this complex and rapid translocation to the nucleus. Fusion proteins of the AR and green fluorescent protein (GFP) (Georget et al., 1997; Tyagi et al., 2000) were shown to translocate to the nucleus within 15-60 minutes after addition of 5α-dihydrotestosteron. Ligand activated AR (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001) was shown to accumulate in bright intranuclear foci. Similar observations were reported for the glucocorticoid receptor (GR) (van Steensel et al., 1995; Htun et al., 1996), oestrogen receptor α (ERα) (Htun et al., 1999; Stenoien et al., 2000) and mineralocorticoid receptor (MR) (Fejes-Tóth et al., 1998). Although several models have been proposed, the functional relevance of this heterogeneous distribution is as yet unclear. Interestingly, only agonistic and partial agonistic ligands were found to concentrate ARs in these potentially functional subnuclear domains (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al.,

2001). It has been proposed that foci are caused by association with an operationally defined nuclear matrix (van Steensel et al., 1995); discussed in (Pederson, 2000)]. However, in nuclei of cells treated with the antagonist bicalutamide no foci were observed whereas AR was also associated with the nuclear matrix (Tyagi et al., 2000).

Knowledge of the mode of action of steroid receptors in the living cell is still limited. The advance of GFP technology and quantitative live cell microscopy has initiated the discovery of novel principles in the mechanisms of action of steroid receptors. Recently, it was shown by fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) that GFP-tagged GRs exchange rapidly between the nucleoplasmic compartment and a mouse mammary tumour virus (MMTV) promoter array (McNally et al., 2000). The p160 coactivator glucocorticoid receptor interacting protein 1 (GRIP1) displayed similar dynamic interactions on this promoter repeat (Becker et al., 2002). GFP-tagged ERα and the p160 coactivator steroid receptor coactivator 1 (SRC-1) in the presence of oestradiol (Stenoien et al., 2001b) showed redistribution kinetics that suggest a highly dynamic interaction with immobile elements in the nucleus similar to the interaction of GRs with the MMTV promoter array. SRC-1 and CREB binding protein (CBP) were shown to rapidly exchange on a *lac* repressor ERα chimera immobilised on an array of *lac* operators (Stenoien et al., 2001a), again demonstrating that interactions between steroid receptors and coactivators are very dynamic. Also, general transcription factors of both RNAP1 and RNAP2 (TFIIB, TFIIH) were reported to interact with transcription preinitiation complexes in a highly dynamic way (Chen et al., 2002; Dundr et al., 2002; Hoogstraten et al., 2002). In contrast, RNAP 1 and 2 were associated with genes much longer (Becker et al., 2002; Kimura et al., 2002), ranging from 3 to 5 and 15 to 45 minutes respectively. In the present study we dissected the dynamics of AR in transcription regulation in the living cell. We applied photobleaching and computer aided analysis to investigate the intranuclear mobility of GFP-tagged AR and the effect of the AR DBD and LBD binding behavior.

4.3. Results

Experimental system

FRAP is a powerful tool to determine transient immobilizations and the average

duration of immobilization of nuclear proteins (Houtsmuller et al., 1999; McNally et al., 2000; Becker et al., 2002; Dundr et al., 2002; Hoogstraten et al., 2002; Kimura et al., 2002). The method is specifically applicable to transcription factors and other DNA-transacting proteins since their main activity requires binding to DNA or to protein-DNA-complexes, leading to a transient immobilization at least during the time required for their action. Here, computer-aided FRAP-analysis was combined with promoter activity assays and high-resolution imaging to study the mechanism of action and reaction kinetics of GFP-AR fusion proteins in living cells. We also studied a GFP-AR mutant carrying an alanine to aspartic acid (A573D) substitution in the DBD (Fig.4.1A). This mutation, found in a patient with complete androgen insensitivity, has been shown to completely abolish the ability of the AR to bind to AREs and is unable to activate target genes (Brüggenwirth et al., 1998). The behaviour of this mutant was compared to wild-type AR. The effect of the LBD on AR subnuclear dynamics was investigated using a truncated AR lacking the LBD and the corresponding DBD mutant.

Transactivating capacity of GFP-AR fusion proteins increases with increasing spacer length

To investigate whether GFP tagging interfered with AR function, both carboxyl-terminal and amino-terminal AR-GFP fusions as well as untagged AR were cotransfected with an androgen inducible MMTV-luciferase reporter construct in Hep3B cells, which lack endogenous AR expression, and luciferase activity was assayed. Both GFP fusion proteins showed a reduced transactivation capacity compared to the untagged AR. GFP tagged to the LBD appeared to interfere with ligand binding, since higher ligand concentrations restored transactivating capacity of the carboxyl-terminal tagged AR (unpublished data). Direct fusion of GFP to the amino-terminus of the AR interfered with its transactivation function (Fig.4.1B) as reported previously (Georget et al., 1997; Tyagi et al., 2000). Therefore, we introduced spacers of different length and composition, Gly_5Ala and $(GlyAla)_6$, between GFP and the amino-terminus of the AR. Transactivation increased with increasing spacer length to 70% of the activity of the untagged AR for the $(GlyAla)_6$ spacer (Fig.4.1B). The construct containing the $(GlyAla)_6$ linker was therefore chosen for further studies. In the remainder of this paper GFP-$(GlyAla)_6$-AR will be referred to as GFP-AR.

Deletion of the ligand binding domain (LBD) results in increased transactivation of androgen inducible promoters

As expected, the A573D mutants, which lack a functional DNA binding domain, were unable to activate transcription of the MMTV-driven luciferase reporter gene (Fig.4.1C). The LBD deletion mutant GFP-ARΔLBD showed a significantly higher transcriptional activity (~4 times) than the GFP-tagged full-length AR (Fig.4.1C). This was also observed with untagged AR (unpublished

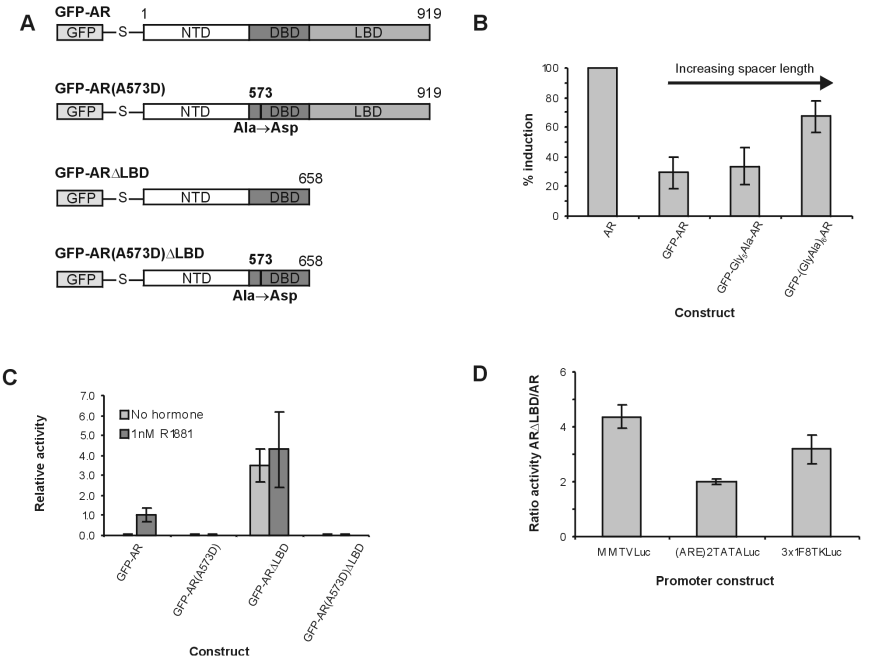


Figure 4.1.
Schematic representation of constructs and their transactivating capacity.
(A) Schematic representation of GFP-AR fusion proteins investigated in this paper. NTD: N-terminal domain; DBD: DNA binding domain; LBD: Ligand binding domain; S: spacer $(Gly\text{-}Ala)_6$. (B) Mouse mammary tumour virus (MMTV)-luciferase cotransfection assay of AR and GFP-AR constructs with different spacer lengths. Inductions are plotted relative to untagged AR. (C) MMTV-luciferase cotransfection assays of GFP-AR and the mutants depicted in A. Activity of mutants is plotted relative to GFP-AR activity in absence and presence of ligand (1nM R1881). (D) Cotransfection assays of GFP-ARΔLBD and GFP-AR with three androgen-regulated promoters controlling the luciferase gene. The ratios of transcriptional activity of GFP-ARΔLBD and GFP-AR in presence of ligand (1nM R1881) are plotted. In all graphs, mean ± 2 times the SEM of at least 3 experiments are plotted.

data), indicating that this increased transactivation function is not an artefact caused by the GFP-tag. Western blot analysis of Hep3B cells transiently transfected with GFP-AR and GFP-ARΔLBD indicated that expression levels were similar. Further quantification by flow cytometric analysis confirmed that

the increased activity observed was neither due to difference in percentage of transfected cells (26 and 22% respectively) nor to a difference in expression levels (57 and 49 units, respectively). To investigate whether the effect of deletion of the LBD was promoter specific, we also assayed a 'minimal' promoter containing two ARE binding motifs, $(ARE)_2$TATA-Luc, and 3x1F8-TKLuc, a construct containing three copies of a 80 bp PSA-enhancer fragment (3x1F8) added to the thymidine kinase promoter. Both promoters were more active in the presence of the constitutively active AR-ΔLBD than in presence of wild-type AR (Fig.4.1D).

Characterization of cell lines stably expressing GFP-tagged ARs or AR mutants

To study wild-type AR and mutant AR mobility *in vivo* at physiological expression levels, Hep3B cell lines were generated each stably expressing one of four GFP-tagged AR constructs. In the absence of hormone GFP-AR and GFP-AR(A573D) were predominantly localized in the cytoplasm, although a fraction of the receptors was already nuclear (Fig.4.2A and B, left panel).

Within 30 minutes after addition of hormone, more than 90% of GFP-AR and GFP-AR(A573D) had translocated to the nucleus (Fig.4.2A and B, right panel), indicating that neither the GFP-tag nor the DBD mutation influenced nuclear transport. GFP-ARΔLBD (Fig.4.2C) and GFP-AR(A573D)ΔLBD (Fig.4.2D) were mainly nuclear in the absence of hormone and their localization was not changed by hormone addition. Interestingly, the mean fluorescence intensities of GFP-ARΔLBD (Fig.4.2C) in all observed stable clones were approximately four times lower than stable clones containing the other constructs, suggesting that cells tolerate only low levels of this protein.

Western blot analysis of the stable clones (Fig.4.2E) showed that all expressed proteins were of the proper size. Expression levels in all stable cell lines were similar to levels in the prostate cancer cell line LNCaP, except for GFP-ARΔLBD, which was expressed at lower levels, as also deduced from the fluorescence intensity (see above). Immunoblotting with an anti-GFP antibody revealed specific bands of the appropriate sizes (~140 kD for full-length and ~110 kD for ΔLBD mutants respectively) in all GFP-AR expressing cell lines. No specific additional bands were observed, indicating that no free GFP (~30kD) was present (Fig.4.2F).

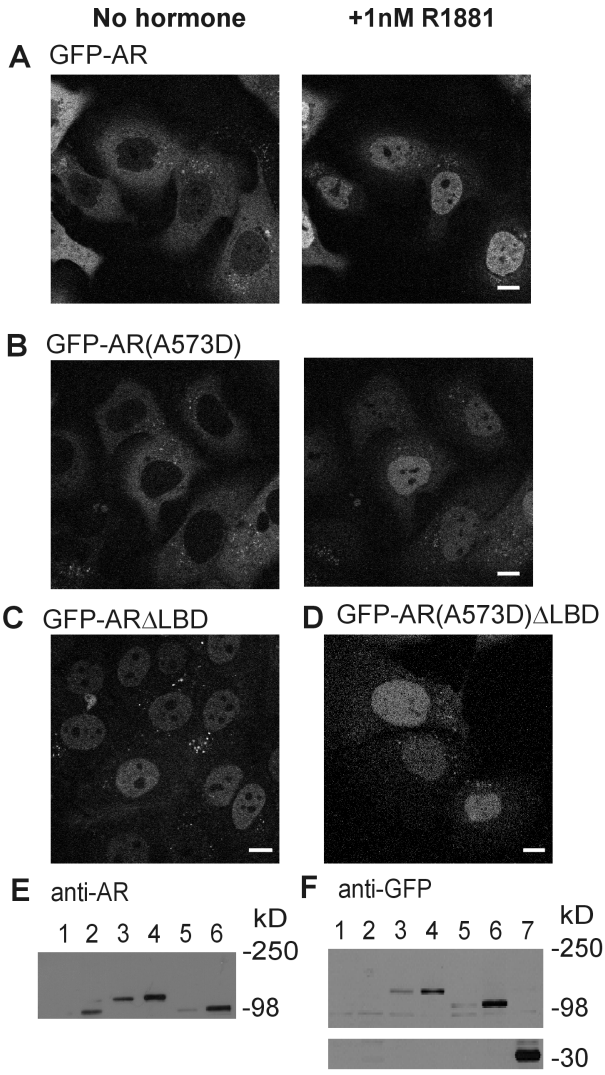


Figure 4.2. GFP-AR cell lines express physiological levels of GFP-AR protein and are functional with respect to translocation to the nucleus.
(A and B) Confocal images of Hep3B cell lines expressing stably integrated GFP-AR (A) or the DNA binding domain mutant GFP-AR(A573D) (B) showing subcellular localization before (left) and 30 minutes after changing the medium to 1 nM R1881 containing medium (right). The DBD mutation did not affect transport to the nucleus. (C and D) Subcellular localization of the deletion mutants lacking the entire LBD, GFP-ARΔLBD (C) and GFP-AR(A573D)ΔLBD (D) Localization did not change upon hormone addition. Cells in C, expressing GFP-ARΔLBD, were imaged with a five times higher laser intensity than the other cell lines. Bars represent 10 μm. (E and F) Western blots of cell lysates from cell lines Hep3B (lane 1), LNCaP (lane 2) and Hep3B cells containing stably integrated constructs GFP-AR (lane 3), GFP-AR(A573D) (lane 4), GFP-ARΔLBD (lane 5) or GFP-AR(A573D)ΔLBD (lane 6) and GFP (lane 7, in (F) only) using anti-AR (E) or anti-GFP (F).

A fraction of the AR pool is transiently immobilised in the presence of R1881

Although it is generally assumed that unliganded AR is largely absent from the nucleus, we observed in GFP-AR stable cell lines that approximately 20% of total GFP-fluorescence was nuclear in the absence of hormone (Fig.4.2A, left panel). This enabled a comparative FRAP study of nuclear AR mobility in the absence or presence of ligand R1881. First, we performed FRAP experiments, in which a narrow strip was bleached (strip FRAP) during 200 ms with an intense bleach pulse and the subsequent redistribution of fluorescence in the bleached strip was monitored for 6 seconds at 100 ms intervals. In the absence of hormone, recovery of GFP-AR fluorescence was rapid. In the presence of hormone, recovery of fluorescence, reflecting mobility characteristics, was incomplete as compared to unliganded AR (Fig.4.3A), indicating that a fraction (~20%) of the tagged ARs was immobilised. To determine the fate of the bound AR fraction we carried out FRAP experiments with extended monitoring time. A secondary redistribution of fluorescence was observed, indicating that the observed immobilization of the liganded AR was transient (Fig.4.3B).

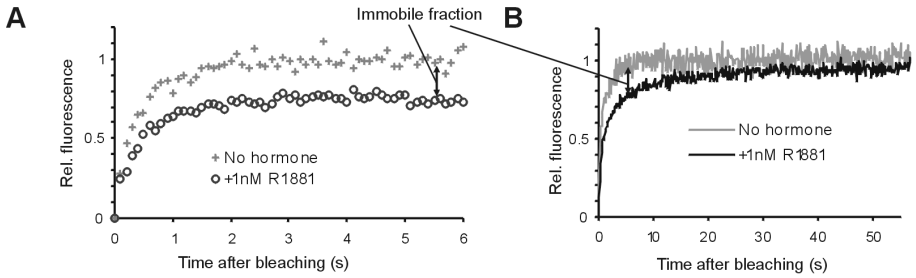
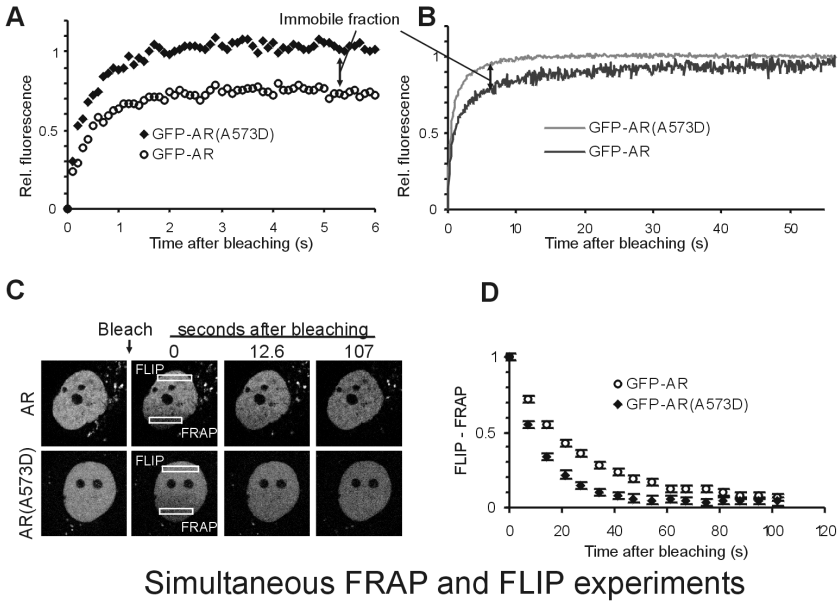


Figure 4.3. Approximately 20% of GFP-AR is immobilized in the presence of 1 nM R1881.

(A) Strip FRAP measurements of GFP-AR in the absence or presence of 1 nM R1881. Fluorescent molecules in a narrow strip were bleached for 200 ms at maximum laser power. Subsequently, fluorescence in the strip (200 nm) was monitored every 100 ms. Fluorescence intensities relative to complete redistribution in the absence of hormone were plotted against time. Mean values of at least 35 cells are plotted. (B) Long term strip FRAP measurements of GFP-AR in the absence and presence of R1881 showed a secondary recovery, indicating that the immobilization in (A) is transient. Conditions were the same as in (A), except a wider strip (1 μm) was monitored for an extended period to follow the fate of the immobile fraction.



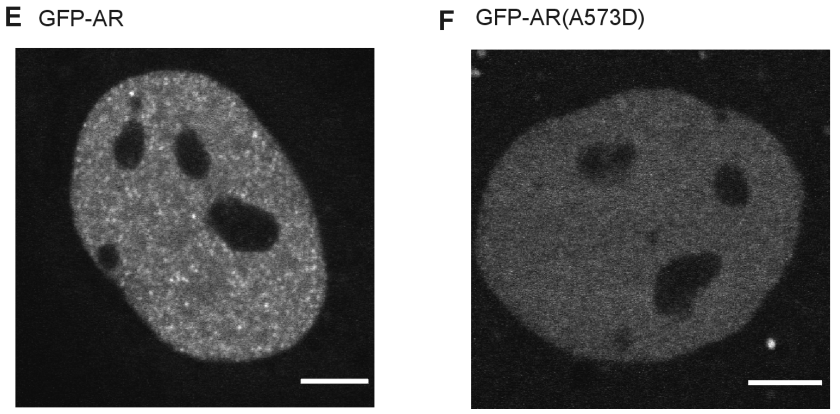


Figure 4.4 The hormone dependent immobilization and granular accumulation of GFP-AR is abolished by a mutation in the DBD that disrupts DNA binding.
(A and B) Short and long term strip FRAP experiments on Hep3B cells expressing GFP-AR and GFP-AR(A573D) in the presence of 1nM R1881. Relative fluorescence intensities of at least 50 cells are plotted. (C and D) Combined FRAP and FLIP on cells expressing GFP-AR and GFP-AR(A573D) in the presence of 1nM R1881. A strip at one pole of the nucleus was bleached (C) The difference between fluorescence signals in the bleached region (FRAP) and a distal region of the nucleus (FLIP) was determined at regular time intervals. Mean values ± two times the SEM of at least 35 cells were plotted against time (D). (E and F) Confocal images of representative nuclei of Hep3B cells stably expressing at physiological levels GFP-AR (E) and the DBD mutant GFP-AR(A573D) (F) in the presence of 1 nM R1881. The wild-type AR shows small irregular shaped foci that are abolished by the DBD mutation. Bars represent 5 μm.

Hormone-induced immobilization of the AR is completely abolished by the A573D mutation in the DNA binding domain

To investigate the nature of the observed immobilization of GFP-AR, FRAP was applied to determine the mobility of the DBD-mutant GFP-AR(A573D), which is unable to bind AREs (Brüggenwirth et al., 1998). In the absence (unpublished data) and presence of hormone, the mobility of GFP-AR(A573D) was similar to the mobility of freely diffusing wild-type GFP-AR in the absence of hormone (Figs. 4.3A and B, 4.4A and B).

To confirm the results of the strip FRAP measurements, we also applied a different bleaching procedure in which FRAP and FLIP were combined (Hoogstraten et al., 2002). In short, a 1.2 μm wide strip was bleached at one pole of the nucleus for 8 seconds at maximum laser power. Beginning 12 seconds after start of bleaching, images were taken at regular intervals (Fig.4.4C). Normalized differences in fluorescence between the bleached strip (FRAP) and a strip at the opposite pole of the nucleus (FLIP) were determined at each time point after bleaching. The distance between the FLIP and FRAP area was kept constant in all measured cells. The curve of activated wild-type AR in the presence of R1881 showed a delay in recovery compared to the DBD mutant (Fig.4.4D), confirming the strip FRAP data (Fig.4.4B).

Interestingly, in the presence of R1881, a marked difference between GFP-AR and GFP-AR(A573D) in intranuclear distribution was also observed. GFP-AR (Fig.4.4E) was distributed inhomogeneously, showing regions with increased fluorescence intensity scattered throughout the nucleus, whereas the DBD mutant showed an almost homogeneous distribution (Fig.4.4F).

Quantification of experimental FRAP data using computer simulations

In order to quantify our observations we fitted experimental FRAP curves to curves obtained by computer simulation of the FRAP procedure (Fig.4.5). The simulation was based on a Monte Carlo approach (Houtsmuller et al., 1999): the program goes through loops representing 100 ms, the same time interval as in the strip-FRAP experiments. In each loop a random number generator determines for each molecule (Typically 30,000 are present in an ellipsoid volume representing the nucleus) whether it is bleached (dependent on the position relative to the laser beam), whether it becomes immobilised (dependent on average binding time and binding percentage), if not immobilised, in which direction it makes a step, the size of the step being dependent on the diffusion coefficient. In the simulated experiments three quantitative mobility parameters

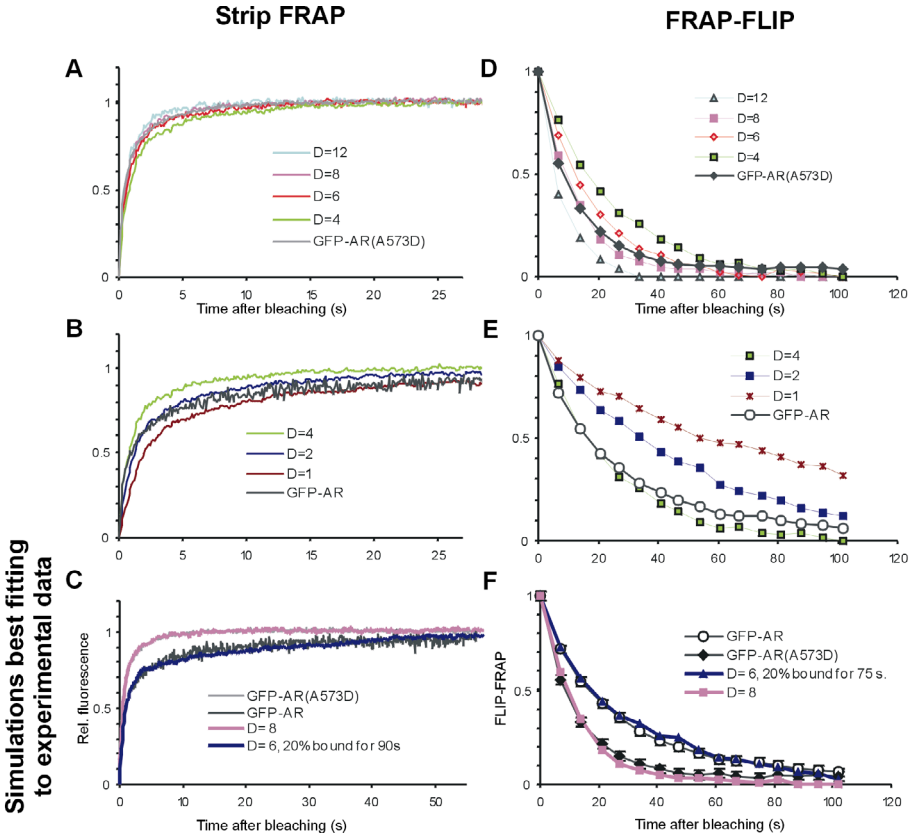


Figure 4.5. Computer modelling of the FRAP assays to estimate diffusion coefficient (*D*), bound fraction and duration of immobilization.
(A and B) Several simulated strip FRAP curves assuming only simple diffusion plotted with the experimental data of fig.4.4B. GFP-AR(A573D) fits best assuming the protein diffuses with D=8 $\mu m^2/s$ (A), whereas simulations assuming only free diffusion fit poorly to FRAP-data of liganded GFP-AR (B). (C) Best fitting simulation curves to strip FRAP of liganded wild-type AR and the DBD mutant (Fig. 4.4 B). (D and E) Several simulated combined FRAP and FLIP curves plotted together with the data of Fig. 4.4 D. GFP-AR(A573D) fits well assuming only free diffusion (D), whereas those simulations fit poorly to the data of GFP-AR in presence of R1881 (E). (F) Best fitting simulation curves of combined FRAP and FLIP to the experimental data of wild-type AR and the DBD mutant in the presence of 1 nM R1881 (Fig. 4.4 D).

were varied: diffusion coefficient, average time of immobilization and immobilised fraction. The average dimensions of the nucleus, the shape and intensity of the laser beam of the confocal microscope used and the fluorescent properties of the GFP-tag were experimentally determined (unpublished data) and kept constant in the simulation. To fit the data, the averages were obtained of three simulated experiments. Ordinary least squares were used to determine the best fit. The data of unliganded GFP-AR and GFP-AR(A573D) fitted best to

simulations of FRAP of freely mobile molecules with a diffusion coefficient of 8 ± 0.5 $\mu m^2/s$ (Fig.4.5A). The FRAP-curves for liganded ARs showed a poor fit to simulated curves of freely mobile molecules (Fig.4.5B). These curves fitted best if an immobile fraction of 20%, with individual molecules being immobilised for 90 seconds was simulated (Fig.4.5C), with the remaining fraction of unbound ARs freely diffusing. Interestingly, this freely mobile fraction was also slowed down compared to the unliganded state, from 8 ± 0.5 to 6 ± 0.5 $\mu m^2/s$ (Fig.4.5C). This could be due to complex formation with other proteins in the nucleoplasm before binding to DNA.

Strip FRAP curves from slowly diffusing molecules may be similar to curves of a situation in which a fraction is transiently bound, and the mobile fraction moves with higher diffusion coefficient. Combined FRAP and FLIP, however, can better distinguish between these scenarios. Combined FRAP and FLIP data of the DBD-mutant confirmed the strip FRAP measurements (Fig.4.5D-F). The activated wild-type AR fitted poorly to simulations of the combined FRAP and FLIP experiment assuming only free diffusion (Fig.4.5E). The curves of activated wild-type AR fitted best to free diffusion at 6 ± 0.5 $\mu m^2/s$ and a bound fraction of 20 % of individual molecules being immobilised for 75 seconds (Fig.4.5F), which is in the same range as the results of the strip-FRAP experiments (Fig.4.5 C).

Androgen receptors lacking the ligand binding domain show increased mobility compared to the full-length AR

The influence of the LBD on nuclear mobility was studied by strip FRAP (Fig.4.6A) and combined FRAP and FLIP (Fig.4.6B) using stable cell lines expressing truncated GFP-AR lacking the LBD. Although more active than full-length AR (Fig.4.1C and D), the GFP-ARΔLBD did not show a long term immobile fraction (Fig.4.6A) as was observed for the full-length GFP-AR. Redistribution of GFP-ARΔLBD was slightly slower than its corresponding DBD mutant (GFP-AR(A573D)ΔLBD) (Fig.4.6A and B), suggesting that ARΔLBD mobility was inhibited due to DNA binding but to a much lesser extent than full-length AR. Using computer simulations, we could not distinguish between a very transient immobile fraction or slower diffusion of GFP-ARΔLBD (D = 10 $\mu m^2/s$ and 20% of proteins immobilised for 2 seconds or D = 8 $\mu m^2/s$ without an immobile fraction respectively) compared to GFP-AR(A573D)ΔLBD (D = 10 $\mu m^2/s$).

The subnuclear distribution of the LBD deletion mutant (Fig.4.6C) showed a

largely homogeneous distribution. The double mutant GFP-AR(A573D)ΔLBD (Fig.4.6D) showed a homogeneous distribution similar to that of the full-length DBD mutant.

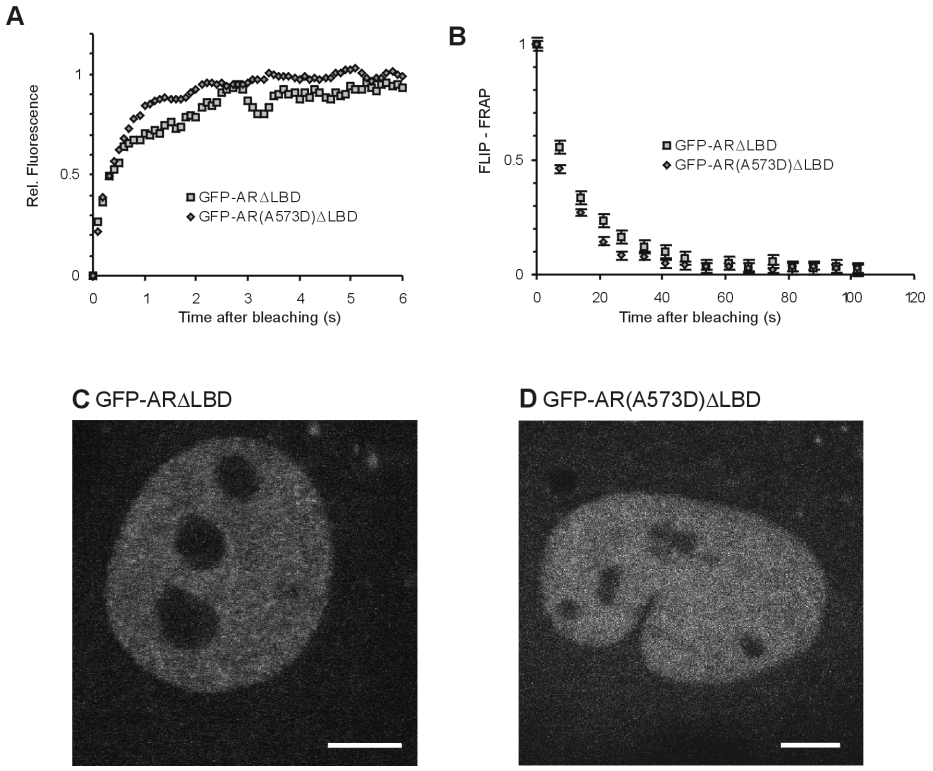


Figure 4.6. Mobility of the constitutively active LBD deletion mutant is slightly reduced by DNA-binding.
(A) Strip FRAP experiments on Hep3B cells stably expressing the LBD deletion mutant without and with the point mutation in the DBD. Mean values of at least 35 cells are plotted. (B) Combined FRAP and FLIP experiments under the same conditions as in (A). Normalized differences between the FRAP and FLIP signals are plotted. Error bars are 2 times the SEM. (C and D) High resolution confocal images showing the intranuclear distribution of GFP-ARΔLBD (C) and GFP-AR(A573D)ΔLBD (D). All experiments were performed in the presence of R1881 (1 nM). Bars represent 5 μm.

4.4. Discussion

We investigated in living cells the transcriptional activity, subnuclear distribution and nuclear mobility of the AR, a member of the steroid receptor family. FRAP experiments, carried out in Hep3B cells stably expressing GFP tagged full-length AR at a physiological level (Fig.4.2), showed that the nuclear mobility of the unliganded wild-type AR is considerably slowed down upon activation by ligand binding. To investigate the nature of this reduced mobility

we first compared wild-type AR and an AR mutant, AR(A573D), that cannot bind its cognate response elements in the promoters of androgen target genes due to an amino acid substitution in the DNA binding domain. Next, we studied the behaviour of a constitutive active AR, lacking the LBD.

Both AR and the non-DNA-binding mutant AR(A573D) reside mainly in the cytoplasm in the absence of ligand, have similar ligand binding characteristics (Brüggenwirth et al., 1998), translocate to the nucleus upon ligand binding at similar rates (Fig.4.2A and B) and are excluded from nucleoli. However, major differences were observed following nuclear translocation. First, as expected, the DBD mutant did not activate androgen target genes (Fig.4.1C). Importantly, the DBD mutant was homogeneously distributed throughout the nucleus, whereas the wild-type AR showed accumulated concentration in small irregular-shaped foci scattered throughout the nucleus (Fig.4.4E and F). Finally, the mobility of wild-type AR was substantially reduced compared to the DBD-mutant AR(A573D) (Fig.4.4B and D) and essentially identical to that of unliganded nuclear AR (Fig.4.3B). These findings strongly suggest that the observed slower mobility of wild-type AR depends on DNA binding and that the AR foci are related to DNA binding and transcriptional activity.

Our FRAP and FLIP-FRAP experiments with full-length AR are in agreement with previous FRAP data showing that (GFP-tagged) GR and ERα undergo changes in nuclear behaviour *in vivo* following binding of agonistic ligands, likely caused by interactions with immobile nuclear elements. For GR and ERα it was speculated that these immobile elements are part of a nuclear matrix (Stenoien et al., 2001b; Schaaf and Cidlowski, 2003). However, we show that disruption of the ability to bind to promoter DNA completely abolishes the transient binding behaviour, strongly suggesting that this is due to AR-promoter interactions. A different experimental approach that visualized the binding of GR to a large array of MMTV promoters (McNally et al., 2000; Becker et al., 2002) also showed that GR and coactivator GRIP1 rapidly exchanged with promoter elements with similar kinetics. Complete recovery of fluorescence was at approximately 30 seconds following photobleaching of the promoter array, in the same range as our data of R1881 activated AR (60 to 120 seconds). Short interactions of transcription initiation factors with the transcription initiation complex were also reported for TFIIH (Hoogstraten et al., 2002) in RNAP2 transcription and also for the RNAP1 transcription machinery (Dundr et al., 2002). In contrast, RNAP2 was immobilised for a much longer period of time (in the order of minutes) (Becker et al., 2002;

Kimura et al., 2002). In all these experiments promoter interaction turned out to be much shorter than previously hypothesized for the formation of stable transcription preinitiation complexes composed of specific transcription factors, cofactors and general transcription factors. The data are in favour of a hit-and-run model of transcription activation by nuclear receptors, coactivators and general transcription factors.

Computer aided analysis of the experimental FRAP and FLIP-FRAP data enabled estimation of the percentage and duration of transient AR immobilization, as well as the apparent diffusion constant of the mobile AR fraction. The experimental FRAP data of the DBD mutant and of unliganded wild-type AR fitted best to simulations assuming free mobility of all ARs (Fig.4.5C and F). The data of the wild-type AR in the presence of R1881 fitted best with 60-120 seconds immobilization of approximately one out of five ARs (Fig.4.5C and F). Since we used cells which stably express AR at a physiological level (Fig.4.2), the percentage of transiently immobile AR and the duration of immobilization might reflect the behaviour of endogenous AR in androgen target cells, like prostate cells.

Fitting of computer-simulated curves to the experimental data also indicated that recovery of the fast freely mobile fraction of ARs was reduced in the presence of hormone (Fig.4.5C and F) from 8 ± 0.5 to 6 ± 0.5 $\mu m^2/s$. There are several explanations for this observation. The liganded receptor might have very transient interactions with immobile nuclear structures that are not distinguishable from free diffusion. Such interactions could represent premature abortion of a preinitiation complex, or binding to non-functional ARE sequences. Another explanation is the presence of multiprotein complexes, for instance composed of AR homodimers (280 kD) and coregulators formed in the nucleoplasm. Such a complex should have a size in the same range as TFIIH (~600 kD), since FRAP-measurements on this transcription/repair factor revealed an apparent diffusion coefficient of 6.2 $\mu m^2/s$ (Hoogstraten et al., 2002).

The transient DNA interactions reported here for AR and in previous studies for GR (McNally et al., 2000; Becker et al., 2002) and ERα (Stenoien et al., 2001b) seem to contrast with recent chromatin immunoprecipitation (ChIP) findings. It was reported that the AR (Kang et al., 2002), ERα (Shang et al., 2000; Reid et al., 2003), and RNAP2 showed proteasome dependent cycling on target promoters, with typical cycling times ranging from 20 to 40 minutes (Reid et al., 2003), whereas our data suggest that AR is associated with

promoters in the order of one to two minutes. However, the ChIP data might represent cycling of the promoter between a repressed and an active state, whereas the dynamic studies with GFP-tagged nuclear receptors represent binding to actually active promoters of steroid hormone regulated genes.

Interestingly, the truncated AR lacking the LBD exhibited much faster redistribution kinetics than the full-length AR. Although very potent in activation of target promoters, we found that interactions of the ARΔLBD mutant with DNA in the nucleus are much more transient, in the order of seconds, than interaction of full-length AR. An additional mutation in the DNA binding domain increased mobility, suggesting the slowed recovery is indeed caused by binding to androgen regulated promoters, although binding times are much shorter than those observed for the liganded wild type AR. Since the ARΔLBD mutant can also activate androgen target genes, it seems that the relatively long immobilization of full-length AR is not necessary for effective transcription initiation. Short interactions in the order of seconds of transcription initiation factors with the transcription initiation complex were also reported for TFIIH in RNAP2 (Hoogstraten et al., 2002) and for RNAP1 regulated transcription (Dundr et al., 2002). The short-lived interactions of the ΔLBD mutant observed here may correspond to these interactions, whereas the longer residence times of full-length AR may be related to a different function of the AR. This additional function may require the formation of a more stable complex on AR regulated promoters. In conclusion, the presented results support a model where the DNA binding domain of the AR has a role in initial AR-DNA interactions, whereas the ligand binding domain has a hitherto unknown role in stabilising these interactions.

4.5. Materials and methods

Constructs

pAR0, expressing human full-length wild-type AR, has been described previously (Brinkmann et al., 1989). To generate plasmids expressing GFP-$(Gly)_5$Ala-AR or GFP-$(GlyAla)_6$-AR, PCR was performed on pAR0 using sense primers to introduce a *Bgl*II restriction site and the spacer sequence (resp. 5'-GCAGAAGATCTGGAGGTGGAGGTGGAGCTGAAGTGCAGTTAGGGCTG-3′ and 5'-GCAGAAGATCTGCAGGTGCTGGAGCAGGTGCTGGAGCAGGTGCTGGAGAAGTGCAGTTAG-3') and an anti-sense primer in the AR cDNA overlapping a *Sma*I site (5'-

TTGCTGTTCCTCATCCAGGA-3'). The PCR product was cloned in pGEM-T-Easy (Promega, Madison, WI) and the sequence was verified. The *Bgl*II-*Sma*I fragment was inserted in corresponding sites of pEGFP-C1 (Clontech, Palo Alto, CA) containing a His_6-HA tag between the *Nhe*I and *Nco*I sites. Next, the *Sma*I fragment from pAR0 was inserted into the *Sma*I site to generate and pGFP-Gly_5Ala-AR. pGFP-$(GlyAla)_6$-AR. pGFP-AR(A573D) was made by exchanging the *Asp*718-*Sca*I fragment from pAR(A564D), previously denoted 564 (Brüggenwirth et al., 1998) in pGFP-AR. Truncated pGFP-ARΔLBD with or without the mutation in the DBD were created by QuickChange™ site-directed mutagenesis (Stratagene, La Jolla, CA) on pGFP-AR and pGFP-AR(A573D) using primers 5'-GACAACCCAGAAGCTGACATAGTAATAGATTGAAGGCTATGAATGTC-3' and 5'-GACATTCATAGCCTTCAATCTATTACTATGTCAGCTTCTGGGTTGTC-3' introducing 3 stop codons resulting in loss of the *Asp*I restriction site. Presence of mutations was verified by sequencing.

3x1F8-TKLuc was generated by annealing of oligos 5'-TCGACATGATCTTGGATTGAAAACAGACCTACTCTGGAGGAACATATTGTATCGATTGTCCTTGACG-3' and 5'-TCGACGTCAAGGACAATCGATACAATATGTTCCTCCAGAGTAGGTCTGTTTTCAATCCAAGATCATG-3' containing *Sal*I restriction sites and the AREIII sequence of the PSA-enhancer (Cleutjens et al., 1997). Three *Sal*I fragments (1F8) were ligated into *Sal*I digested pTZ19. The *Hind*III/*Sma*I fragment, containing 3x1F8, from pTZ19 was blunt-ended and cloned into the *Pvu*II site of TKLuc. The minimal promoter $(ARE)_2$TATA-Luc was a generous gift from Dr. G. Jenster (Erasmus MC, Rotterdam, The Netherlands).

Cell culturing and transactivation assays

Hep3B cells were cultured in αMEM (Bio-Whittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 5% FBS (PAN Biotech GmbH, Aidenbach, Germany). Cells were passaged every 3-4 days.

For transactivation assays cells were plated at a density of 100,000 cells/well in 24-well plates. For nuclear import and FRAP mobility studies, cells were plated at 300,000 cells/well on glass cover slips in 6-well plates.

Hep3B cells were transiently transfected with 250 ng/well of AR expression construct and 500 ng/well of a promoter construct driving a luciferase reporter gene, using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Four hours prior to transfection the medium was changed to medium containing 5% dextran charcoal depleted FBS minus or plus 1nM R1881. Twenty-four hours

after transfection cells were lysed in lysis buffer (15% glycerol, 25mM TrisPhosphate (pH7.8), 1mM DTT, 1% Triton X-100 and 8 mM $MgCl_2$). Luciferase activity in cell lysates was determined using Fluoroscan Ascent FL (Labsystems Oy, Helsinki, Finland). Mean and SEM of at least 3 experiments were plotted. For MMTV-Luc studies fold inductions by 1nM R1881 were calculated. For GFP-AR and GFP-ARΔLBD studies ratios of ARΔLBD and AR activity in the presence of 1nM R1881 were calculated.

Generation of stable cell lines

Stable cell lines containing GFP-AR constructs were generated to ensure GFP-AR protein was expressed at physiological levels. Hep3B cells were transfected using FuGENE 6 in 6 wells plates with 1 µg/well plasmid DNA 1 day after plating. After 24 hours cells were trypsinised and plated in medium containing 1 mg/ml Geneticin (G418 sulphate, Sigma, St. Louis, MO) in 10 cm tissue culture dishes. Clones were selected and checked for appropriate GFP-AR distribution and expression by confocal microscopy and Western blotting. Stable cell lines were maintained as normal Hep3B cells in medium containing 1 mg/ml Geneticin.

Western blotting

Stable clones were cultured in a 75 cm^2 flask and allowed to grow fully confluent. Cells were washed with DPBS and lysed in 1 ml lysis buffer (40mM Tris, 1 mM EDTA, 10% glycerol, 10mM DTT, 1 % Triton X-100, 0.08 % SDS, 0.5 % deoxycholate and complete mini protease inhibitor cocktail (Roche)). 5 µl Laemmli sample buffer was added to 5 µl cell lysate, samples were boiled for 5 minutes and separated by electrophoreses on a 10% SDS-polyacrylamide gel. Following electrophoresis proteins were transferred to a nitrocellulose membrane. Blots were either incubated with 1:2000 anti-AR (F39.4 I) or 1:2000 anti-GFP (Ab 290, Abcam Ltd., Cambridge, UK). Blots were subsequently incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). Signal was visualized using Super Signal® West Pico Luminol solution (Pierce, Rockford, IL). Blots were exposed to X-ray film to visualize the proteins.

Nuclear import and FRAP studies

Nuclear import of GFP-AR and GFP-AR(A573D) stable cell lines was tested by addition of 1 nM R1881 to the medium and making sequential images every 30 seconds during 30 minutes. Nuclear import and FRAP studies were performed

using a Zeiss LSM410 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) using a 40x/1.3 NA oil objective. Images were scanned at a lateral resolution of 102 nm, using the 488 nm laser line of a Argon laser, a 488/543 nm dichroic beam splitter and a 515-540 nm band pass filter. Cells were imaged in culture medium at 37°C.

Mobility of the different GFP-AR constructs in the nucleus of the stable cell lines Hep3B cells before and after addition of 1 nM R1881 was studied using fluorescence recovery after photo bleaching (FRAP) techniques (Houtsmuller et al., 1999; Houtsmuller and Vermeulen, 2001). Fluorescence in a small strip in the nucleus was monitored every 0.1 seconds for 6 or 60 seconds. After 2 seconds a bleach pulse of 0.2 seconds at maximum laser power was given. To correct for monitor bleaching curves were made without the bleach pulse. Mean of all cells before and after induction by androgen were calculated and plotted, correcting for differences in cell fluorescence by setting the fluorescence before bleaching to 1. All graphs were normalized to GFP-AR equilibrium fluorescence in the absence of hormone. In the long term FRAP experiments a wider strip (1 μm) was used than in the short term experiments (0.2 μm).

The combined FRAP and FLIP method has been described previously (Hoogstraten et al., 2002). In short, a strip of 1.2 μm was bleached at one pole of the nucleus for 8 sec. at maximum laser power. Subsequent images were taken at regular intervals after bleaching. Fluorescence intensities as a ratio of the image before bleaching were calculated and averaged. Differences in fluorescence between the bleached strip (FRAP) and a strip at the opposite pole of the nucleus (FLIP) were calculated, on each time point after bleaching. The distance between FRAP and FLIP regions was kept constant in all cells. Average of at least 35 cells ± 2 · SEM were plotted.

Computer Simulation

For analysis of FRAP assays we developed computer software to simulate FRAP applied to fluorescent molecules inside a finite ellipsoid volume representing the nucleus. Simulations were performed using fixed, experimentally obtained parameters, describing lens (beam shape and 3-D intensity distribution, during monitoring and during bleach pulse), GFP (quantum yield, susceptibility to bleaching) and nuclear properties (size and shape). Three protein mobility parameters, diffusion coefficient, bound fraction and duration of binding of individual molecules were varied. Diffusion was simulated by randomly picking a direction to step into with a stepsize derived from the equation $D = \text{stepsize}^2 \cdot \text{cycletime}^{-1}$, where the cycletime in our

simulation was 100 ms. The bound fraction was established by giving molecules a chance to bind at each cycle, derived from the equation: $P_{\text{bind, 1 cycle}}$ = (immobile fraction) · (mobile fraction)$^{-1}$ · cycletime^{-1} .

To obtain best fit with experimental data we used least square fitting to the average of three simulated curves. In a Monte Carlo set-up best fit is estimated by varying the variables to be fitted in a stepwise fashion. Here, diffusion was varied with steps of 1 $\mu m^2/s$, bound fraction with steps of 5% and binding time with steps of 15 seconds in the case of the wild type androgen receptor and 2 seconds in the case of the shortly bound LBD deletion mutant.

4.6. Acknowledgements

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Chapter

5

Antiandrogens prevent stable DNA-binding of the androgen receptor

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Adapted

5.1. Summary

The androgen receptor (AR) is essential in development of the male gender and in growth of the majority of prostate cancers. Agonists as well as most antagonists induce translocation of the receptor to the nucleus, whereas only agonists can activate AR function. Antagonists are therefore applied in therapy of metastasized prostate cancer. To obtain insight into the mechanism by which antagonists block AR function in living cells, we studied nuclear mobility and localization of green fluorescent protein (GFP)-tagged AR in the presence of either the agonist R1881 or the antagonists bicalutamide and hydroxyflutamide, respectively. As controls we investigated a non-DNA-binding AR mutant (A573D) and two mutants (W741C and T877A) with broadened ligand specificity. We demonstrate that in presence of R1881 AR localizes in numerous intranuclear foci and, using complementary fluorescence recovery after photobleaching (FRAP) approaches and computer modeling, that a fraction of ARs (~10-15%) is transiently immobilized in a DNA-binding dependent manner (individual ARs being immobile for ~45 s). In contrast, antagonist-bound GFP-AR showed no detectable immobile fraction and the mobility was similar to that of the R1881-liganded non-DNA-binding mutant (A573D), indicating that antagonists do not induce the relatively stable DNA-binding dependent immobilization observed with agonist-bound AR. Moreover, in presence of bicalutamide and hydroxyflutamide GFP-AR was homogeneously distributed in the nucleus. Binding of bicalutamide and hydroxyflutamide to GFP-AR(W741C) and GFP-AR(T877A), respectively, resulted in similar mobility and heterogeneous nuclear distribution as observed for R1881-liganded GFP-AR. The presented live cell studies indicate that the investigated antagonists interfere with events early in the transactivation function of AR.

Keywords: Androgen Receptor – Antiandrogens – Fluorescence Recovery After Photobleaching – Prostate Cancer – DNA-binding

5.2. Introduction

The androgen receptor (AR) is a member of the family of steroid receptors. Functional ARs are required for development of the male gender (Cunha et al., 1987). In addition, ARs play a role in growth of prostate cancer (Feldman and Feldman, 2001; Trapman, 2001). Therefore, metastasized prostate cancers are frequently treated with antiandrogens, such as flutamide or bicalutamide (Casodex) (Small and Vogelzang, 1997). However, in spite of initial success, all patients eventually show tumor relapse. There may be several causes for therapy resistance, including changes in cell signaling pathways, AR overexpression and mutation of the AR (Feldman and Feldman, 2001; Trapman, 2001). The latter may lead to activation of the AR by ligands other than androgens testosterone and 5α-dihydrotestosterone (DHT), including estrogens, glucocorticoids and adrenal androgens (Veldscholte et al., 1992; Brinkmann and Trapman, 2000; Zhao et al., 2000; Mizokami et al., 2004). Therapy also selects for AR-mutants that are activated by the applied antiandrogens (Veldscholte et al., 1990; Hara et al., 2003). One of the AR mutations most frequently found in antiandrogen treated patients is a mutation in codon 877, resulting in replacement of threonine by alanine AR(T877A) (Veldscholte et al., 1990; Taplin et al., 2003). The mutation results in agonistic activity of OH-flutamide (Veldscholte et al., 1990), the active metabolite of flutamide (Katchen and Buxbaum, 1975). Recently, in a bicalutamide treated patients a novel mutation was found, resulting in substitution of tryptophan at position 741 by cysteine AR(W741C) (Haapala et al., 2001; Taplin et al., 2003). It was demonstrated that this mutation enabled bicalutamide to act as an agonist (Hara et al., 2003).

The intracellular distribution of ARs in absence and presence of ligand has been extensively studied in cell lines using both immunocytochemistry (Jenster et al., 1991; Simental et al., 1991) and green fluorescent protein (GFP)-tagging (Georget et al., 1997; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001; Farla et al., 2004). In the absence of ligand, ARs are predominantly localized in the cytoplasm associated with a chaperone complex containing heat shock proteins (Smith and Toft, 1993; Pratt and Toft, 1997; Stenoien et al., 1999), keeping the AR in a high-affinity ligand-binding conformation (Vanaja et al., 2002). Ligand binding induces release from this complex and rapid translocation of AR to the nucleus within 15-60 minutes after addition of androgen (Georget et al., 1997; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001; Farla et al., 2004). In the nucleus ARs bind as dimers to androgen

response elements (AREs) in promoters of target genes (Roche et al., 1992; Claessens et al., 2001). Addition of antiandrogens OH-flutamide and bicalutamide also resulted in translocation of ARs from the cytoplasm to the nucleus (Jenster et al., 1993; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001), although the translocation in the presence of bicalutamide was slower and incomplete (Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001).

Ligand-activated steroid receptors, including estrogen receptor α (ERα) (Htun et al., 1999; Stenoien et al., 2000), glucocorticoid receptor (GR) (van Steensel et al., 1995; Htun et al., 1996) and mineralocorticoid receptor (MR) (Fejes-Tóth et al., 1998) were found to be distributed in the nucleus in a focal pattern. Likewise agonist liganded ARs were shown to accumulate in foci in the nucleus (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001; Ochiai et al., 2003; Farla et al., 2004). Interestingly, ARs only accumulated into foci with agonistic and partial agonistic ligands, whereas antagonist-bound ARs showed a more homogeneous nuclear distribution (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001). Recently, we showed that ARs carrying a mutation in the DNA-binding domain show a homogeneous intranuclear distribution, indicating that the focal pattern depends on the DNA-binding ability of the AR (Farla et al., 2004).

GFP technology and quantitative live cell imaging have provided new insights in the mechanism of gene activation by steroid receptors. We showed using fluorescence recovery after photobleaching (FRAP) that agonist bound ARs are immobilized in a DNA-binding dependent manner, the average immobilization of a single AR being 1-2 minutes (Farla et al., 2004). Others have shown that GFP-tagged GRs exchange rapidly between the nucleoplasmic compartment and a mouse mammary tumor virus (MMTV) promoter array (McNally et al., 2000). The p160 coactivator glucocorticoid receptor interacting protein 1 (GRIP1) displayed similar dynamic interactions on this promoter repeat (Becker et al., 2002). GFP-tagged GR (Schaaf and Cidlowski, 2003; Elbi et al., 2004), ERα and the p160 coactivator steroid receptor coactivator 1 (SRC-1) (Stenoien et al., 2001b) showed reduced intranuclear mobility in the presence of agonistic ligands suggesting they dynamically interact with immobile elements in the nucleus, similar to the interaction of GRs with the MMTV promoter array. The coactivators SRC-1 and CREB binding protein (CBP) were shown to rapidly exchange on a *lac* repressor ERα chimera immobilized on an array of *lac* operators (Stenoien et al., 2001a), again demonstrating that

interactions between steroid receptors and coactivators are very dynamic. Likewise in chromatin immuno precipitation (ChIP) experiments, ERα and AR as well as associated coactivators have been shown to associate with transcription initiation complexes in a cyclic manner, albeit with much longer cycling times (in the order of minutes) (Kang et al., 2002; Métivier et al., 2003; Reid et al., 2003) as compared to the residence times observed with FRAP (in the order of seconds). In addition, ChIP results suggest that the AR in the presence of bicalutamide binds to promoter regions of androgen regulated genes, although it is unable to form an active transcription complex (Kang et al., 2002; Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004), in contrast to DHT activated ARs.

To investigate the mechanism of action of antagonists, we studied the intranuclear dynamics and localization of GFP-tagged ARs in the presence of the non-steroidal antagonists OH-flutamide and bicalutamide, and compared them with the effects of the agonistic ligand R1881. We also studied mutant ARs containing mutations in codons 741 or 877 of the AR found in prostate cancer patients (Taplin et al., 1999; Haapala et al., 2001; Taplin et al., 2003). As mentioned above, certain AR antagonists can activate transcription by these mutant ARs (Veldscholte et al., 1990; Hara et al., 2003). Mobility, transcriptional activation and intranuclear focal distribution pattern of wild-type and mutant androgen receptors in presence of activating ligands were highly correlated. We found the behavior of wild-type AR in presence of bicalutamide or OH-flutamide to be similar to that of the non-DNA-binding mutant AR(A573D), suggesting that antiandrogens act by interfering with the stable DNA-binding of the AR.

5.3. Results

In previous work we studied the transcriptional activity, intranuclear distribution and mobility of the GFP-tagged wild-type AR using live cell microscopy and FRAP (Farla et al., 2004; Houtsmuller, 2005). To determine the role of DNA-binding, we compared the wild-type AR with an AR containing a mutation in the DNA-binding domain (A573D) that disrupts promoter binding, but is unaffected with respect to ligand binding (Brüggenwirth et al., 1998) and transport from cytoplasm to the nucleus (Farla et al., 2004). To investigate the mechanism of action of AR antagonists, we investigated the effect of bicalutamide and OH-flutamide on the intranuclear mobility and localization of

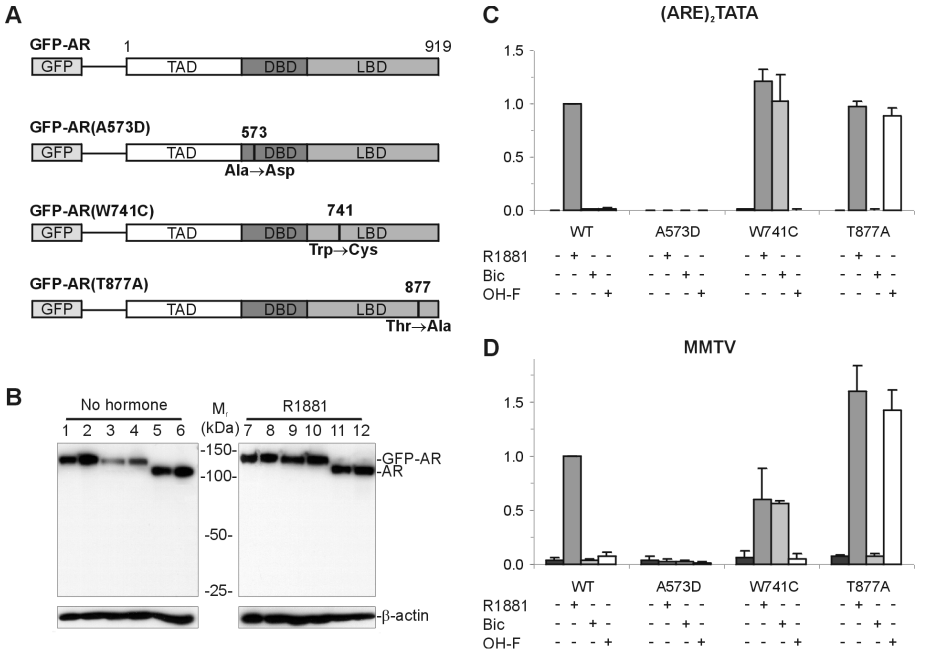
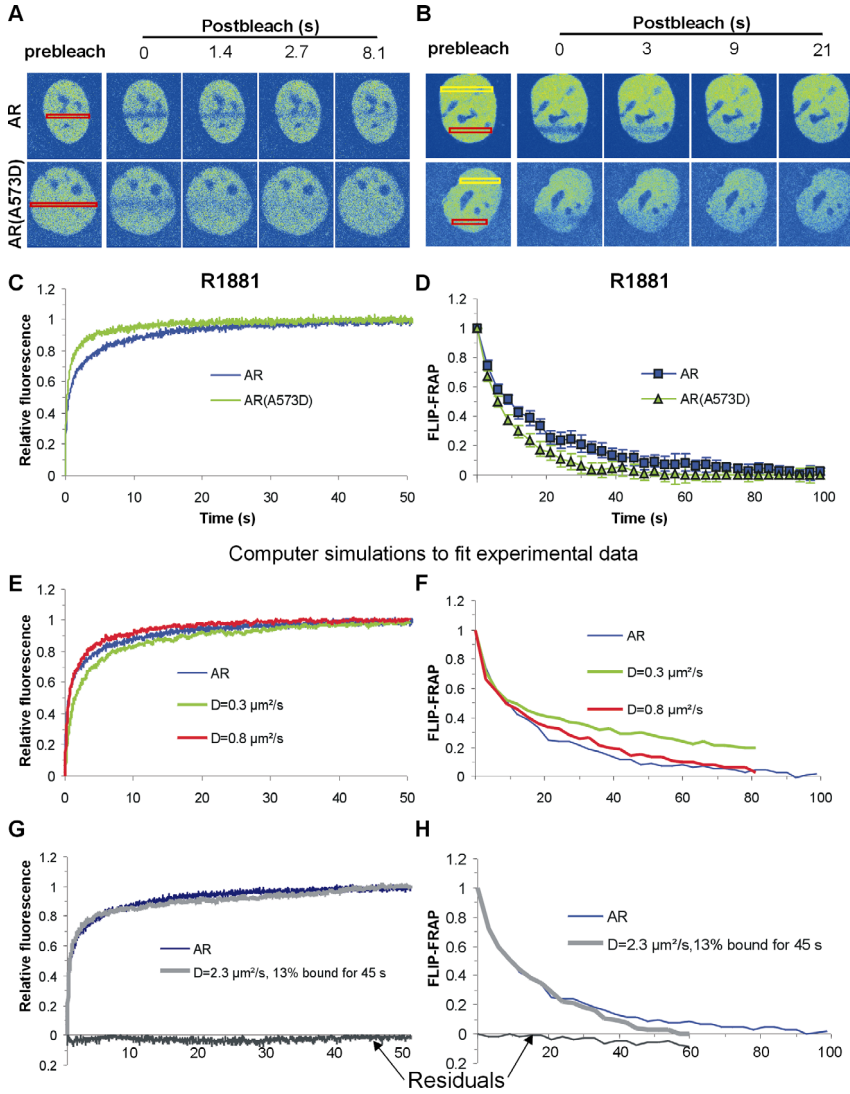


Figure 5.1. Schematic representation, expression and transactivating capacity on androgen regulated promoters of GFP-AR proteins investigated.
(A) TAD: Transactivating domain; DBD: DNA-binding domain; LBD: Ligand binding domain. Next to GFP-tagged wild-type AR, ARs containing a mutation in the DBD disrupting DNA-binding (Brüggenwirth et al., 1998), or in helix 3 (W741C) or helix 12 (T877A) of the AR-LBD which result in altered ligand specificity (Veldscholte et al., 1990; Hara et al., 2003) were studied. (B) Hep3B cells containing stably integrated GFP-AR expression constructs and AR expressing prostate cancer cell lines were cultured for 1 day in the absence (lanes 1 to 6) or presence of 1nM R1881 (lanes 7 to 12). Cell lysates were prepared and subjected to Western blot. Western blots of cell lysates from Hep3B cells (AR negative) containing GFP-AR (lanes 1 and 7), GFP-AR(A573D) (lanes 2 and 8), GFP-AR(W741C) (lanes 3 and 9) or GFP-AR(T877A) (lanes 4 and 10 and LNCaP (lanes 5 and 11) and PC346 (lanes 6 and 12) using an anti-AR or β-actin antibody. β-actin expression was used as loading control. (C and D) Cotransfection assays of GFP-AR and the mutants depicted in A with androgen regulated promoter constructs (ARE)2-TATA-luciferase (C) and mouse mammary tumor virus (MMTV)–luciferase (D) in the presence of 10-9 M R1881, 10-6 M bicalutamide (Bic), 10-6 M OH-flutamide (OH-F) or no ligand as indicated. Luciferase activity of the GFP-AR proteins is plotted relative to activity of GFP-AR in presence of 10-9 M R1881. Mean ± S.E.M. of at least 3 independent experiments are shown.

Figure 5.2. Combined strip-FRAP and FLIP-FRAP reveal that a fraction of agonist liganded GFP-ARs is transiently immobilized.
(A) The strip-FRAP method: a strip in the center of a nucleus is bleached (red rectangle) with high laser power. Subsequently, fluorescence in the strip is measured at regular time intervals. Images are shown in false color to visualize fluorescence differences more clearly. (B) Combined FLIP and FRAP method (FLIP-FRAP): a strip at one pole of the nucleus was bleached for a relatively long period. The difference between fluorescence signals in the bleached region (FRAP, red rectangle) and a distal region at 10 μm from the bleached region of the nucleus (FLIP, yellow rectangle) was determined at regular time intervals. (C and D) strip-FRAP and FLIP-FRAP experiments of GFP-AR or the non-DNA-binding mutant GFP-AR(A573D) in the presence of 10-9 M R1881. →



(C) Graph showing fluorescence intensities relative to complete redistribution of the non-DNA-binding mutant GFP-AR(A573D) in the presence of R1881 plotted against time. Mean values of at least 10 cells of a representative experiment are plotted. All experiments were performed at least thrice. (D) Graph showing the difference between fluorescence intensity in the FLIP and FRAP regions (rectangles in B) relative to the difference directly after bleaching, plotted against time. Mean values ± two times the S.E.M. of 2 independent experiments on at least 10 cells are plotted. (E-F) Computer simulations (see materials and methods) of strip-FRAP and FLIP-FRAP of freely diffusing molecules do not explain the experimental FRAP-data obtained with both methods. Experimental strip-FRAP data on wild type GFP-AR lies in between curves representing indicated scenarios of free diffusion (E), whereas experimental FLIP-FRAP data on wild type GFP-AR lies outside these boundaries (F). (G and H) Computer simulations representing a model where, next to freely diffusing molecules, a fraction is transiently immobilized, fit to both strip-FRAP and FLIP-FRAP experimental curves on wild-type GFP-AR. Computer simulations shown correspond to the average of best fits of FRAP and FLIP-FRAP experiments respectively (Table I), so are not necessarily the best fits of the individual experiments. Absolute value of residuals of the computer simulation fit and the experimental data on each time point are plotted below the x-axis.

GFP-AR and GFP-AR(A573D) (Fig. 5.1A). In addition, we studied two AR mutants, implicated in resistance to prostate cancer treatment with these non-steroidal AR antagonists (Veldscholte et al., 1990; Taplin et al., 1999; Haapala et al., 2001; Hara et al., 2003; Taplin et al., 2003). The first mutant has a base substitution in codon 741 of the AR coding sequence, resulting in substitution of tryptophan on position 741 by cysteine (Hara et al., 2003). The second mutant has a base substitution resulting in substitution of threonine 877 by alanine (Veldscholte et al., 1990). The cDNA expression constructs coding for GFP-tagged versions of these AR mutants were transfected in the AR negative cell line Hep3B and allowed to stably integrate. Western blot analyses revealed that all stable cell lines expressed full-length GFP-AR (Fig. 5.1B). Expression in the GFP-AR stable cell line is comparable to the levels of AR in the prostate cancer cell lines LNCaP and PC346 (Fig. 5.1B and Farla et al., 2004), indicating that GFP-AR is expressed at physiological levels. Since expression levels of the mutants GFP-AR(W741C) and GFP-AR(T877A) were similar or slightly less than that of wild-type GFP-AR, the effects we observed were unlikely to be caused by overexpression.

To test the transactivating capacity of the GFP-tagged AR mutants, their corresponding cDNA expression plasmids were co-transfected with androgen regulated promoters in AR-negative Hep3B cells. Activation of either the minimal promoter $(ARE)_2TATA$ (Fig. 5.1C) or the mouse mammary tumor virus (MMTV)-promoter (Fig. 5.1D) in the presence of R1881, or the antiandrogens OH-flutamide or bicalutamide were measured. As expected, the non-DNA-binding mutant GFP-AR(A573D) was inactive. Wild-type AR could activate transcription of the $(ARE)_2TATA$ promoter in presence of R1881, whereas OH-flutamide and bicalutamide did not activate transcription, as expected. The prostate cancer related GFP-tagged W741C mutant (Haapala et al., 2001; Taplin et al., 2003) was activated by bicalutamide to the same extent as by R1881, whereas GFP-AR(T877A) in addition to R1881 was also activated by OH-flutamide (Fig. 5.1C). The same ligand specificity was observed on the MMTV promoter, although there were some quantitative differences in transactivating capacity between the mutants (Fig. 5.1D). These results show that the GFP-tag does not interfere with the transactivating properties of the studied AR-mutants, since the response is similar to results reported previously with untagged ARs (Veldscholte et al., 1990; Hara et al., 2003).

Antiandrogens OH-flutamide and bicalutamide do not reduce the mobility of wild-type androgen receptors

Previously we have shown using FRAP that binding of R1881 to GFP-AR resulted in a strongly reduced mobility of nuclear GFP-AR (Farla et al., 2004), as compared to a mutant GFP-AR(A573D), which is unable to bind DNA (Brüggenwirth et al., 1998). In this investigation we repeated these experiments as controls. Using two complementary FRAP assays (Fig. 5.2A-D) we show that fitting the data of the two assays to a model of free diffusion yields different diffusion coefficients, indicating that the results cannot be explained by a simple model of free diffusion. Therefore, the observed slower mobility is probably not the result of an overall slow-down of diffusion (Fig. 5.2E,F). In contrast, a scenario where a ~ 15% fraction of ARs in the nucleus was immobilized for ~45 seconds (Fig. 5.2G,H and Table 5.1) fitted well to both strip-FRAP and FLIP-FRAP curves. The R1881 liganded DBD-mutant GFP-AR(A573D) was freely mobile, similar to unliganded wild-type ARs (Farla et al., 2004), suggesting that the immobilization of wild-type AR was related to binding to its cognate sequences in the DNA.

To investigate the mechanism by which AR antagonists OH-flutamide and bicalutamide interfere with proper transcription activation we set out to compare the behavior of R1881-associated wild-type AR with that of antagonist liganded wild-type ARs. First, we performed strip-FRAP and FLIP-FRAP assays on cells expressing wild-type AR in presence of OH-flutamide (Fig. 5.3A,B). Fitting of the experimental data to computer simulated curves revealed no significant slow-down of AR mobility, compared to the non-DNA-binding GFP-AR(A573D) (Fig. 5.3A,B; Table 5.1). Diffusion constants in both strip-FRAP and FLIP-FRAP experiments were similar to that of GFP-AR(A573D). Similar to these results, the combined FRAP analysis of wild-type AR in presence of the antagonist bicalutamide showed almost identical recovery kinetics as GFP-AR(A573D) indicating that no substantial immobilization occurred (Fig. 5.3C,D; Table 5.1). These results suggest that antagonist liganded ARs have a similar mobility as the non-DNA binding GFP-AR(A573D) and show no immobilization, whereas the agonistic ligand R1881 induces a transient immobilization of a fraction of ARs in a DNA-binding dependent manner. These data suggest that antagonist-bound ARs cannot stably bind DNA.

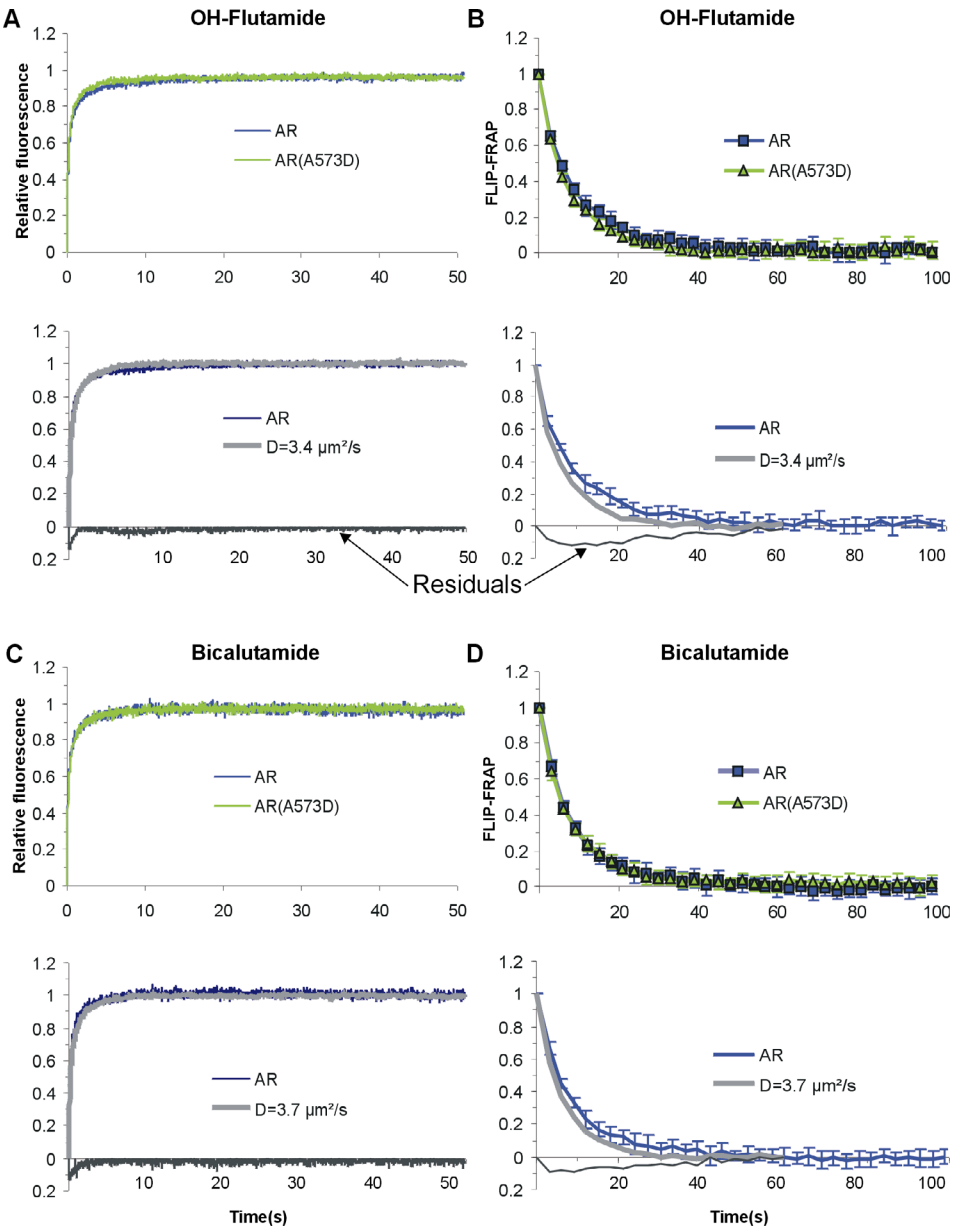


Figure 5.3. In presence of antagonists OH-flutamide and bicalutamide GFP-AR shows no or little DNA dependent immobilization.
Strip FRAP (A and C) or combined FLIP and FRAP (B and D) of GFP-AR or the non-DNA-binding mutant GFP-AR(A573D) in the presence of 10-6 M OH-flutamide (A and B) or 10-6 M bicalutamide (C and D). Experimental settings were identical to those described in Fig. 5.2. Lower graphs show computer simulations corresponding to the average of best fits of strip-FRAP and FLIP-FRAP models of wild-type GFP-AR (see Table 5.1). The absolute value of the residuals of the fit and the experimental data are plotted below the x-axis. Larger residuals in the first second of strip-FRAPs are probably due to larger variation in the beginning of the experiment, when fluorescence changes rapidly.

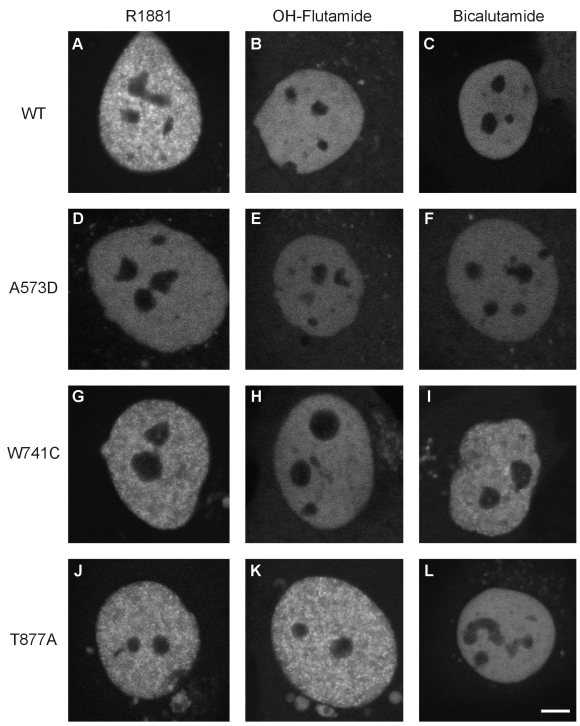


Figure 5.4. Activation of AR by agonistic ligands results in intranuclear localization in foci.
Confocal laser scanning microscope images showing representative nuclei of Hep3B cell lines stably expressing GFP-AR (A to C), the non-DNA-binding mutant GFP-AR(A573D) (D to F), or GFP-AR proteins with mutations in the LBD which result in altered ligand specificity GFP-AR(W741C) (G to I) and GFP-AR(T877A) (J to L) showing subnuclear localization in the presence of 10-9 M R1881 (A, D, G and J), 10-6 M OH-flutamide (B, E, H and K) or 10-6 M bicalutamide (C, F, I and L). With all ligands androgen receptors are localized in the nucleus, but are excluded from nucleoli (dark areas in the nucleus). In situations where AR is able to activate transcription intranuclear foci are observed (A, G, I, J and K, see Fig. 5.1). Bar corresponds to 5 µm.

Androgen receptor agonists induce intranuclear foci

In absence of ligand, GFP-AR as well as GFP-AR(A573D) are predominantly localized in the cytoplasm, although not completely absent from the nucleus (Farla et al., 2004). As shown previously, R1881 induced translocation to the nucleus and in addition to AR immobilization, induced intranuclear AR accumulation in a focal pattern (Fig. 5.4A and Farla et al., 2004). In contrast the DBD-mutant GFP-AR(A573D) was homogeneously distributed in the nucleus (Fig. 5.4D and (Farla et al., 2004)). We studied the intranuclear distribution of GFP-AR and GFP-AR(A573D) in presence of OH-flutamide or bicalutamide by high-resolution confocal microscopy. Addition of the antagonists translocated the receptor to the nucleus although at a slower rate and resulted in a homogeneous intranuclear distribution of wild-type AR (Fig. 5.4B and C, see also Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001; Farla et al., 2004) as well as the DBD mutant GFP-AR(A573D) (Fig. 5.4E and F), indicating the binding of antagonists prevented foci formation.

Next, we studied the intracellular distribution of GFP-AR(W741C) and GFP-AR(T877A). In the absence of ligand, those mutants similar to wild-type

Table 5.1. Fit of experimental data to curves generated by computer simulation

		Ligand		
Construct	Parameter	R1881	OH-F	Bic
GFP-AR	Diffusion constant†	2.3±0.3**	3.4±0.2*	3.7±0.2
	Immobile fraction‡	0.13±0.01**	-	-
	Binding time§	45±5	-	-
GFP-AR(A573D)	Diffusion constant	3.0±0.2	3.7±0.2	3.5±0.2
	Immobile fraction	-	-	-
	Binding time	-	-	-
GFP-AR(W741C)	Diffusion constant	2.2±0.3**	3.6±0.3	2.8±0.3**
	Immobile fraction	0.13±0.01**	-	0.10±0.02**
	Binding time	52±7	-	33±5
GFP-AR(T877A)	Diffusion constant	2.4±0.3**	2.6±0.2**	3.5±0.2
	Immobile fraction	0.14±0.01**	0.11±0.01**	-
	Binding time	45±6	39±5	-

Data are the mean ± two times s.e.m. of best fitting parameters from Strip-FRAP and FLIP-FRAP. Shades indicate conditions with transcriptionally active ARs (see Fig.5.1).
†Diffusion constant in μm^2/second of mobile fraction.
‡Fraction of receptors immobilized owing to interaction with subnuclear structures. –, no detectable immobile fraction (fraction < 0.05). §Mean immobilization of individual ARs in seconds. P-values of Mann-Whitney U-test comparing the value of the parameter with that of GFP-AR(A573D) with the same ligand *P< 0.05 and **P< 0.005.

receptors were predominantly cytoplasmic. Exposure to R1881 as well as the antagonists OH-flutamide and bicalutamide resulted in translocation of the mutant receptors to the nucleus (Fig. 5.4G-L). In the presence of R1881 GFP-AR(W741C) and GFP-AR(T877A) displayed a very similar focal distribution (Fig. 5.4G,J). GFP-AR(W741C), which activates transcription on androgen regulated promoters in presence of bicalutamide (Fig. 5.1C,D and Hara et al., 2003), in addition showed bicalutamide-induced intranuclear accumulations (Fig. 5.4I), whereas OH-flutamide treatment resulted in a homogeneous intranuclear distribution (Fig. 5.4H), supporting the hypothesis that lack of transactivating capacity results in homogeneous distribution. Treatment with OH-flutamide of the mutant GFP-AR(T877A) induced intranuclear accumulations (Fig. 5.4K), but bicalutamide did not result in accumulation of this AR mutant (Fig. 5.4L), consistent with transactivation of androgen regulated target genes by OH-flutamide and not by bicalutamide (Fig. 5.1 and (Veldscholte et al., 1990)).

Prostate cancer related androgen receptor mutants show reduced mobility in presence of their agonistic ligands

The intranuclear mobility of the mutant receptors GFP-AR(W741C) and GFP-AR(T877A) in presence of R1881 was similar to wild-type AR (compare Fig. 5.5A,B with Fig. 5.2C,D). Fitting to computer simulated curves revealed

approximately 10-15% of ARs were immobilized for approximately 45 seconds (Table 5.1). In addition, diffusion of the mobile fraction was slowed down significantly compared to GFP-AR(A573D). The antiandrogen OH-flutamide retarded redistribution of GFP-AR(T877A) (Fig. 5.5C,D), in agreement with OH-flutamide acting as an agonist of this mutant (Fig. 5.1C,D). Computer simulation fits showed a fraction (13%) of OH-flutamide liganded GFP-AR(T877A) was immobilized in a similar manner as R1881-bound wild-type AR (Table 5.1). In addition, diffusion of the mobile fraction was significantly slower compared to the other GFP-ARs in the presence of OH-flutamide (Table 5.1). Similarly, bicalutamide slowed down nuclear redistribution of GFP-AR(W741C) (Fig. 5.5E,F). In contrast, GFP-AR(T877A) showed the same FRAP kinetics as GFP-AR(A573D), suggesting that no substantial stable DNA-binding occurs in presence of bicalutamide. In conclusion, the slower recovery of fluorescence in presence of agonistic ligands is most likely caused by DNA binding dependent immobilization of ~10-15 % of ARs for approximately 45 seconds, usually accompanied by a slow down in diffusion of the mobile fraction (Table 5.1, Fig. 5.5).

5.4. Discussion

To obtain insight in the mechanism of blocking of AR transcription activation by antagonists, we investigated the behavior of the AR in living cells in presence of bicalutamide and OH-flutamide. Using FRAP on GFP-tagged ARs to determine intranuclear mobility, we show that a fraction (~10-15%) of agonist R1881 liganded ARs are immobilized for ~45 seconds (Fig. 5.2C-H, Table 5.1). Immobilization is dependent on DNA-binding, since the mutant GFP-AR(A573D), containing a mutation that completely disrupts DNA-binding (Brüggenwirth et al., 1998), did not show an immobile fraction (Fig. 5.2C,D and (Farla et al., 2004)). Similar high mobility was observed for other AR-DBD mutants (V581F and R585K), which do not bind DNA (data not shown). Binding of the AR antagonists bicalutamide and OH-flutamide resulted in a similar relatively high mobility as observed for the non-DNA-binding mutant AR (Fig. 5.3), strongly suggesting that the antagonists interfere with early steps in the mechanism of AR transcription activation i.e. stabilizing binding to promoters and enhancers of androgen regulated genes. This is supported by previous observations in vitro showing that bicalutamide-liganded AR, but not R1881-liganded AR, could be removed from the nuclear fraction by detergent

treatment (Berrevoets et al., 1993).

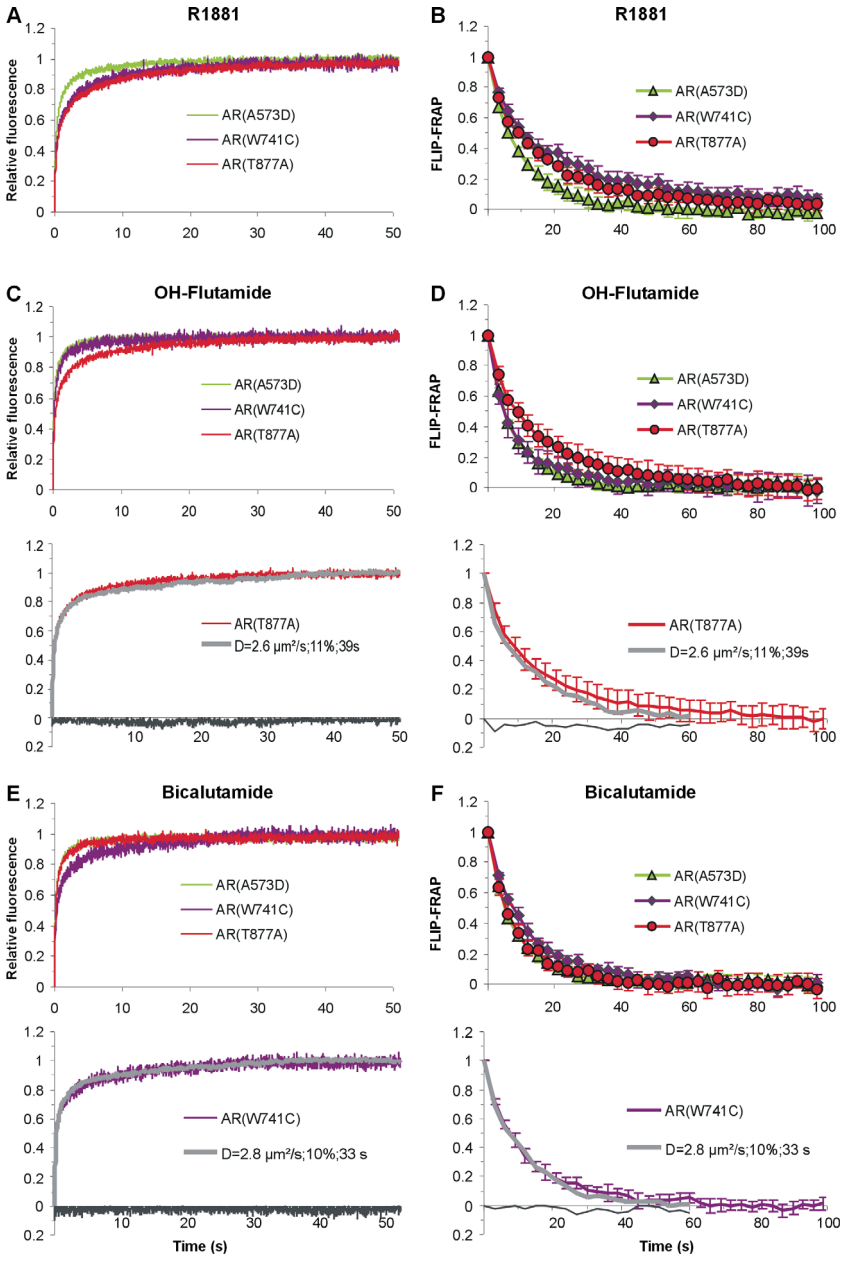


Figure 5.5. Prostate cancer related AR-LBD mutants display reduced mobility in presence of their agonistic ligands.
Nuclear mobility of antiandrogen resistant prostate cancer mutants AR(T877A) and AR(W741C) was investigated using two complementary FRAP assays (see also Fig. 5.2): strip-FRAP (A, C and E) and combined FLIP and FRAP (B, D and F). Intranuclear mobility of these mutants in the presence of 10^{-9} M R1881 (A and B), 10^{-6} M OH-flutamide (C and D) or 10^{-6} M bicalutamide (E and F) was studied. Mobility of non-DNA-binding GFP-AR(A573D) is plotted as a reference. Experimental settings were identical to those described in Fig. 5.2. Lower graphs in C-F show computer simulations corresponding to the average of best-fits of strip-FRAP and FLIP-FRAP models (data in Table I) of the experimental curves of GFP-AR(T877A) (C and D) and GFP-AR(W741C) in presence of 1 μM OH-flutamide (C and D) respectively bicalutamide (E and F). Absolute value of the residuals of computer simulated curves and experimental data are plotted below the x-axis.

However, studies using ChIP suggested that bicalutamide-bound ARs (Kang et al., 2002; Masiello et al., 2002) were present in DNA-protein complexes containing the corepressors N-CoR and SMRT on the promoter/enhancer region of androgen regulated prostate specific antigen (PSA) (Cleutjens et al., 1997; Shang et al., 2002; Kang et al., 2004). Assuming that the formation of DNA-bound repressor complexes is not a unique feature of the PSA gene, it is expected that antagonist-bound ARs also bind to other AR-specific promoters or enhancers. Although this seems to contrast to the data presented here (Fig. 5.3C,D and Table 5.1), it may be that repressor complexes on promoters are very short-lived (<1 s) and escape detection by FRAP.

In addition to the observed transient immobilization, R1881 also induced a slow-down of the effective diffusion of the mobile AR fraction, which was not observed after antagonist binding (Table 5.1). There are two explanations for this observation. First, mobile agonist-bound ARs in the nucleoplasm may, in contrast to antagonist-bound ARs, associate with coactivators forming large complexes exhibiting slower diffusion due to their size. This view is supported by published data indicating that binding of antagonists results in a conformation of the AR-LBD that does not allow AR amino/carboxyl-terminal (N/C)-interaction (Doesburg et al., 1997; Chang and McDonnell, 2002) and interactions with coactivators (Chang and McDonnell, 2002; Shang et al., 2002). A second explanation may be that the R1881-induced ability to bind DNA not only leads to relatively stable binding to AR-regulated promoters, but also to very transient, highly frequent binding to non-specific regions in the DNA. Such a scenario in which DNA interacting proteins 'scan' DNA in order to find their cognate binding sites has been suggested previously (Karpova et al., 2004; Phair et al., 2004; Sprague et al., 2004). For instance, it was reported that the glucocorticoid receptor binds very transiently (<200ms) to DNA with a

very high frequency, such that on average 80% of the GR is associated with DNA (Sprague et al., 2004). Although such a model does not completely explain our results of combined strip-FRAP and FLIP-FRAP experiments (since our data fit better to a model in which a small fraction is more stably immobilized), it is possible that the observed large mobile AR fraction (85-90%) exhibits these type of rapid interactions, resulting in the measured slow-down of this mobile fraction.

We further studied the effect of OH-flutamide and bicalutamide on the nuclear behavior of two mutant ARs (T877A and W741C) (Figs. 5.4 and 5.5), which were found in prostate cancer patients treated with OH-flutamide and bicalutamide, respectively (Haapala et al., 2001; Taplin et al., 2003). OH-flutamide can activate the transcription function of the T877A mutant and similarly, bicalutamide can activate the W741C mutant (Fig. 5.1C,D and Veldscholte et al., 1990; Hara et al., 2003). It has been suggested that binding of OH-flutamide to AR containing the T877A mutation induces a conformation of the AR-LBD that allows AR N/C-interaction and coactivator interaction (Doesburg et al., 1997; Chang and McDonnell, 2002; Shang et al., 2002), which results in agonistic activity of OH-flutamide. A similar mechanism may explain the agonist effect of bicalutamide on AR(W741C). Our data are in agreement with this hypothesis since both mutants, when exposed to bicalutamide (W741C) or OH-flutamide (T877A), showed the same kinetics as the R1881 liganded AR, i.e. all three mobility parameters measured here were in the same range (~10-15 % transiently immobile for 30-40 s, as well as a slow-down of the mobile fraction (Fig. 5.5 and Table 5.1)). As discussed above this slow down of diffusion can either be explained by very transient binding events or engagement of AR in larger complexes, or both.

The focal nuclear distribution pattern observed for agonist-bound wild-type AR is a common feature of steroid receptors. It has been described not only for AR (Tyagi et al., 2000; Tomura et al., 2001), but also for the ERα (Stenoien et al., 2000) and GR (Htun et al., 1999). Here we show that there is a direct relationship between transactivating capacity, reduced mobility (as a consequence of transient binding) and the occurrence of the focal pattern: in the presence of an agonistic ligand all three features are observed together, irrespective of whether the agonist is an antiandrogen activating a mutant or R1881 activating wild-type AR or AR(W741C) and AR(T877A) mutants. In contrast, in presence of an antagonist all three features were absent, i.e. the nuclear distribution was diffuse, mobility was not reduced, and no

transactivating capacity was observed. Previously, it was suggested that the focal pattern reflects binding to the nuclear matrix (Stenoien et al., 2000; Schaaf and Cidlowski, 2003; Stavreva et al., 2004). However, this is contradicted by observations that bicalutamide bound ARs were also found in the operationally defined nuclear matrix fraction (Tyagi et al., 2000), although they did not appear in foci (Fig. 5.4 and (Tyagi et al., 2000; Tomura et al., 2001)). For the aryl hydrocarbon receptor it was shown that foci correlated with transcription sites (Elbi et al., 2002) whereas for the GR no clear correlation of foci with pre-mRNA synthesis sites could be demonstrated (van Steensel et al., 1995). Our results (Fig. 5.4 and that of our previous studies (Farla et al., 2004)) strongly suggest a role for DNA binding in foci formation.

In conclusion, we have shown that, in contrast to R1881 binding, binding of the antagonists bicalutamide and OH-flutamide to AR: 1) did not induce detectable DNA-binding related immobilization, 2) did not give rise to a slow-down of the effective diffusion of the mobile fraction and, 3) did not induce the formation of a heterogeneous intranuclear distribution (foci). These three observations strongly suggest that the investigated antagonists interfere with events early in the transactivation function of AR leading to absence of stable DNA-binding dependent immobilization. This may be due to absence of appropriate stabilizing interactions with cofactors. In cell lines expressing AR mutants GFP-AR(W741C) and GFP-AR(T877A) bicalutamide and OH-flutamide, respectively, induce intranuclear immobilization and localization in numerous irregular shaped foci, suggesting these mutations restore the appropriate AR configuration and the capacity to stably bind to DNA.

5.5. Materials and methods

Constructs

Generation of pGFP-AR and pGFP(A573D) constructs, coding for amino-terminally tagged GFP-AR fusion proteins of which the expression is driven by a CMV promoter, has been described previously (Farla et al., 2004). pGFP-AR(W741C) and pGFP-AR(T877A) were generated by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) on pGFP-AR using sense primers 5'-CTGTCATTCAGTACTCCTGTATGGGGCTCATGGTGTTTG-3' and 5'-CTGCATCAGTTCGCTTTTGACCTGCTA-3' respectively and antisense primers 5'-CAAACACCATGAGCCCCATACAGGAGTACTGAATGACAG-3' and 5'-

TAGCAGGTCAAAAGCGAACTGATGCAG -3'. Presence of mutations was verified by sequencing.

Stock solutions of hormones

R1881 (Methyltrienolone) was purchased from NEN (Boston, MA); OH-flutamide was obtained from Schering (Bloomfield, NJ). Bicalutamide (Casodex) was a gift from AstraZeneca (Macclesfield, UK). R1881 and OH-flutamide were diluted to 1 µM and 1 mM stock solutions respectively in ethanol. Stocks were stored at –20 ˚C. Bicalutamide stocks of 1 mM in ethanol were freshly prepared directly before use.

Cell culturing and transactivation assays

Hep3B cells were cultured in αMEM (Cambrex, East Rutherford, NJ) supplemented with 2 mM L-glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin and 5% FBS (PAN Biotech GmbH, Aidenbach, Germany). For confocal microscopy, cells were seeded on glass cover slips in 6-well plates.

For transactivation assays Hep3B cells were seeded at a density of 100,000 cells/well in 24-well plates. Cells were transfected with 250 ng/well of AR expression construct and 500 ng/well of a luciferase reporter expression vector gene using FuGENE 6 (Roche, Indianapolis, IN). Four hours prior to transfection the medium was changed to medium containing 5% dextran charcoal treated hormone depleted FBS in the absence or presence of 1 nM R1881, 1 µM bicalutamide or 1 µM OH-flutamide. Twenty-four hours after transfection cells were lysed in lysis buffer (15% glycerol, 25 mM TrisPhosphate (pH 7.8), 1 mM DTT, 1% Triton X-100 and 8 mM $MgCl_2$). Luciferase activity was measured by addition of an equal volume of lysis buffer containing luciferine to cell lysates using Fluoroscan Ascent FL (Labsystems Oy, Helsinki, Finland). Luciferase activities were normalized to the activity in presence of 1 nM R1881.

Generation of stable cell lines

Stable cell lines expressing GFP-AR (mutants) were generated to ensure GFP-AR protein was expressed at physiological levels. Hep3B cells were transfected with 1 µg/well plasmid DNA using FuGENE 6 one day after plating in 6 wells plates. After 24 hours cells were trypsinized and plated in medium supplemented with 800 µg/ml Geneticin (G418 sulfate, Sigma, St. Louis, MO) in 10 cm tissue culture dishes. Clones were selected and checked for appropriate GFP-AR distribution and expression by confocal microscopy and Western

blotting. Stable cell lines were maintained as normal Hep3B cells in medium supplemented with 800 µg/ml Geneticin.

Western blotting

Stable cell lines expressing GFP-tagged AR constructs were cultured in 25 cm^2 flasks and allowed to grow fully confluent. Cells were washed with DPBS and lysed in 250 µl Laemli buffer (50 mM Tris, 10 mM DTT, 10% glycerol, 2% SDS and 0.001% bromophenolblue). Lysates were boiled and stored at –20 ˚C. Lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide gel using β-actin expression as loading control. Following electrophoresis proteins were transferred to nitrocellulose membranes. Blots were incubated with monoclonal antibodies F39.4.1, directed against the AR N-terminal domain (Zegers et al., 1991), or anti-β-actin (Sigma). Blots were subsequently incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark). Proteins were visualized using Super Signal West Pico Luminol solution (Pierce, Rockford, IL), followed by exposure to X-ray film.

Confocal microscopy

Cell imaging and FRAP studies were performed using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany) using the 488 nm laser line of a 200 mW Ar laser with tube current set at 6.1 A. All images and FRAP results were obtained using a 40x/1.3 NA oil immersion lens using filters which pass emission light between 505 and 530 nm. One day prior to confocal microscopy, media were changed to αMEM containing 5% dextran charcoal treated FBS. Prior to confocal microscopy, cell media were changed to αMEM containing 5% dextran charcoal treated FBS plus or minus 1 nM R1881, 1 µM bicalutamide or 1 µM OH-flutamide. Cells were incubated with the ligands for at least 1 hour before they were imaged or used for FRAP analysis.

FRAP nuclear mobility studies

Nuclear mobility in the presence of the various ligands was studied using two different FRAP methods (Houtsmuller et al., 1999; Houtsmuller and Vermeulen, 2001; Farla et al., 2004). In the first method (strip-FRAP) fluorescence in a narrow strip (~0.75 µm) spanning the width of the nucleus was monitored every 21 ms using 0.5% laser power of the 488 nm laser line, an intensity at which no significant monitor bleaching was observed. After 4 seconds the strip was bleached for 42 ms at maximum laser power. Fluorescence intensity in the strip was expressed relatively to the fluorescence

intensity before bleaching. All graphs were normalized to relative fluorescence of GFP-AR(A573D) in presence of 1nM R1881 after complete redistribution (Farla et al., 2004).

The second FRAP method uses a combination of FRAP and fluorescence loss in photobleaching (FLIP), of which the principle has been described previously (Hoogstraten et al., 2002; Farla et al., 2004). Briefly, a strip of ~1.1 µm was bleached at one pole of the nucleus for 0.6 s at maximum laser power. Subsequent post-bleach images were taken at 3 seconds intervals. Fluorescence intensities in the bleached strip and in a strip 10 µm from the bleached area were normalized to prebleach intensities. Differences in fluorescence ratio between the bleached strip (FRAP) and distal region of the nucleus (FLIP) were calculated, on each time point after bleaching. Maximal difference was set to 1 and values of the individual cells in the experiments were averaged. For both FRAP methods nuclei were selected with similar expression levels and similar dimensions.

For analysis of FRAP assays, experimental data were fitted to curves generated by computer software we developed to simulate FRAP of fluorescent molecules inside a finite ellipsoid volume representing the nucleus. Simulations were performed using fixed, experimentally obtained parameters, describing lens (beam shape and 3-D intensity distribution, during monitoring and during bleach pulse), GFP (quantum yield, susceptibility to bleaching) and nuclear properties (size and shape). Details on the simulations can be found in (Farla et al., 2004). Three protein mobility parameters, diffusion coefficient, bound fraction and duration of binding of individual molecules were varied. The three-dimensional diffusion constant (D_{eff}) was defined as stepsize^2/(6 × cycletime). The values of the parameters reported here are smaller than reported previously. This is because of the use of an improved microscope system, which enabled us to measure sooner after the bleach pulse. Furthermore the improved sensitivity of the detector allowed monitoring of the cells at low laser intensities, where no monitor bleaching occurs, whereas previously correction for monitor bleaching may have resulted in overestimation of the binding times. The D_{eff}'s reported here differ from those reported previously (Farla et al., 2004), because a one-dimensional model was used to fit the data. All simulations were performed five times and averaged, the average SD in each point of the simulation curves was < 0.01. Least square fitting of averaged simulated curves was used to determine which curves fitted best to the experimental data. Mobility parameters for all curves with $\sum (x_t - y_t)^2 / n <$ 0.002 were averaged, with x_t and y_t

representing the value of the experimental data and the simulation at a given time point of the curve, respectively, and n the number of time points. Mann-Whitney U-test were performed to assess statistical significance of differences in mobility parameters of GFP-AR (mutants) compared with GFP-AR(A573D).

5.6. References

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Chapter

6

Involvement of the molecular chaperone hsp90 in release of DNA-bound androgen receptors

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Submitted

6.1. Abstract

The androgen receptor (AR) is a member of the family of steroid receptors that activate transcription in a hormone-dependent manner. The molecular chaperone hsp90 plays a role in maturation of unliganded AR complexes with high-affinity hormone binding in the cytoplasm and most likely in translocation of ligand-bound receptor to the nucleus. Less is known about its role in the function of liganded AR in the nucleus. Therefore, we studied the behavior of the AR in the nucleus in absence and presence of the hsp90 inhibitor geldanamycin (GA). Fluorescence recovery after photobleaching (FRAP) revealed a DNA-binding dependent immobile fraction of ~10% in presence of GA, similar to that in absence of GA. In untreated cells ARs exchange between the mobile and immobile pool leading to a secondary recovery of fluorescence, whereas in presence of GA there is no exchange, suggesting that ARs are trapped in a DNA-binding dependent immobilized pool when hsp90 is inhibited. Remarkably, OH-flutamide bound AR was immobilized to a similar extent in presence of GA, whereas in its absence no immobilization could be detected. Our results indicate that molecular chaperone hsp90 plays a role in dissociation of agonist and antagonist liganded AR from DNA and possibly in nuclear recycling of receptors, which is prevented by GA.

6.2. Introduction

Androgens are essential in male sex development (Cunha et al., 1987) and stimulate the growth of the majority of prostate cancers (Feldman and Feldman, 2001; Trapman, 2001). Androgens exert their actions through the androgen receptor (AR), a member of the steroid receptor subfamily of nuclear receptors (Wahli and Martinez, 1991). In the cytoplasm, steroid receptors are in complex with chaperones and assemble in a stepwise fashion in a mature steroid receptor complex that can bind hormones with high affinity (reviewed in (Pratt and Toft, 1997; Pratt and Toft, 2003)). Functionally mature steroid receptor competent to bind hormones are thought to be in complex with heat shock protein (hsp) 90, p23 and an immunophillin (e.g. FKBP52) (Pratt and Toft, 1997). In addition to its role in receptor maturation functional hsp90 was shown to prevent degradation of nuclear receptors (Whitesell and Cook, 1996; Vanaja et al., 2002).

Geldanamycin (GA) binds specifically to hsp90 and inhibits its chaperone function (Whitesell et al., 1994). Inhibition of hsp90 activity by GA has been shown to inhibit nuclear import of glucocorticoid receptor (GR) (Galigniana et al., 1998) and AR (Georget et al., 2002). Crystal structures revealed that GA binds to an ATP-binding site in the amino-terminus of hsp90, which suggested GA acts by blocking ATP-binding to hsp90 (Prodromou et al., 1997; Stebbins et al., 1997). This inability to bind ATP may block binding of co-chaperones to hsp90, the co-chaperone p23 for example can only bind to hsp90 in its ATP-bound state (Sullivan et al., 1997).

Accumulating evidence indicates that molecular chaperones are not only involved in maturation of receptors, but also in nuclear import of steroid receptors via interaction with components of the cytoskeleton (Galigniana et al., 1998; Silverstein et al., 1999; Ozanne et al., 2000; Whitaker et al., 2004). Cytoplasmic dynein, a component of microtubules (Walker and Sheetz, 1993), was co-immunoprecipitated with GR and FKBP52 (Silverstein et al., 1999), which can bind to AR and GR complexes directly (Silverstein et al., 1999) as well as via hsp90 (Young et al., 1998; Cheung-Flynn et al., 2005), suggesting FKBP52 mediates GR and AR (Cheung-Flynn et al., 2005) import via interactions with the dynein component of microtubules. Indeed, GR retrograde movement from cytoplasm to the nucleus was shown to be dynein dependent (Harrell et al., 2004). The AR was also shown to interact with the actin-binding protein filamin and GFP-AR remained cytoplasmic in filamin deficient cells

(Ozanne et al., 2000). Reintroduction of filamin in these cells restored androgen induced nuclear translocation (Ozanne et al., 2000), indicating that the cytoskeleton is required for nuclear import of the AR.

It has been reported that the function of hsp90 is not limited to stabilization of steroid hormone receptors and cytoplasmic transport, but that hsp90 also influences steroid regulated processes in the cell nucleus. Hsp90 at low concentration allowed the estrogen receptor (ER) to form complexes with its cognate estrogen response element (ERE) whereas a high concentration of hsp90 specifically prevented *in vitro* ER binding to an ERE. Furthermore, it was shown that at high concentrations hsp90 could dissociate ER-ERE complexes and that dissociated ERs retain their ERE-binding ability (Sabbah et al., 1996). *In vitro* GA inhibited GR release from chromatin after hormone withdrawal, indicating that hsp90 might be involved in release of GR from chromatin (Liu and DeFranco, 1999). The hsp90 cochaperone p23 was found to inhibit transcriptional activity of AR, thyroid receptor (TR) and mineralocorticoid receptor (MR), whereas it stimulated transcription activated by GR (Freeman et al., 2000), although in another study p23 was reported to also inhibit transcriptional activity of GR (Wochnik et al., 2004). *In vitro*, p23 preferentially interacted with holo-TR and stimulated the dissociation of DNA-bound TR (Freeman et al., 2000). P23 and hsp90 also disrupted TR/retinoid X receptor (RXR) α and GR as well as some other transcriptional regulatory complexes (Freeman and Yamamoto, 2002). These observations indicate that chaperones hsp90 and p23 might play a role in disassembly of transcriptionally regulatory complexes and recycling of nuclear receptors in the nucleus.

Using fluorescence recovery after photobleaching (FRAP), green fluorescent protein (GFP)-tagged steroid receptors have been shown to display high mobility, both in total nuclei as well as on an array of MMTV-promoters. In presence of an agonist, slower recovery of fluorescence was observed and was shown to be DNA-binding dependent (Chapters 4 and 5 and (McNally et al., 2000; Stenoien et al., 2001; Schaaf and Cidlowski, 2003)). In cells depleted of soluble factors by permeabilization, nuclear GR and PR were completely immobilized. A mixture of purified molecular chaperones (including hsp90 and p23) and ATP restored nuclear mobility of GR and PR, whereas GA completely abolished the mobility of these receptors (Elbi et al., 2004). Therefore, it was proposed that molecular chaperones function as nuclear mobility factors. Remarkably, inhibition of hsp90 activity by GA in the same cell line resulted in faster recovery of GFP-GR fluorescence at an array of MMTV-promoters

(Stavreva et al., 2004).

Previously we have shown using a combination of FRAP methods and computer modeling, that in presence of an agonist a fraction of nuclear GFP-ARs is immobilized for ~1 minute (Chapter 4). Here we investigated using confocal microscopy and FRAP the effect of hsp90 inhibition by GA on nuclear mobility and localization of the AR. We found that a fraction of the receptors is permanently immobilized and dependent on the ability to bind DNA and required the AR-LBD. Surprisingly, we also detected a permanent immobilization of AR liganded with the antagonist OH-flutamide, accompanied by localization into foci, although previously we have shown that OH-flutamide liganded AR does not show stable DNA-binding (Chapter 5). Our results suggest a role for hsp90 in dissociation of DNA-bound ARs, in concordance with the results of *in vitro* studies (Freeman et al., 2000; Freeman and Yamamoto, 2002).

6.3. Results

AR translocation to the nucleus is inhibited by geldanamycin

Previously, we have generated Hep3B cells stably transfected with GFP-AR. In these cell lines GFP-AR is expressed at physiological levels (Chapters 4 and 5). GFP-AR is functional with respect to nuclear translocation after hormone addition and transactivation of target genes (Chapter 4). In the present study this cell line was used to investigate the role of hsp90.

GA has been reported to impair nuclear import of steroid receptors, including the AR, by blocking hsp90 activity (Georget et al., 2002). To confirm and extend those findings, we first investigated the behavior of the GFP-tagged ARs in Hep3B cells after addition of the androgen R1881 in absence or presence of GA (1.25 μg/ml). In absence of GA, addition of ligand R1881 resulted in translocation of AR from the cytoplasm to the nucleus, with equal concentrations of GFP-AR in the cytoplasm and nucleus being reached within 5 minutes after androgen addition (Fig. 6.1). After 20 minutes, ARs were almost exclusively localized in the nucleus, similar to previous reports (Chapter 4 and Georget et al., 1997). In the presence of GA, after addition of R1881, fluorescence remained cytoplasmic and hardly any import of the AR was observed within the 30 minutes time frame of the experiment (Fig. 6.1). Even after 3 hours, hardly any translocation of GFP-AR into the nucleus was

observed (data not shown), clearly indicating that GA blocks nuclear translocation of the AR.

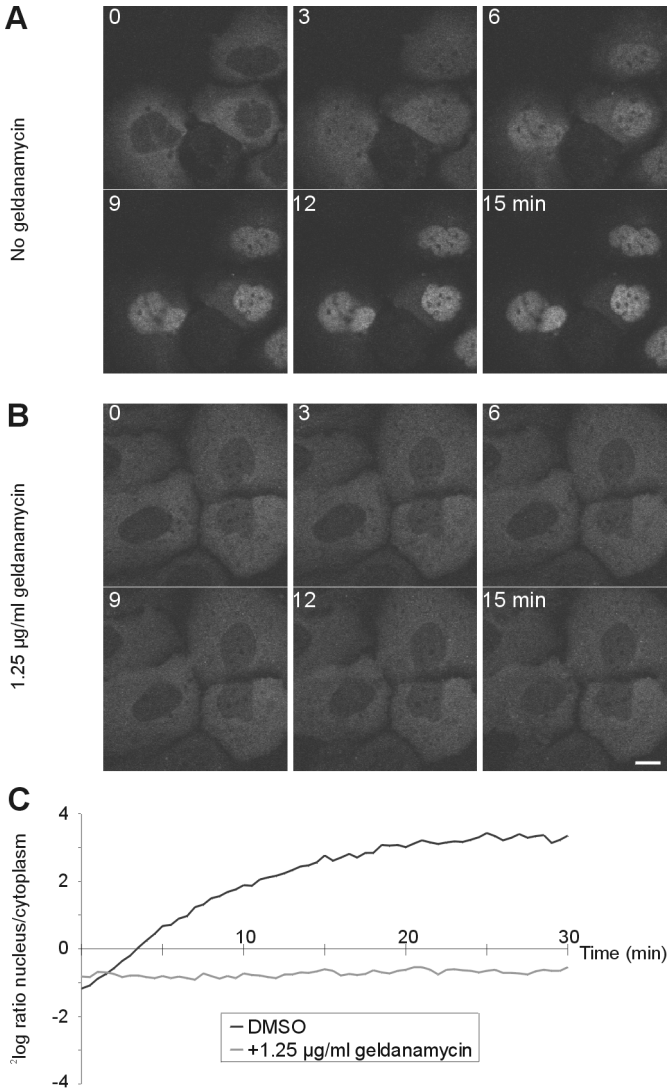

Figure 6.1. GA inhibits cytoplasmic to nucleus translocation of the AR.
(A and B) Cells expressing GFP-AR were treated with either 0.2% DMSO (vehicle) alone (no GA) (A) or 1.25 µg/ml GA (B). After 10 minutes incubation, 1nM R1881 was added to the medium. Subsequently cells were imaged every 30 seconds. Scale bar represents 10 µm. (C) Fluorescence intensities were measured in a region of the nucleus and a region just outside the nucleus of the cells. Graph showing the averages of the ^{2}log of the ratios of the background substracted fluorescence intensities in the cytoplasm and the nucleus of the three cells in the images in A and B.

Hsp90 inhibition by geldanamycin blocks release of the AR from DNA-binding and transcription related immobile nuclear complexes

Although it is well known that hsp90 plays a role in maturation of unliganded AR complexes with high-affinity hormone binding in the cytoplasm, less is known about its role in the function of liganded AR in the nucleus. To investigate this, we first exposed Hep3B cells stably expressing GFP-AR to agonist R1881 to allow translocation of receptors to the nucleus and subsequently studied nuclear mobility of GFP-AR in the absence or presence of GA. In absence of GA, a fraction of GFP-AR was transiently immobilized, but after approximately 1 minute fluorescence recovered to the same level as a non-DNA-binding mutant AR(A573D), as previously reported (Chapter 4). In presence of GA, fluorescence initially recovered to the same lower level as in its absence (up to 10s in Fig. 6.2A), but did not show a secondary fluorescence recovery (Fig. 6.2A).

Hsp90 is involved in folding and maturation of many proteins. To exclude that the reduced recovery of fluorescence observed in the presence of GA is caused by inappropriate folding of GFP which might result in a fluorescent protein more prone to bleaching, the parental Hep3B cell line was transfected with DNA coding for GFP-$(NFP)_4$-NLS, a fluorescent protein consisting of 5 copies of GFP of which only one is fluorescent and containing nuclear localization signals to target the protein to the nucleus (see Materials and Methods for details). The molecular weight of this protein is similar to that of GFP-AR. GA did not affect recovery of fluorescence of GFP-NFP_4-NLS (Fig. 6.2B), indicating that the reduced fluorescence recovery of GFP-AR observed in the presence of GA is likely caused by immobilization of a fraction of the GFP-AR protein and not by GA-induced artifacts, such as increased bleaching due to inappropriate folding of GFP.

To investigate if DNA-binding was involved in this immobilization we performed FRAP on Hep3B cells expressing GFP-AR(A573D), a GFP-tagged AR with a mutation in the DNA-binding domain preventing binding to androgen response elements in the DNA (Chapter 4 and Brüggenwirth et al., 1998). GFP-AR(A573D) showed dynamics similar to GFP-$(NFP)_4$-NLS, supporting our previous observations that it moves according to a model of (effective) diffusion. GA did not affect mobility of GFP-AR(A573D) indicating a role for DNA-binding in the GA induced permanent immobilization of a fraction of GFP-AR (Fig. 6.2C).

Since GFP-AR in presence of GA is immobilized in a DNA-binding dependent way we questioned whether this fraction could be identical to the fraction we previously observed after agonist induction. To quantify this fraction we performed computer simulations to fit the experimental data in Fig. 6.2A. As previously reported, our data of R1881 liganded AR in absence of GA fit best to a model in which a fraction of ~10-15% immobilized for ~40 seconds (Chapter 4). Curves generated by computer simulations fit best to FRAP curves of GA exposed wild-type cells, with a scenario where a similar fraction (~10%) of ARs is permanently immobilized (Fig. 6.2D). Therefore GA most likely results in permanent immobilization of the DNA-associated fraction.

Agonistic ligands result in a DNA-binding dependent intranuclear localization of the AR into foci (Fig. 6.2E, left panels and Chapter 5). GA did not alter the intranuclear localization of GFP-AR, nor did it result in formation of foci of GFP-AR(A573D) (Fig. 6.2E, right panels).

GA does not immobilize a constitutively active AR lacking the LBD

The region of interaction with hsp90 on the AR was mapped to amino acids 704-758 in the rat AR-LBD (Marivoet et al., 1992), which corresponds to amino acids 721-775 in the human AR. To assess if the immobilization of GFP-AR is due to direct interaction, we investigated the effect of GA on the mobility of an AR deletion mutant lacking the LBD. Previously we have shown that although this mutant can constitutively activate transcription from androgen regulated promoters, hardly any immobilization could be observed with FRAP (Chapter 4). FRAP did not reveal any effect of hsp90 inhibition by GA on the mobility GFP-ARΔLBD (Fig. 6.3A), nor did the homogeneous intranuclear distribution change (compare Fig. 6.3B with 6.3C). This suggests the permanent immobilization of GFP-AR observed with GA is caused by direct interaction of hsp90 via the AR-LBD.

GA does not affect ligand-dependent N/C-interaction of the AR

The AR contains an FXXLF-motif in its NTD (He et al., 2000; Steketee et al., 2002) that resembles LXXLL-motifs present in steroid receptor coactivators (Chen et al., 1997; Heery et al., 1997; Torchia et al., 1997; Voegel et al., 1998). Coactivators interact via these LXXLL-motifs with AF-2 in the LBD of steroid receptors in a ligand-binding dependent way. Similarly, the FXXLF-motif (^{23}FQNLF^{27}) in the AR-NTD can ligand-dependently interact with the carboxyl terminal AR LBD (N/C-interaction). To investigate whether hsp90 plays a role

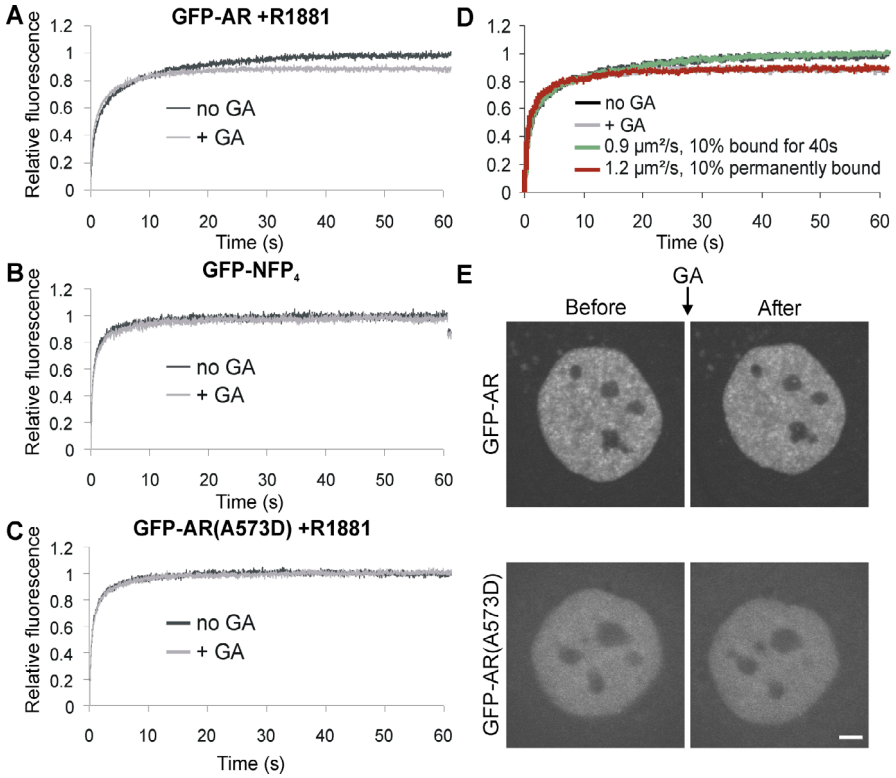

Figure 6.2. Inhibition of nuclear hsp90 by GA results in DNA-binding dependent permanent immobilization of a fraction of R1881 liganded receptors.
(A-C) Cells expressing GFP-AR (A) GFP-$(NFP)_4$, a GFP protein consisting of a single fluorescent GFP and 4 mutated GFPs which do not fluoresce (NFPs, see M&Ms for details) (B), or the non-DNA-binding mutant GFP-AR(A573D) (C) (Chapter 4 and (Brüggenwirth et al., 1998) were treated with R1881 for at least 1 hour, to allow import of the AR into the nucleus. Cells were subsequently treated with 1.25 µg/ml GA or vehicle (DMSO) only. Cells were incubated for 10 minutes before the first FRAP measurement was performed. Strip-FRAP was performed on the cells as previously described (Chapter 4). In short: a strip in the center of the nucleus was bleached with maximum laser intensity and fluorescence recovery in the bleached region was measured at an intensity at which hardly any bleaching occurs. All data are normalized to equilibrium fluorescence recovery of GFP-AR(A573D). (D) Computer simulations of the data in A and C showing reveal that a permanently DNA-binding dependent immobile fraction in the presence of GA. (E) Intracellular localization of AR in the presence of R1881, before and after exposure to GA. Scale bar represents 5 µm.

in the ligand dependent N/C-interaction of the AR, we checked if this interaction was influenced by inhibition of hsp90 activity by GA. To measure N/C-interaction, we performed fluorescence resonance energy transfer (FRET) experiments (Fig. 6.4A) using ARs tagged with yellow fluorescent protein YFP to its NH_2-terminal transactivating domain and cyan fluorescent protein CFP to the C-terminal ligand binding domain (Fig. 6.4B). Wild-type AR had a FRET efficiency of ~30% of the FRET observed with a fusion in which CFP and YFP

are linked (CFP-YFP). In presence of GA, YFP-AR-CFP showed FRET to a similar degree as in the absence of GA, indicating that FRET and thus N/C-interaction of the AR are not influenced by inhibition of hsp90 activity. Mutation of the AR ^{23}FQNLF^{27}-motif to ^{23}LQNLL^{27} results in reduced N-C interaction as previously reported (Dubbink et al., 2004), and accordingly in reduction of FRET (Fig. 6.4C and van Royen et al., unpublished data).

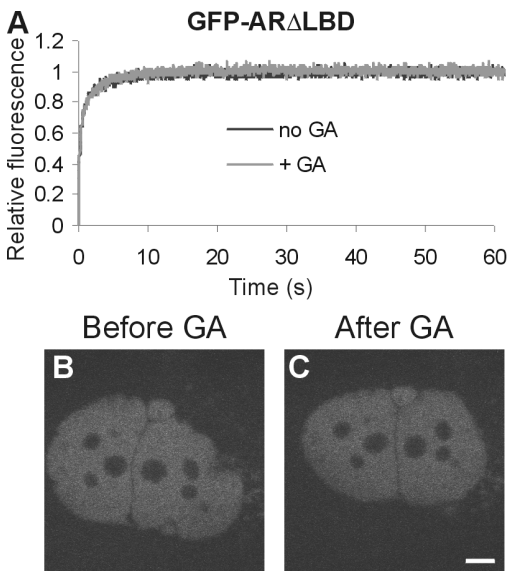

Figure 6.3. GA does not influence mobility and intranuclear distribution of a constitutively active AR mutant lacking the LBD.
(A) Strip-FRAP in nuclei of cells expressing GFP-ARΔLBD, a constitutively active AR mutant that does not require hormone for activation of transcription (Chapter 4), in the absence or presence of 1.25 µg/ml GA was performed on these nuclei to measure the mobility of this protein. Experimental setup was identical to that of Fig. 6.2. Data were normalized to maximum recovery in the absence of GA. Data represent average of at least 10 cells. (B and C) Representative images of a cell expressing GFP-ARΔLBD before (B) and after (C) addition of GA. Scale bar represents 5 µm.

GA exposure results in permanent immobilization of a fraction of OH-flutamide liganded AR

As previously reported, FRAP did not reveal immobilization of the AR in presence of the antagonists bicalutamide and OH-flutamide (Fig. 6.2A and Chapter 5), although in ChIP assays AR was reported to form inactive repressor complexes on promoter/enhancer regions of AR-regulated promoters (Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004). Surprisingly, exposure of GFP-AR expressing Hep3B cells to GA after primary exposure to OH-flutamide, resulted in permanent immobilization of a fraction of the receptors to a similar extent as in R1881 treated cells (Compare Fig. 6.5A with Fig. 6.2A).

Previously, we have shown that an agonist such as R1881, in addition to transient immobilization of a fraction of receptors, resulted in a focal intranuclear distribution of GFP-AR. However GFP-AR bound by an

antagonist, such as OH-flutamide showed a homogeneous intranuclear distribution of receptors (Chapter 5). Therefore, we wondered if GA would also alter the intranuclear distribution of OH-flutamide liganded AR. Addition of GA changed the homogeneous intranuclear distribution of OH-flutamide liganded wild-type AR (Fig. 6.5B) to a pattern showing focal accumulations (Fig. 6.5C). In contrast GA did not alter the intranuclear distribution of GFP-AR(A573D) (Fig. 6.5D and E). Our data suggest that OH-flutamide bound AR fails to stably bind/immobilize in a DNA-dependent manner, but that hsp90 is required for rapid release of both agonist and antagonist bound receptor. Due to the very transient nature of the immobilization of OH-flutamide liganded AR in absence of GA it does not accumulate to an extent where foci are visible, whereas an agonist such as R1881 results in transient DNA-binding dependent immobilization of the receptor and foci formation. GA treatment of cells in the presence of OH-flutamide results in fixation of transcriptionally unproductive AR and the formation of GFP-AR foci (Fig. 6.6).

6.4. Discussion

Previously we have shown that binding of an agonist results in a decreased mobility of GFP-AR, caused by the transient immobilization of a 10-15% fraction of nuclear ARs for 40-60 seconds (Fig. 6.2 and Chapters 4 and 5). In this report we show that inhibition of the hsp90 chaperone activity by GA, results in a DNA-binding dependent permanent immobilization of a fraction of nuclear ARs (Fig. 6.2), suggesting that GA prevents the dynamic exchange of ARs of the transient immobile fraction with binding sites in DNA observed in unexposed cells. Indeed, curve fitting based on computer simulated FRAP curves indicated that an immobile fraction of ~10%, similar to the transiently immobile fraction found in untreated cells.

In addition the observation that not all available ARs are eventually immobilized suggests that the number of binding sites for ARs is limited. We estimate a nucleus contains $\sim 3 \times 10^4$ ARs, if there are $\sim 5 \times 10^2$ AR regulated genes with on average 3 AREs to which ARs bind as dimers, this would result in 3×10^3 binding sites for AR. Recently, chrosome wide mapping of chromosomes 21 and 22 revealed 57 binding sites for ER (Carroll et al., 2005). Since genes on these chromosomes represent about 2% of the total number of genes, it is estimated that the total genome contains $\sim 3 \times 10^3$ ER binding sites. This would be in the same order of magnitude as the number of AR binding

sites assessed in our calculation.

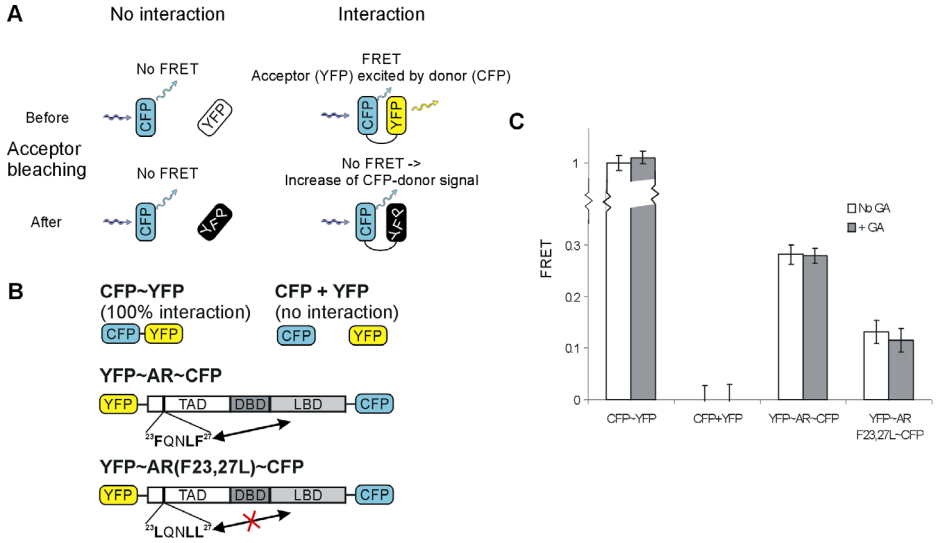

Figure 6.4. GA does not influence ligand-dependent amino-carboxyl terminal interactions of the AR.
(A) Fluorescence resonance energy transfer (FRET) is a method to measure interaction between two fluorescent molecules (e.g. CFP and YFP). If two proteins tagged with CFP and YFP are in close proximity, non-radiative energy transfer of CFP to YFP will occur and emission light with a wavelength specific to YFP will be observed. Acceptor photobleaching was used to measure FRET. In this method YFP is bleached, thereby preventing the energy transfer of CFP to YFP, which results in increased emission of light at a CFP specific wavelength. The acceptor bleaching method has the advantage that an increase in CFP emission light can be directly attributed to loss of energy transfer to YFP. (B) Schematic representation of constructs measured in (C). NTD: amino-terminal domain, DBD: DNA-binding domain, LBD: ligand binding domain. The AR-NTD ligand dependently interacts with the AR-LBD through an inter- or intra-molecular interaction. Mutation of phenylalanines on positions 23 and 27 in the AR-NTD into leucines has been shown to result in loss of N-C interaction in yeast- and mammalian-hybrid studies (Dubbink et al., 2004). (C) FRET in nuclei of cells stably expressing wild-type AR or the mutant AR(F23,27L) (Dubbink et al., 2004) tagged with YFP to the amino- and CFP to the carboxyl-terminus. Cells were exposed to 100 nM R1881 for at least 1 hour and subsequently treated with 1.25 µg/ml GA or vehicle (DMSO). FRET was quantified by measuring the increase in CFP-emission after bleaching of YFP. The FRET value was normalized to maximum FRET (CFP-YPP fusion) and minimal FRET (separate CFP and YFP) signals. Data present average and 2*SEM of 3 independent experiments performed on different days on at least 10 cells.

We have previously reported that antagonist bound ARs, in contrast to agonists, do not show stable DNA-binding dependent immobilization (Chapter 5). However, we could not exclude that very transient binding by antagonist liganded receptors occurs. Our present data indicate that similar to agonist liganded receptors, in presence of GA, a fraction of the OH-flutamide liganded

ARs is permanently immobilized (Fig. 6.5A). Moreover GA also induced DNA-binding dependent intranuclear accumulations (Fig. 6.5B-E), similar to the situation in the presence of an agonist (Fig. 6.2D and Chapters 4 and 5). This would explain the seemingly contrasting findings of our previous FRAP studies and chromatin immunoprecipitatin (ChIP) studies by others, in which antagonists liganded ARs were shown to bind to promoter/enhancer regions of androgen regulated genes, although no transcriptionally active complex could be detected in this case (Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004).

Since hsp90 in the cytoplasm is required to obtain a mature AR with high ligand binding affinity, it may be argued that GA also influences ligand dependent events in the nucleus. However, GA exposure did not affect ligand-dependent N/C-interaction (Berrevoets et al., 1998; He et al., 2000; Dubbink et al., 2004) of the AR (Fig. 6.3). Furthermore, in presence of GA, agonist dependent focal accumulations of GFP-AR (Chapter 4 and (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001)) that have also been reported for ER (Htun et al., 1999; Stenoien et al., 2000), GR (Htun et al., 1996; Schaaf et al., 2005) and mineralocorticoid receptor (MR) (Fejes-Tóth et al., 1998) and were

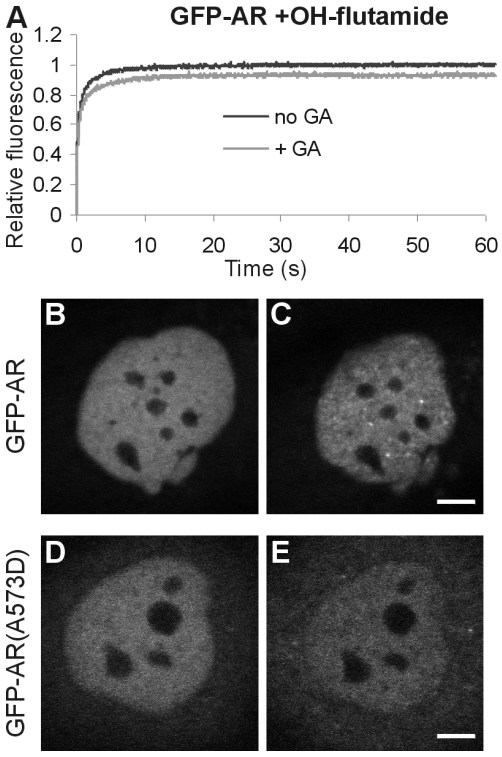

Figure 6.5. OH-flutamide liganded ARs are permanently immobilized and show nuclear foci in presence of GA.
Cells expressing GFP-AR were exposed to 1 µM OH-flutamide and subsequently treated with 1.25 µg/ml GA or vehicle (DMSO) only (no GA). (A) Mobility of GFP-AR in the presence of antagonist OH-flutamide in the absence or presence of GA was measured by strip-FRAP as described in Fig. 6.2. Data were normalized to maximum recovery in the absence of GA. (B-E) Intranuclear localization of GFP-AR (B and C) and GFP-AR(A573D) (D and E) in presence of OH-flutamide before (B and C) and after (D and E) treatment with GA. GA results in intranuclear foci of wild-type AR, whereas the AR(A573D) still shows a homogeneous intranuclear distribution. Scale bar represents 5 µm.

shown to correlate with ligand affinity (Schaaf et al., 2005), were still observed (Fig. 6.2D). Moreover, it has been reported that GA does not dissociate ligand from receptors (Czar et al., 1997; Liu and DeFranco, 1999). Therefore it is not likely that the permanent immobilization observed in presence of GA is caused by disruption of ligand binding.

GA prevents binding of ATP to hsp90 and thereby disrupts its interaction with p23 (Sullivan et al., 1997). Overexpression of p23 was inhibited transcriptional activity of AR, thyroid receptor (TR) and MR, whereas it stimulated transcription activated by GR (Freeman et al., 2000). Although another study also reported inhibition of GR transcriptional activity by p23 (Wochnik et al., 2004). GR transcription inhibition by p23 required interaction of p23 with hsp90 (Wochnik et al., 2004). *In vitro* experiments have shown that hsp90 or its cochaperone p23 could promote dissociation of ER (Sabbah et al., 1996), GR (Liu and DeFranco, 1999; Freeman and Yamamoto, 2002) and TR (Freeman et al., 2000; Freeman and Yamamoto, 2002) from their cognate response elements. p23 preferentially interacted with holo-TR and stimulated the dissociation of DNA-bound TR (Freeman et al., 2000; Freeman and Yamamoto, 2002). These observations and the results presented here support a model in which chaperones hsp90 and p23 play a role in disassembly of complexes and possibly recycling of receptors.

In cells which had been depleted of soluble factors, including chaperones, by permeabilization with digitonin, GFP-tagged GR and PR were completely immobilized (Elbi et al., 2004). Reintroduction of a chaperone mixture including hsp90 and p23 was shown to restore mobility of the receptors. In addition, a high concentration of GA (10 μg/ml) resulted in complete immobilization of the receptors, with almost no recovery of fluorescence being observed after photobleaching. Therefore, it was suggested that molecular chaperones function as nuclear mobility factors. In contrast, we never observed permanent immobilization of the total nuclear pool of receptors by GA even at high concentrations (data not shown). Remarkably, in the same cell line, in presence of lower GA concentrations (2.5 μg/ml) on an array of MMTV-promoters more rapid exchange of GRs was reported (Stavreva et al., 2004), suggesting that hsp90 decreases receptor mobility rather than increases it. In concordance with this latter observation we also seem to observe slightly more rapid recovery of fluorescence of the freely diffusing fraction of agonist liganded GFP-AR in presence of GA. In contrast, GA did not result in an observable immobile fraction on the promoter repeat. It might be argued that

receptor dynamics in total nuclei are different from that on the promoter repeat. However, dynamics of the PR on the repeat were shown to be identical to the dynamics in total nuclei (Rayasam et al., 2005).

Chromatin remodeling complexes of the SWI/SNF-family have been shown to coactivate transcription by steroid receptors, including the AR (Rouleau et al., 2002; Marshall et al., 2003). On a mouse mammary tumor virus (MMTV) promoter nucleosomal array, GR and PR were shown to bind and attract SWI/SNF chromatin remodeling complexes, resulting in instantaneous loss of GR and PR complexes from the promoter (Fletcher et al., 2002; Rayasam et al., 2005). Moreover using a UV-laser crosslinking assay GR and SWI/SNF-complexes were shown to be cyclically associated with specific nucleosome regions and to result in H2A/H2B-core rearrangement of nucleosomes (Nagaich et al., 2004). Therefore it is likely that both hsp90 activity as well as SWI/SNF-family members regulate the dynamic behavior of steroid receptors on promoters.

Based on our findings, we propose a model for hsp90 action depicted in Fig.6.6. Our findings suggest that action of the molecular chaperone hsp90 and/or its associated cochaperones (e.g. p23) is required to remove ARs from DNA. Remarkably, antagonist liganded receptors are also immobilized by GA treatment, supporting the findings of ChIP assays (Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004), where antagonist liganded receptors were shown to bind to promotor regions of androgen regulated although no active transcription complexes were formed. It appears that the action of hsp90 or its associated chaperones (e.g. p23) is required to remove ARs from DNA and acts before activation of transcription.

6.5. Materials & Methods

Constructs

Generation of cDNA constructs coding for N-terminally GFP-tagged ARs GFP-AR and the non-DNA-binding GFP-AR(A573D) has been described previously (Chapter 4). The GFP-AR constructs were allowed to stablyintegrate into AR^- Hep3B cells using G418 as selecting agent (Chapter 4).

pGFP-$(NFP)_4$-NLS, coding for a protein of the size of 5 GFPs, of which only one is fluorescent and the remainder are non fluorescent proteins (NFPs). NFPs are GFP proteins in which a mutation has been introduced in amino acid 67 (Gly67Val), which is essential for an active GFP-chromophore (Cubitt et al.,

1995). pGFP-(NFP)$_4$-NLS was generated by insertion of multiple *Pin*AI-*Xma*CI-fragments containing NFP without the start codon (ATG) from pGEM-

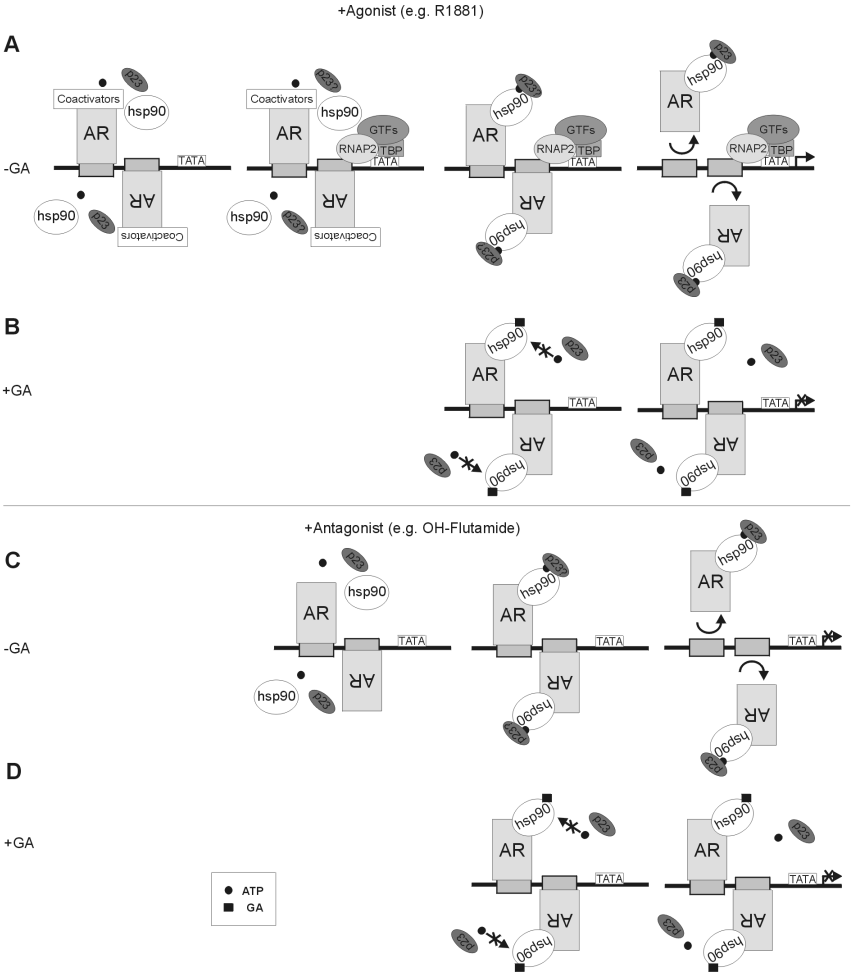

Figure 6.6. Model for the role of hsp90 in removing DNA-bound AR from DNA.

(A) In presence of an agonist the AR dimers bind to AREs in promoter/enhancer regions of androgen regulated genes, together with coactivators. This results in a transient immobilization of AR in the order of 1 minute and attraction of TATA-binding protein (TBP) and general transcription factors (GTFs) that facilitate gene transcription by RNA-polymerase II (RNAP2). The AR is then removed from the DNA by ATP-dependent action of hsp90, which may involve the co-chaperone p23, resulting in activation of transcription by RNAP2. (B) The presence of GA prevents ATP binding to hsp90 and therefore renders it dysfunctional and AR cannot be removed from DNA, resulting in permanent immobilization of DNA-bound receptors (which is observed as reduced recovery of FRAP and intranuclear accumulations of AR). (C) In presence of an antagonist such as OH-flutamide ARs interact very transiently with AREs, however no functional transcription complex is formed. ARs are removed from DNA by in an hsp90 dependent way. (D) In presence of GA, hsp90 is not functional and cannot remove ARs from DNA, resulting in permanent immobilization of DNA-bound ARs.

NFP in the *Xma*CI-site of pGFP-NLS. The ATG of NFP was removed by PCR on pEGFP-C1 (Clontech, Palo Alto, CA) using forward primer 5'-GCGCTACCGGTCGCCACCGTGAGCAAGGGC-3' and a reverse primer priming downstream of the GFP-coding region. The resulting PCR-fragment was cloned into p-GEM-T-Easy (Promega, Madison, WI) to result in pGEM-GFP. pGEM-NFP was generated by QuikChange (Stratagene, La Jolla, CA) mutagenesis on pGEM-GFP using primers 5'-GTGACCACCCTGACCTACGTAGTGCAGTGCTTCAGCCGC-3' and 5'-GCGGCTGAAGCACTGCACTACGTAGGTCAGGGTGGTCAC-3'. pGFP-NLS contains three tandem copies of the SV40 T-antigen nuclear localization signal, which were inserted by cloning the *Bgl*II/*Bam*HI-fragment from pEYFP-Nuc (Clontech) in the *Bam*HI site of pEGFP-C1(Clontech). Hep3B cells were transfected with pGFP-$(NFP)_4$-NLS, pCYFP, pEYFP (Clontech) or pECFP (Clontech) using FuGENE6 (Roche, Indianapolis, IN) one day before they were subjected to FRET analysis.

The cDNA construct coding for AR with YFP and CFP tags (pYFP-AR-CFP) and its F23,27L variant used for the FRET assay were generated by combining sequence of an N-terminally YFP-tagged AR (pYFP-AR) or F23,27L-mutant AR with a C-terminally CFP-tagged AR (pAR-CFP). N-terminally YFP-tagged AR (pYFP-AR) was generated by replacing EGFP in pGFP-AR (Chapter 4) by a *Nhe*I/*Bgl*II fragment from pEYFP-C1 (Clontech), with additional His_6 and haemaglutinin (HA) tags between the *Nhe*I and *Nco*I sites. The F23,27L variant was generated by QuikChange mutagenesis on pYFP-AR using previously described primers (Dubbink et al., 2004). pAR-CFP coding for carboxyl-terminally CFP-tagged AR was generated from pAR-GFP. pAR-GFP in which two AR fragments, a *Hind*III/*Kpn*I C-terminal AR fragment from pcDNA-AR0mcs (Sui et al., 1999) lacking the AR stop codon and a N-terminal *Hind*III AR fragment from pAR0 (Brinkmann et al., 1989) were sequentially inserted in pEGFP-N3, followed by the introduction of a spacer sequence coding for a hexamer repeat of alanines and glycines in the *Sac*II site between the AR and CFP, using annealed oligos (5'-GGGTGCTGGAGCAGGTGCTGGAGCAGGTGCTGGAGCCGC-3' and 5'-GGCTCCAGCACCTGCTCCAGCACCTGCTCCAGCACCCGC-3'). pAR-CFP was generated by insertion of a *Bam*HI/*Not*I fragment containing ECFP coding sequence from pECFP-N3 (Clontech, Palo Alto, CA) to replace EGFP in pAR-GFP pAR-ECFP. By insertion of the *Nhe*I/*Asp*718I fragment of pYFP-AR and the F23,27L variant, in the corresponding sites of pAR-CFP cDNA constructs coding for YFP and CFP tagged AR was obtained. pCYFP encoding an ECFP-

EYFP chimera was kindly provided by dr. Wim Vermeulen (Erasmus MC, Rotterdam, The Netherlands).

Cell culture and transfections

Previously, we have described the generation of the AR^- Hep3B cell line stably transfected with GFP-AR using G418 as selecting agent (Chapter 4). For FRET experiments Hep3B cells were transfected with pGFP-$(NFP)_4$-NLS, pCYFP or pEYFP (Clontech) and pECFP (Clontech) using FuGENE6 (Roche, Indianapolis, IN) one day before they were subjected to FRET analysis. Cells were plated on glass cover slips in 6-wells plates at approximately 300,000 cells/well 2-3 days before experiments. One day prior to confocal microscopy, media were changed to αMEM containing 5% dextran charcoal treated FBS. Prior to FRAP studies cell media were changed to αMEM containing 5% dextran charcoal treated FBS plus 1 nM R1881 or 1 μM OH-flutamide. Cells were incubated with ligand for at least 2 hours before they were used for FRAP or FRET analysis.

Stock solutions of chemicals

R1881 (methyltrienolone (NEN, Boston, MA) and OH-flutamide (Schering, Bloomfield, NJ) stock solutions were prepared in ethanol. GA was obtained from Sigma (St. Louis, MO) and stored as 2.5 μg/ml stock solution in DMSO. All stock solutions were stored at –20°C.

Confocal microscopy

Cells were allowed to warm up to 37°C on the microscope stage for five minutes. GA or an equal amount of vehicle (DMSO) was then added to the medium. Cells were incubated for another 10 minutes, before the first measurements were performed.

Cell imaging, FRAP and FRET studies were performed using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany) using the 488 nm laser line of a 200 mW Ar laser with tube current set at 6.1 A. For FRET the 458 and 514 nm laser lines of the same laser were used (see below). All images and FRAP results were obtained using a 40x/1.3 NA oil immersion lens using filters resulting in detection of 505-530 nm emission light.

FRAP

FRAP was performed as described previously (Chapters 4, 5 and Houtsmuller et al., 1999; Houtsmuller and Vermeulen, 2001). In short fluorescence in a narrow

strip (~0.75 μm) spanning the width of the nucleus was monitored every 21 milliseconds using the 488 nm laser line at 0.8 μW, an intensity at which hardly any monitor bleaching was observed. After 2 seconds the strip was bleached for 42 milliseconds at maximum laser power. Fluorescence intensity in the strip was expressed relatively to the fluorescence intensity before bleaching. All graphs were normalized to relative fluorescence of GFP-AR(A573D) in presence of 1nM R1881 after complete redistribution (Chapter 4).

For analysis of FRAP assays, experimental data were fitted to curves generated by computer software developed to simulate FRAP of fluorescent molecules inside a finite ellipsoid volume representing the nucleus. Details on the simulations can be found in (Chapters 4 and 5). Three protein mobility parameters, diffusion coefficient, bound fraction and duration of binding of individual molecules were varied. All simulations were performed five times and averaged. Least square fitting of averaged simulated curves was used to determine which curves fitted best to the experimental data. Mobility parameters for all curves with $\sum (x_t - y_t)^2 / n < 0.002$ were averaged, with x_t and y_t representing the value of the experimental data and the simulation at a given time point of the curve, respectively, and n the number of time points.

FRET measurement by acceptor photo bleaching

CFP and YFP images were collected in separate sequential images using laser intensities that do not result in bleaching. CFP was excited with the 458 nm laser lines of an Ar laser and emission light of 470-500 nm was detected. YFP was excited with the 514 nm laser line at moderate laser power and its emission light with a wavelength >560 nm was detected. 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 (~50%) of the nucleus using the 514 nm argon laser line at high laser power. Directly after acceptor (YFP) photobleaching a second YFP and CFP image pair was collected. CFP and YFP images were analyzed using the Carl Zeiss' LSM510 software. After background subtraction the apparent FRET efficiency was calculated as:

$$FRET = \frac{(CFP_{after} - CFP_{before}) * YFP_{before}}{(YFP_{before} - YFP_{after}) * CFP_{after}}$$

in which the relative CFP increase due to YFP bleaching is corrected for the fraction of YFP bleached. FRET efficiencies were normalized relative to the values obtained with co-expressed CFP and YFP (No FRET) were and corrected for CYFP values (100% FRET).

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Chapter

7

Involvement of the microtubule cytoskeleton in nuclear import of the androgen receptor

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7.1. Abstract

The androgen receptor (AR) is an intracellular receptor that activates transcription after binding of androgen. In absence of androgen ARs are predominantly cytoplasmic and binding of androgen induces translocation of receptors to the nucleus. However, little is known about the molecular mechanism of receptor translocation. Microtubules (MTs) are structural components of the cytoskeleton and among other functions have been shown to be involved in transport of organelles. We investigated the role of the MT cytoskeleton on hormone induced nuclear import of green fluorescent protein (GFP)-tagged AR. Exposure of cells stably expressing GFP-AR to the MT destabilizing agent nocodazole in the absence of androgens, already resulted in some nuclear translocation, suggesting MTs may play an active role in cytoplasmic retention. Furthermore, nocodazole treatment resulted slowed down nuclear import of GFP-AR after activation by ligand. To investigate the role of MT based transport in more detail, we studied translocation of GFP-AR in mouse embryonic fibroblasts (MEFs) that are deficient for the MT plus-end interacting proteins cytoplasmic linker proteins 115 and 170 ($CLIP115^{-/-}$/$CLIP170^{-/-}$) or CLIP-associated protein 2 ($CLASP2^{-/-}$). Ligand induced import of GFP-AR was identical in $CLASP2^{-/-}$ and wild-type MEFs, suggesting that CLASP2 stabilization of MTs is not required for AR nuclear import. GFP-AR import in $CLIP115^{-/-}$/$CLIP170^{-/-}$ cells was severely impaired and sometimes completely absent. Our preliminary results suggest that efficient nuclear import of the AR requires an intact MT cytoskeleton and may involve a role for CLIPs in loading of ARs on MTs.

7.2. Introduction

The androgen receptor (AR) is an intracellular receptor that functions as a transcription factor activated by androgens such as testosterone and dihydrotestosterone. The AR is essential for development and maintenance of the male phenotype and is involved in the pathogenesis of prostate cancer. The AR is a member of the family of steroid receptors, which also includes the estrogen receptor (ER) α and β, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and progesterone receptor (PR).

In absence of hormone, AR, GR, MR and PR are mainly localized to the cytoplasm and are in complex with chaperone proteins, including heat shock proteins, p23 and a large immunophilin (e.g. FKBP51 or 52) (Pratt and Toft, 1997). To be able to exert their function as a transcription factor, those receptors need to translocate to the nucleus. It is assumed that the chaperone proteins keep steroid receptors in the cytoplasmic compartment, possibly by masking of a bipartite nuclear localization signal (NLS) in the hinge region of the AR (Jenster et al., 1993). Upon hormone binding these factors dissociate from the receptors, allowing the receptors to translocate to the nucleus.

However, more recent observations have shown that the chaperones are most likely also involved in the active (facilitated) transport of the receptors to the nucleus and involves interactions with components of the cytoskeleton (Galigniana et al., 1998; Ozanne et al., 2000; Harrell et al., 2004; Whitaker et al., 2004). The AR was shown to interact with the actin-binding protein filamin and GFP-AR remained cytoplasmic in filamin deficient cells, whereas reintroduction of filamin restored androgen induced nuclear translocation (Ozanne et al., 2000), indicating that the actin-cytoskeleton most likely is also involved in nuclear import of the AR. In 3T3 cells in which the cytoskeleton was disrupted by cytoskeleton destabilizing agents, in contrast to intact cells, the hsp90 inhibitor geldanamycin did not impair dexamethosone induced import of GFP-GR. Withdrawal of the cytoskeletal destabilizing agents restored normal cytoskeletal architecture and geldanamycin prevented dexamethasone induced GFP-GR translocation (Galigniana et al., 1998). Cytoplasmic dynein, a minus-end directed MT motor (Walker and Sheetz, 1993), was co-immunoabsorbed with GR and the immunophilin FKBP52 (Silverstein et al., 1999), a protein that can bind to AR and GR complexes directly (Silverstein et al., 1999) as well as via hsp90 (Young et al., 1998; Cheung-Flynn et al., 2005), suggesting FKBP52 might mediate GR and AR import via interactions with dynein. Indeed, GR

retrograde movement from cytoplasm to the nucleus was shown to be dynein dependent (Harrell et al., 2004).

MTs are an important component of the cytoskeleton. They are involved in chromosome segregation during cell division, organelle transport, cell movement and cell-cell contact. MTs have a polarized structure with slow growing minus ends and fast growing plus ends. Most minus ends are organized in the MT organizing center (MTOC), whereas the plus ends are directed to the outside of the cell. MT plus ends are constantly elongating and shrinking, a process referred to as dynamic instability (Desai and Mitchison, 1997; Galjart, 2005). Several proteins, some of which are destabilizing whereas others promote elongation of MTs, regulate the dynamic properties of MTs. Proteins specifically associated with the plus ends of MTs are referred to as plus-end tracking proteins (+TIPs) (Schuyler and Pellman, 2001). One of these +TIPs is cytoplasmic linker protein (CLIP)-170. A green fluorescent protein (GFP) fusion of CLIP170 was shown to localize with growing tips of MTs (Perez et al., 1999). CLIP170 has also been shown to be involved in attachment of endocytic vesicles to MTs (Pierre et al., 1992) and to transiently associate with prometaphase chromosome kinetochores (Dujardin et al., 1998). In fibroblasts CLIP170 colocalized with dynein and dynactin at MT plus ends (Valetti et al., 1999; Vaughan et al., 1999) and deficiency of the protein deregulates dynactin localization (Lansbergen et al., 2004). Since it was suggested that dynactin functions as a cargo loading protein for minus-end directed transport, CLIP170 might play a role in dynein-mediated functions. CLIP115 is a CLIP170 homologue that is mainly expressed in neurons. When expressed in fibroblasts, it localizes to MT plus-ends, like CLIP170 (Hoogenraad et al., 2000). In a yeast-two-hybrid screen for proteins that interact with MTs as well as with CLIP115 and CLIP170, two closely related proteins were identified: CLIP-associating proteins (CLASPs) 1 and 2,. CLASP1 and 2 were found to stabilize distal ends of MTs at the leading edge of the fibroblasts (Akhmanova et al., 2001).

Here, we investigated the role of MTs in general, and the +TIPs CLIP115/CLIP170 and CLASP2 in particular in the transport of ARs to the cell nucleus. Our findings suggest a role for MTs and CLIPs in efficient translocation of AR, whereas stable MTs seem not to be required.

7.3. Results

The MT disrupting agent nocodazole decreases the rate of nuclear import

Previously we have shown that in Hep3B cells that stably express transfected GFP-AR at physiological levels, the fluorescently labeled AR is predominantly localized in the cytoplasm (Fig. 7.1A, Chapters 4 and 5). It has been suggested that an important aspect of the mechanism of action that governs AR localisation is the shielding/exposure of the NLS in a ligand dependent manner. In addition, the cytoplasmic MT network may play a facilitating role in translocation of AR to the nucleus. To assess the role of MTs in AR import we exposed these cells to the MT disrupting agent nocodazole and imaged fluorescent ARs using confocal microscopy. Nocodazole treatment resulted in some translocation of the receptor into the nucleus (Fig. 7.1B). Addition of agonist R1881 resulted in translocation of AR to the nucleus in both untreated and nocodazole treated cells (Fig. 7.1A and B), although in nocodazole treated cells import was slower (Fig. 7.1C). These data indicate that MTs facilitate transport to the nucleus but are not essential, since all AR is eventually translocated to the nucleus.

Characterization of mouse embryonic fibroblast cell lines

CLIP115 and CLIP170 are MT plus-end interacting proteins that function as positive regulators of MT growth and that have been shown to play a role in dynactin localization at MT ends (for review, see Galjart, 2005) CLASP2 is a protein that associates with CLIP115 and CLIP170 and plays a role in the stabilization of highly specific subsets of MTs (Akhmanova et al., 2001). To further investigate the role of MTs and the associated proteins in nuclear import of the AR, we studied the import of the AR in mouse embryonal fibroblast (MEF) cells of mice that are deficient for CLASP2 (CLASP2$^{-/-}$) or for both CLIP115 and CLIP170 (CLIP115$^{-/-}$/CLIP170$^{-/-}$). In addition to those cell lines we also derived wild-type MEFs from littermates.

To determine whether the cell lines were derived from male or female embryos, we performed a PCR using Y-chromosome specific primers (Fig. 7.2A). Y-chromosomal DNA was detected in all MEF cell lines, indicating that all cell lines were derived from genotypic male mice embryos and any differences observed are not sex-related. Western blot analysis revealed that CLASP2$^{-/-}$ and CLIP115$^{-/-}$/CLIP170$^{-/-}$ cell lines did not express CLASP2 or the CLIPs, respectively (Fig.2B). Expression of CLIP115 and CLIP170 in

CLASP2$^{-/-}$ MEFs was similar to levels in wild-type MEFs, indicating that the

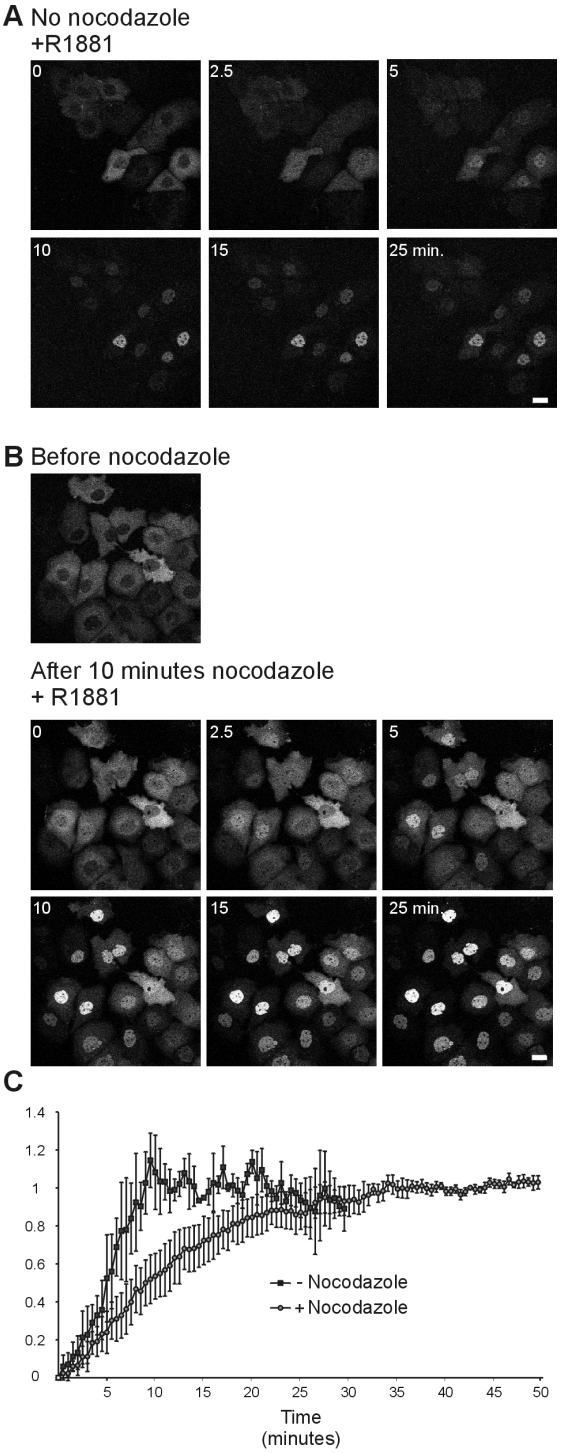

Figure 7.1. Disruption of MTs by nocodazole decreases nuclear translocation rate of the AR

(A and B) Representative images of Hep3B cells stably expressing GFP-AR either untreated (A) or treated for at least 10 minutes with 10 µM nocodazole (B) at several time points after addition of 1nM R1881_to the medium. Scale bar represents 20 µm. (C) Graph showing the relative intensity of fluorescence in the nucleus at different time points after addition of R1881.

CLASP2$^{-/-}$ genotype does not influence expression of CLIP115 and CLIP170. Accordingly, CLASP2 expression was at wild type levels in CLIP115$^{-/-}$ /CLIP170$^{-/-}$ cells. To verify whether these MEF cell lines express endogenous AR, cell lysates of the MEFs, and the cell line LNCaP that endogenously expresses AR, were subjected to Western blotting (Fig. 7.2C). No expression of AR in MEFs was detected and therefore endogenous AR will not compete for ligand with transfected GFP-AR

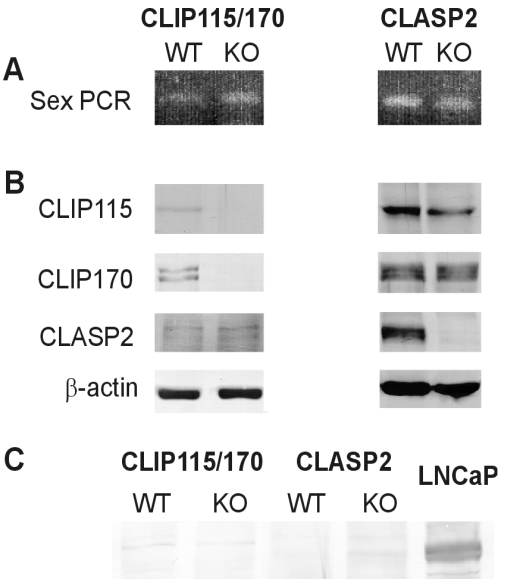

Figure 7.2. Characterization of mouse embryonic fibroblast (MEF) lines
Wild-type (WT), CLASP2 knock-out (KO), or CLIP115/CLIP170 double knock-out mouse embryonic fibroblasts (MEFs) were derived as described in the Materials and Methods section. (A) Sex PCR using Y-chromosome specific primers showed a specific band in all lines. (B) Western blots of total cell lysates showing expression of CLIP115, CLIP170 or CLASP2 in the different MEF lines. Beta-actin expression was used as loading control. (C) Western blot of total cell lysates showing endogenous AR expression in the MEF lines. Cell lysate of the endogenously expressing AR prostate cancer cell line LNCaP was used as a control for AR-antibody functionality.

Nuclear import of the AR in wild-type and CLASP2$^{-/-}$ MEF cells

Wild-type and CLASP2$^{-/-}$ MEF cells were transfected with an expression vector encoding GFP-AR. In absence of androgen in both wild-type and CLASP2$^{-/-}$ cells the AR was predominantly localized to the cytoplasm, similar to the subcellular localization in Hep3B (Fig. 7.1A and Chapter 4) and other cell lines (Georget et al., 1997; Poukka et al., 2000; Tyagi et al., 2000; Avancès et al., 2001). After addition of R1881, in wild-type MEFs the AR translocated to the nucleus (Fig. 7.3A), where it showed the typical agonist-dependent intranuclear localization into foci that has been reported previously for other cell lines (Chapters 4, 5 and (Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001; Ochiai et al., 2003)). Translocation kinetics of GFP-AR in CLASP2$^{-/-}$ MEFs following R1881 addition (Fig. 7.3B and C) were identical to wild-type and Hep3B cell lines, indicating that stable MTs are not essential for nuclear translocation of AR.

CLIP115$^{-/-}$/CLIP170$^{-/-}$ cell lines show altered import of the receptor after hormone addition

Next, we studied the intracellular localization of GFP-AR in wild-type or CLIP115/CLIP170 deficient (CLIP115$^{-/-}$/CLIP170$^{-/-}$) MEFs transfected with DNA coding for GFP-AR (Fig. 7.4A-C). Nuclear import in wild-type MEFs was similar to that in the formerly used wild-type cell-line (Fig. 7.3A and C), indicating there are no differences in wild-type cell lines derived from different mice (Fig. 7.4A and C). In absence of hormone in CLIP115/CLIP170$^{-/-}$ cells

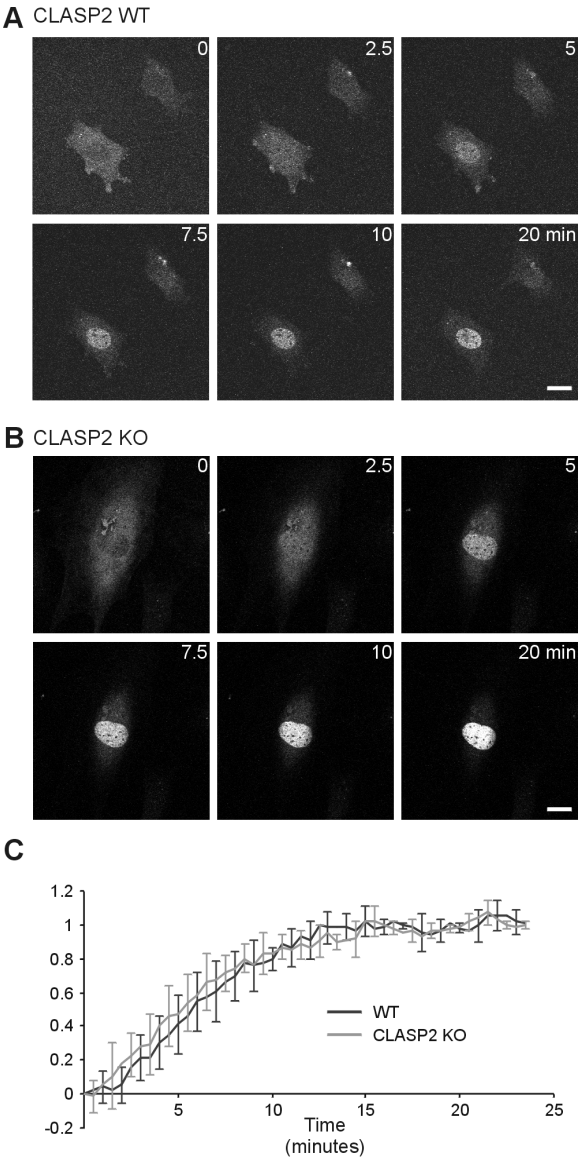

Figure 7.3. Nuclear import of the AR is identical in wild-type and CLASP2$^{-/-}$ cells
(A and B) Representative images of GFP-AR transfected wild-type (WT) (A) or CLASP2$^{-/-}$ (CLASP2 KO) MEFs (B) at different time points after addition of R1881 to the medium. Scale bar represents 20 µm. (C) Graph showing the normalized relative intensity of fluorescence ±2*SEM in nuclei at different time points after addition of R1881 in wild-type and CLASP2$^{-/-}$ MEFs. For visibility SEMs are alternately shown in every other point of the WT and KO curves.

GFP-AR was predominantly localized to the cytoplasm, similar to wild-type cells. In contrast to wild-type cells addition of agonist R1881 to the medium did not or hardly result in nuclear translocation of the AR (Fig. 7.4B and C) in these cells, showing the requirement of CLIP115 and/or CLIP170 for efficient nuclear import of the AR. However, it has to be noted that a lot of heterogeneity between cells was observed.

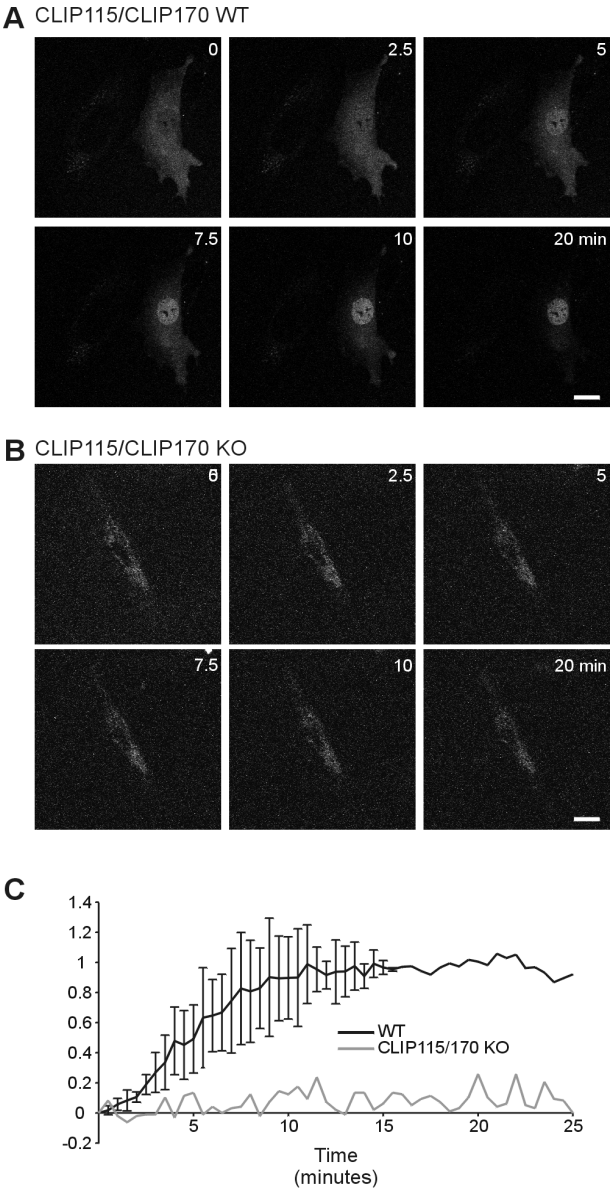

Figure 7.4. Nuclear import of the AR is impaired in CLIP115$^{-/-}$/CLIP170$^{-/-}$ MEFs
(A and B) Representative images of GFP-AR transfected wild-type (WT, A) and CLIP115$^{-/-}$/CLIP170$^{-/-}$ (KO, B) MEFs at different time points after addition of R1881 to the medium. Scale bar represents 20 µm. (C) Graph showing the normalized relative intensity of fluorescence ±2*SEM in nuclei of wild-type cells (N=2) at different time points after addition of R1881. At later time points only WT cell was measured, therefore, in this part of the curve no SEM is shown. CLIP115/CLIP170 KO cells do not show import; therefore the data were normalized to intensity just after addition of R1881 (t=0).

7.4. Discussion

The mechanisms that govern translocation of steroid receptors, such as AR, to the nucleus are largely unknown. There have been several reports that cytoskeletal components are involved in import of steroid receptors (Galigniana et al., 1998; Ozanne et al., 2000; Harrell et al., 2004; Whitaker et al., 2004). GR (Galigniana et al., 1998) and AR (Chapter 6 and Georget et al., 2002) translocation to the nucleus is prevented by the hsp90 inhibitor geldanamycin. Remarkably in cells in which the MT cytoskeleton has been disrupted nuclear tranlocation of GR was no longer prevented. We observed that disruption of the MT cytoskeleton by nocodazole resulted in slower translocation of AR after addition of androgen (Fig.7.1), which suggests that normally receptors are actively transported to the nuclear membrane via MTs, whereas in absence of MTs ARs reach the nuclear membrane by diffusion. At the nuclear membrane receptors are imported into the nucleus by the NLS related import system. Previously cell fractionation studies of AR expressing cell lines in presence of nocodazole suggested that the MT cytoskeleton is required for nuclear translocation of the AR (Whitaker et al., 2004). Our results suggest that the MT cytoskeleton is not essential but assists in efficient translocation of AR to the nucleus.

In presence of the MT disrupting agent nocodazole we observed already some nuclear localization of the AR in the absence of androgen (Fig.7.1B). This suggests that the MT skeleton is not only involved in translocation of the liganded receptor, but also in keeping the unliganded AR in the cytoplasm. Although it is not yet known what proteins might mediate this interaction, they most likely include immunophilins, which are known to bind to hsp90. The immunophilin FKBP51 competes for binding to hsp90 with FKBP52 (Davies et al., 2002; Riggs et al., 2004) and elevated levels of FKBP51 have been associated with decreased sensitivity to glucocorticoids and progestins in squirrel monkeys (Denny et al., 2000; Hubler et al., 2003). The immunophilin FKBP52 which binds to hsp90 as well as dynamitin (Galigniana et al., 2004) mediates nuclear import of the GR (Harrell et al., 2004). FKBP52 is also important for AR activity since FKBP52 knock-out mice showed a phenotype corresponding to androgen insensitivity (Cheung-Flynn et al., 2005). Interestingly, dynamitin is an essential component of the dynactin complex, which in turn regulates processivity of the dynein motor complex (Schroer, 2004). These data provide a link between AR nuclear import and a minus-end directed motor complex.

To investigate if proteins that are known to interact with plus-ends of MTs are involved in nuclear import of the AR, we investigated the import of GFP-AR in MEFs in which the genes coding for both CLIP115 and CLIP170 or the interacting protein CLASP2 were knocked-out. No differences in nuclear import of AR were observed in CLASP2$^{-/-}$ MEFs compared to wild-type cells (Fig. 7.3). Therefore CLASP2 stabilization of distal ends of MTs (Akhmanova et al., 2001) does not seem to be essential for nuclear translocation of AR. In contrast, nuclear import of AR after hormone addition was absent in most CLIP115$^{-/-}$/CLIP170$^{-/-}$ MEFs cells (Fig. 7.4, whereas in some cells import was observed, although at a much slower rate. However, the results indicate that nuclear import of AR in CLIP115$^{-/-}$/CLIP170$^{-/-}$ MEFs is at least impaired. This suggests that AR import resembles the active transport mechanism used for retrograde transport of endocytic vesicles (Pierre et al., 1992), in which CLIP170 is involved in linking vesicles to MTs.

In conclusion, our preliminary results suggest that MTs facilitate efficient nuclear import of ARs. Loading of AR on MTs most likely, similar to endocytotic vesicle transport along MTs may require action of CLIP115 and/or CLIP170.

7.5. Materials and Methods

Cell lines and constructs

The construction of a pGFP-AR, which codes for an N-terminally GFP-tagged AR of which the expression is driven by a CMV promoter has been described previously (Chapter 4). Hep3B cells that stably express GFP-AR were generated and maintained as previously described. Expression levels in these cells are comparable to cell lines that endogenously express AR (Chapters 4 and 5). For confocal micropic import studies cells were grown on glass cover slips. One day before nuclear import studies the medium was replaced by medium containing 5% dextran charcoal hormone-depleted FBS.

Mouse embryonic fibroblasts (MEFs) were derived from 13.5-day old embryos. Embryos were removed from the yolk sac and transferred to cell culture dishes containing 5 ml 1:1 DMEM/Ham's F10 (Cambrex, Verviers, Belgium) with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Cambrex). After removal of the head and liver, the rest of the embryo was cut up into small pieces. Cells were allowed to attach and spread overnight. 5 ml of fresh medium was added and cells were grown for an

additional 24 to 72 hours. Cells were split and grown for further use.

Wild-type and CLASP2 (CLASP2$^{-/-}$) and CLIP115/CLIP170 (CLIP115$^{-/-}$/CLIP170$^{-/-}$) knock-out MEFs were transfected with 2.5 to 5 µg of pGFP-AR using the MEF2 Nucleofector Kit (Amaxa GmbH, Cologne, Germany) according to the manufacturers instructions in medium containing 10% dextran charcoal stripped FBS. After transfection cells were directly put on glass cover slips.

Western blots

Total cell lysates were prepared from each MEF line. Proteins were separated on a 10 % SDS-PAGE gel and blotted onto PDVF membranes (Millipore Corporation, Bedford, MA). Membranes were blocked using PBS containing 2% BSA and 0.05% Tween-20 followed by incubation with primary antibodies recognizing CLIP115 (#2238 (Hoogenraad et al., 2000)), CLIP170 (#2360, (Coquelle et al., 2002)) or CLASP2 (#2358 (Akhmanova et al., 2001)) in block buffer. After three washes with PBS containing 0.05% Tween-20, membranes were incubated with secondary anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma, St. Louis, MO). Protein bands were visualized using BCIP/NBT alkaline phosphatase substrate (Sigma). To check for endogenous AR expression in MEFs, total cell lysates were subjected to SDS-PAGE, Western blotting and incubation with a polyclonal antibody recognizing amino acids 1-20 of both human and mouse AR (Sp 197 (Kuiper et al., 1993)). Protein bands were visualized as described above. Beta-actin expression was used as loading control for Western blots.

Nuclear import studies

Nuclear import in Hep3B cells stably expressing GFP-AR or MEFs transiently transfected with the same construct was imaged using a Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany), with a 63x 1.4 NA oil immersion objective. GFP-AR was visualized by excitation with the 488 nm laser line of an Ar-laser and detection of emission light between 500 and 530 nm. Cells were imaged every 30 seconds using four times averaging. Average fluorescence intensities in the nucleus were measured at each time point. Fluorescence intensity values were normalized to initial and steady state intensities. Mean and SEM of normalized nuclear import curves were calculated and plotted.

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Chapter

8

General discussion

In this thesis dynamics of the AR in the nucleus of the cell, where the receptor is involved in regulation of transcription of androgen-regulated genes and a preliminary study into the role of MTs in nuclear translocation of the receptor have been described. In this final chapter a general reaction scheme of AR action in the living cell is provided (Fig. 8.1) and the most important implications for fundamental and clinical understanding of the findings are discussed.

8.1. Interaction of AR with DNA/promoters

Steroid receptors bind to response elements in promoter and enhancer regions of the genes they regulate and recruit coactivators that remodel chromatin and modify histones. This enables binding of gene specific and general transcription factors and the formation of a transcription initiation complex. Until recently, very little was known with respect to the binding kinetics of steroid receptors and other factors involved in the activation of transcription in living cells. The results described in this thesis on the AR (Chapters 4, 5 and 7) and reports on other steroid receptors, such as the GR (McNally et al., 2000; Schaaf and Cidlowski, 2003), ERα (Stenoien et al., 2001) and PR (Rayasam et al., 2005) show that agonist- as well as antagonist-liganded receptors in the nucleus display highly dynamic behavior. In presence of an agonist mobility of all investigated steroid receptors is reduced (Chapters 4, 5 and Stenoien et al., 2001). The decrease in mobility was shown to be related to agonistic potency of a ligand (Schaaf and Cidlowski, 2003; Rayasam et al., 2005) and DNA binding was shown to be required for the agonist-induced decrease in mobility (Chapter 4 and 5). This suggests the slower mobility is caused by immobilizing binding events that are at least transcription related. However, it has been proposed that it cannot only represent specific binding in transcription, but rather represents binding to immobile elements in the nucleus other than chromatin, such as for instance the operationally defined (but never independently visualized) nuclear matrix (see Pederson, 2000 and Nickerson, 2001 for reviews, Stenoien et al., 2001; Schaaf and Cidlowski, 2003).

The slower kinetics of GR in presence of an agonist have also been explained by a mechanism of scanning where the majority of receptors (~85%) shows interactions in the order of milliseconds with DNA, which would represent scanning of GRs for binding sites (Sprague et al., 2004). We have shown that a model of diffusion can explain dynamics of antagonist-liganded AR, or AR with a mutation in the DBD that cannot bind DNA. However, using

a combination of two complementary FRAP methods (Strip-FRAP and FLIP-FRAP) and computer modeling we show the reduced mobility of wild-type AR cannot be explained merely by a model of very transient interactions (which yields identical FRAP curves as slower diffusion). Although a model of diffusion may fit the data obtained with one type of FRAP quite well, a diffusion model with the same parameters cannot properly explain the data of the other FRAP method with the same diffusion speed (Chapter 4, 5 and Houtsmuller, 2005). Using the combination of the two complementary FRAP methods, we showed that a fraction of 10-20% of ARs is immobilized for ~1 minute in a DNA-binding dependent way, with the rest of the receptors freely diffusing. Diffusion of this "free" fraction is delayed by binding of an agonist, which may be explained by "scanning"-interactions that are shorter than those proposed by Sprague et al. or the residence of AR in larger complexes (see 8.2).

Can this 10-20% immobile fraction represent specific binding of AR to binding sites in promoter/enhancer regions of androgen-regulated genes? Assuming there are $\sim 5 \times 10^2$ androgen regulated genes with on average 3 binding sites to which ARs bind as dimers, it follows that the nucleus of an individual cell contains $\sim 3 \times 10^3$ binding sites. We estimate that our cells contain on average 3×10^4 GFP-AR molecules. This would mean that in theory, an average of 10% of nuclear AR can be bound to specific binding sites, which is consistent with the fraction of receptors immobilized in FRAP.

The immobilization of ARs in the order of 1 minute seems reasonable, since the general transcription factor TFIIH, which interacts with the AR-NTD (Lee et al., 2000), was immobilized for ~10s when involved in RNAP2 transcription (Hoogstraten et al., 2002), whereas RNAP2 itself was immobilized for much longer periods of time (10-20 min) (Becker et al., 2002; Kimura et al., 2002).

One way to address the problem of specific or aspecific binding might be to study mobility of steroid receptors at an array of promoter repeats of a single gene with multiple specific binding sites for steroid receptors, such as the MMTV-promoter. On this promoter repeat, steroid receptors are visible as an accumulation and FRAP revealed that GR exchanged with this array with a half maximal recovery time of ~5s (McNally et al., 2000; Becker et al., 2002). Remarkably, mobility of PR measured by FRAP in total nuclei and on an MMTV-promoter array was similar (Rayasam et al., 2005). This similar mobility in total nuclei and the MMTV-repeat suggests that on the many more binding sites for steroid receptors in a nucleus, receptors show similar binding behavior as on the promoter repeat.

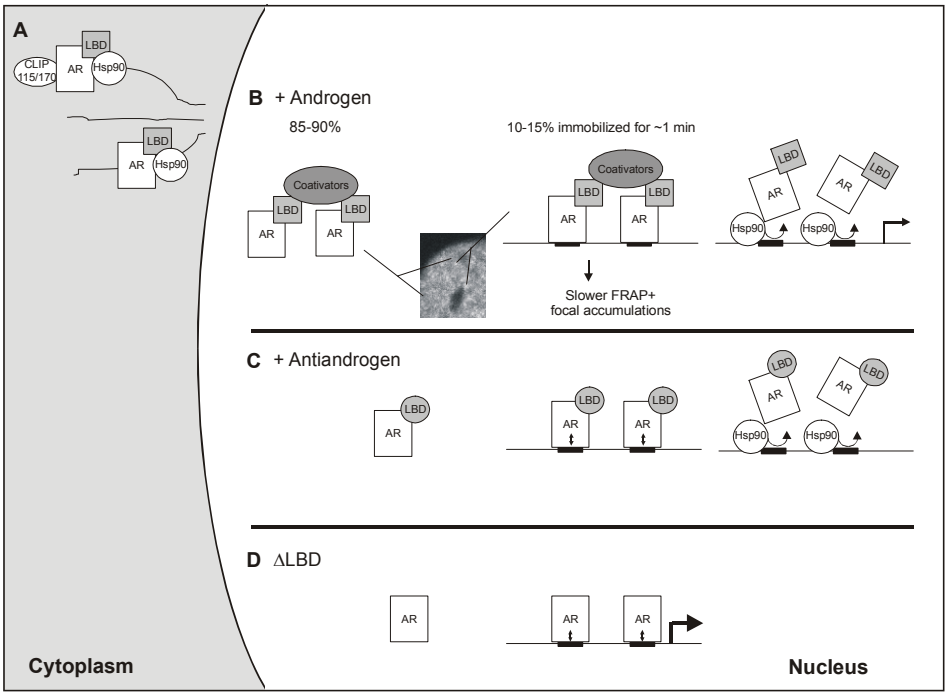

Figure 8.1 Scheme of the *in vivo* mechanisms of action involved in AR transcription regulation

A. In absence of hormone, AR is predominantly localized in the cytoplasm and presumably present in a multi-protein complex. Upon hormone addition AR is translocated to the nucleus. Efficient transport to the nucleus requires intact MTs and active Hsp90 (Chapters 6 and 7) and most likely its co-chaperones (FKBPs and p23). CLIP115 and/or CLIP170 may play a role in loading AR on MTs (Chapter 7). B. In presence of an androgen such as (DH)T, intranuclear foci are observed. A fraction of AR is immobilized in a DNA-binding and LBD-dependent manner, whereas the remaining free fraction of receptors shows slower mobility, suggesting the presence of preformed receptor-coactivator complexes or scanning of DNA for specific binding sites (Chapter 4 and 5). Release of DNA-bound AR most likely requires action of Hsp90 (Chapter 6) C. In presence of an antiandrogen, such as OH-flutamide or bicalutamide, ARs are highly dynamic and do not show stable DNA-binding (Chapter 5). Hsp90 inhibition results in DNA-binding dependent immobilization of antiandrogen-bound receptors, suggesting a role for hsp90 in release of DNA-bound receptors (Chapter 7). D. A constitutively active AR mutant lacking the entire LBD is localized in the nucleus and more potent in activating transcription, but does not show stable DNA-binding dependent immobilization (Chapter 4).

8.2. Is slower mobility of agonist-bound AR due to interaction with cofactors or scanning?

As argued above, our FRAP data are explained best by assuming the presence of both a relatively stable immobile fraction and a slowed down diffusion of the remaining free fraction. This slow-down of diffusion could either be caused by

very transient interactions with DNA ("scanning") or alternatively by assuming AR is part of a large protein complex that is expected to display slower diffusion. It is not possible to distinguish these two scenario's by FRAP, and further research is required to be able to determine which is favorable. Below the pros and cons of both scenarios are discussed.

The slow-down of the mobile fraction of ARs cannot be explained by dimerization of the receptor alone, since this would not result in a significant change in diffusion. Scanning may slightly slow down the receptor as indicated by the decrease in diffusion speed of wild-type receptor compared to DBD-mutant. However, the differences in diffusion between agonist and antagonist-liganded receptors are much bigger. Antagonist-bound receptors can most likely also bind DNA, since OH-flutamide liganded ARs in presence of geldanamycin are immobilized in a DNA-binding dependent manner (Chapter 6). Moreover, ChIP-studies revealed association of bicalutamide bound AR with promoter regions of androgen regulated genes (Kang et al., 2002; Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004). Therefore, the differences in diffusion of the free fraction of agonist-bound and antagonist-bound receptors are likely not solely explained by scanning.

There is evidence that in presence of an agonist, AR conformation is suitable for binding of co-activators, whereas antagonists such as OH-flutamide and bicalutamide do not induce such a conformation and coactivator proteins cannot bind the AR (Doesburg et al., 1997; Chang and McDonnell, 2002). Our findings support this view and in addition suggest that agonist-liganded ARs are already in preformed complexes with cofactors prior to DNA-binding. In support of this the liganded PR was shown to exist in stable complexes with the p160 coactivators SRC1 and TIF2/GRIP1 (McKenna et al., 1998). Moreover, those coactivators showed similar dynamics as agonist liganded ER and GR, respectively (Stenoien et al., 2001; Becker et al., 2002). In presence of antagonists OH-flutamide and bicalutamide AR do not obtain a conformation that is suitable for binding of coactivators, which results in smaller AR complexes and therefore a more mobile receptor.

8.3. Intranuclear foci

Binding of an agonist to the AR and other steroid receptors results in focal accumulations (foci or "punctate pattern") in the nucleus (Chapters 4, 5, 6 and (van Steensel et al., 1995; Htun et al., 1996; Htun et al., 1999; Stenoien et al.,

2000; Tyagi et al., 2000; Avancès et al., 2001; Tomura et al., 2001; Schaaf et al., 2005)). The presence of these foci was always accompanied by slow-down of receptors as measured by FRAP (Chapter 5 and Schaaf et al., 2005). Moreover, both phenomena were dependent on the ability of the AR to bind DNA, as neither immobilization nor foci were observed with receptor mutants that cannot bind DNA, due to a mutation in the DBD. Furthermore, foci formation required the presence of the LBD (Chapters 4, 5 and 6). Since the foci are quite numerous it may be considered unlikely that all foci represent specific DNA-binding sites (Stenoien et al., 2000; Schaaf et al., 2005). Moreover, RNAP2 did not colocalize with GR (van Steensel et al., 1995) or only partly with the arylhydrocarbon (or dioxin) receptor (Elbi et al., 2002) foci. As an alternative explanation, it was suggested that receptors interact with the operationally defined nuclear matrix, leading to the focal pattern. The interaction with the nuclear matrix could for instance function as storage for receptors that are not involved in transcription. However, the latter is not in agreement with observations in which both agonist and antagonist liganded AR were recovered from the nuclear matrix fraction, whereas foci were only observed with agonist liganded AR (Tyagi et al., 2000). Analogous to the clustering of replication factors and DNA polymerases in replication foci, multiple RNAP2s associated with different gene promoters have been suggested to cluster in transcription foci or factories (Cook, 1999). The foci observed with RNAP2 appear similar to those observed with the AR. Therefore it could also be that GFP-AR foci represent clusters of multiple androgen-regulated genes. However, to prove this more research is required.

8.4. Role of the AR-LBD

We observed that a constitutively active AR mutant lacking the LBD (ARΔLBD) was more potent in activating transcription from a number of cotransfected androgen-regulated gene promoter constructs (Fig.8.1, Chapter 4). Others have reported a similar increased constitutive transactivation by a truncated AR protein with a deletion of the LBD (Céraline et al., 2004). We have not been able to obtain stable cell lines expressing ARΔLBD at levels similar to endogenous wild-type AR expression. All surviving clones displayed significantly lower levels, suggesting that constitutive expression of AR-induced genes at high levels, in line with the observed higher expression of reporter genes from transfected androgen-regulated promoters, may eventually

be disadvantageous for the cell. Using an inducible expression system it may be possible to obtain cells with ARΔLBD expression at wild-type levels. Although agonistic ligand-bound full-length receptor is slowed down, most likely by DNA-binding dependent immobilization, and forms intranuclear foci, this is not observed with ARΔLBD (Chapter 4), strongly suggesting that the AR-LBD, has a modulating function in regulation of AR activated transcription.

8.5. Role of hsp90

Hsp90 has been associated with folding and maturation of numerous proteins involved in signal transduction, including nuclear receptors and protein kinases, thereby regulating their stability and biological functions. Hsp90 has been implicated in maturation of the unliganded steroid receptor to a form that can bind hormone with high affinity (reviewed in (Pratt and Toft, 1997; Pratt and Toft, 2003). Moreover a role for hsp90 and the associated immunophilin FKBP52 (Davies and Sánchez, 2005) in nuclear import of GRs involving the dynein component of the cytoskeleton (Harrell et al., 2004; Pratt et al., 2004) has been suggested. Recently, FKBP52 has also been shown to be present in AR complexes and increase AR transcriptional activity in an hsp90 dependent manner. Male FKBP52 knock-out mice have several defects in reproductive tissues which resemble androgen insensitivity; including ambiguity of external genitalia and dysgenesis of the prostate (Cheung-Flynn et al., 2005). Although it was suggested that this was due to a defective folding of the AR it might well be that FKBP52 is implicated in AR nuclear import as well.

In addition to these roles for hsp90 on AR functionality in the cytoplasm, hsp90 also affects AR (Chapter 6) and GR (Elbi et al., 2004; Stavreva et al., 2004) mobility in the nucleus in DNA-binding dependent manner (Chapter 6, Fig. 8.1). Our findings support a model where hsp90 acts to remove receptors from DNA as has previously been suggested for other steroid receptors using *in vitro* systems (DeFranco and Csermely, 2000; Freeman and Yamamoto, 2002). Proteasomes have also been suggested to play a role in transcription regulation of GR and ERα, since the proteasome inhibitor MG132 resulted in an immobile fraction with FRAP (Stenoien et al., 2001; Deroo et al., 2002; Schaaf and Cidlowski, 2003; Stavreva et al., 2004) and inhibited cycling on promoters in ChIP experiments (Reid et al., 2003). However, preliminary results of FRAP experiments on GFP-AR expressing cells exposed to MG132 show that both wild-type receptors and ARs with deficient DNA-binding are immobilized. This

suggests that immobilization is not specific for DNA-bound receptors and considering the long incubation times with MG132 may represent a secondary effect, which is supported by the observation that MG132 resulted in an immobile fraction of unliganded GR in the nucleus (Schaaf and Cidlowski, 2003).

8.6. Role of findings for disease/therapy

The AR is implicated in a number of diseases (see Chapter 1), including prostate cancer. Advanced prostate cancers are treated with antiandrogens to block AR signaling. We have shown that two antiandrogens frequently used to treat advanced prostate cancers, flutamide and bicalutamide (Casodex), do not induce stable DNA-dependent binding of the AR. However, our study with the hsp90 inhibitor geldanamycin (Chapter 6) and ChIP experiments (Kang et al., 2002; Masiello et al., 2002; Shang et al., 2002; Kang et al., 2004) suggest that antiandrogen bound receptors can bind DNA, although this does not result in activation of transcription (Fig. 8.1). A number of mutations mainly in the AR-LBD domain are known to confer resistance to antiandrogens (see Chapters 1 and 5). We showed that AR-mutants AR(W741C) and AR(T877A) in presence of the antiandrogens bicalutamide and OH-flutamide, respectively, activate transcription and induce foci and stable DNA-dependent immobilization of the receptor, similar to wild-type receptor with an agonist (Chapter 5). This suggests that these mutations change the conformation of the AR-LBD, enabling the binding of cofactors and activation of transcription. Recently, crystal structures of a mutant AR containing a substitution of the tryptophan (W) on position 741 to leucine, which similar to cysteine on this position has been shown to result in transcription activation by bicalutamide (Hara et al., 2003), revealed that this mutation results in a conformation of the AR-LBD that positions the B-ring of bicalutamide at the location of the indole ring of W741 in the wild-type receptor bound to DHT (Bohl et al., 2005).

We observed that ARs lacking the LBD are more potent in activating transcription from a number of AR target promoters (Chapter 4). There is at least one report of a patient with a nonsense mutation in the AR gene, resulting in a truncated receptor. Similar to our experiments, this AR mutant was shown to be constitutively active and was about 2x fold more active than the wild-type receptor in activating transcription from an MMTV-promoter (Céraline et al., 2004). Nevertheless, it seems unlikely that such an event where prostate cancer

cells are selected that express an AR with a complete deletion of the ligand binding domain would frequently occur. However, mutations in the AR-LBD may similarly affect interactions with cofactors that bind to it, resulting in loss of modulation and a higher transcriptional activity. This altered transcriptional activity might eventually contribute to the development of prostate cancer.

As mentioned above hsp90 acts at multiple levels to enhance steroid receptor transcription. This, combined with its role in other signal transduction pathways that promote cell survival makes hsp90, an attractive target for prostate cancer therapy. Although, the hsp90 inhibitor geldanamycin itself has been proven to be quite toxic, currently phase I clinical trials with geldanamycin analogs, such as 17-allylaminogeldanamycin (17-AAG) are being performed (Isaacs et al., 2003; Workman, 2003). 17-AAG has been shown to have approximately 100 fold higher affinity for hsp90 in tumor cells than in normal cells, most likely because in tumor cells more hsp90 is present in multichaperone protein complexes with high ATPase-activity (Kamal et al., 2003). 17-AAG is thought to be particular useful for treatment of HER-2 positive breast and prostate cancers (Isaacs et al., 2003), where it might interfere with androgen/estrogen signaling as well as HER-2 signaling.

8.7. Future research

The results described in this thesis reveal highly dynamic behavior of ARs in the living cell, which most likely reflects a rapid turnover on promoter DNA. In addition it was shown that chaperones like hsp90 might play a role in this dynamic on-off binding behavior. Future studies may focus on the nature and dynamics of interactions with cofactors and inter-and intramolecular changes in the AR and interactions with cofactors. An appropriate method to study this is FRET, which has already been used to show molecular interactions of steroid receptors ((Michalides et al., 2004; Nishi et al., 2004; Schaufele et al., 2005) and van Royen et al., unpublished data) as well as interactions of steroid receptors with coactivators (Llopis et al., 2000; Weatherman et al., 2002; Bai and Giguere, 2003) in living cells.

The preliminary work presented in Chapter 7, which focused on the nuclear import of the AR, indicates that MTs and CLIPs are required for efficient translocation of AR to the nucleus. The role of the MT and proteins that bind to it and link it to the dynein/dynamitin transport system has to be investigated more closely.

In conclusion, the fundamental knowledge on AR function in living cells gained by experiments presented in this thesis may contribute to a better understanding of the molecular principles that govern transcription regulation by steroid receptors. Taken together with the wealth of information coming from many other groups, also in other fields like DNA replication and repair this fundamental knowledge may be applied to identify new drug targets for treatment of diseases in which the AR is involved.

8.8. References

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Summary

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Samenvatting

Summary

The action of androgens, such as testosterone or dihydrotestosterone is required for development and maintenance of the male phenotype. Androgens exert their action through the androgen receptor (AR), a member of the steroid receptor family of nuclear receptors that function as ligand activated transcription factors. In this thesis we investigated the dynamics of ARs in its most relevant context: the living cell.

Chapter 1 is an introduction is given into the molecular structure of the AR and the diseases associated with dysfunction of the receptor. Like all other steroid receptors, the AR has a modular structure and consists of an N-terminal transactivating domain (NTD), a central DNA binding domain (DBD), which is linked by a hinge region containing nuclear localization signal (NLS) sequences to the C-terminal ligand-binding domain (LBD). The three diseases in which the AR plays a major role are androgen insensitivity syndrome, Kennedy's disease and prostate cancer.

Binding of ligand to the AR triggers a cascade of events that eventually result in the activation of transcription of androgen regulated genes. In **Chapter 2** the current picture of the events that occur before activation of transcription are described. In the absence of hormone the AR is in complex with chaperone proteins and localizes predominantly in the cytoplasm. Activation by androgens results in transport to the nucleus, which most likely involves actions of chaperone proteins and the cytoskeleton. In the nucleus access to promoters of androgen-regulated genes is prevented by the compactness of chromatin. To overcome the repressive nature of compact chromatin, ARs probably bind as dimers to androgen response elements and recruit coactivator proteins that remodel and modify histone tails in nucleosomes, the structural protein component of chromatin. The coactivator complexes in chromatin remodeling and histone modification are introduced. Remodeling of the local chromatin structure by coactivators allows the binding of other transcription factors, which eventually together with the AR, general transcription factor and RNA-polymerase II (RNAP2) result in formation of a transcription initiation complex on the promoter and activation of transcription.

In **Chapter 3** current knowledge on the spatiotemporal dynamics of chromatin remodeling and histone-modification is discussed, with a focus on transcription activation by steroid receptors. Three techniques have recently revolutionized the studies of dynamics of steroid receptors and their

coactivators: 1) The discovery of the genetically encoded green fluorescent protein (GFP) has the study of GFP-tagged proteins in living cells 2) Fluorescence recovery after photobleaching (FRAP), a method to study the mobility of fluorescent molecules or proteins in living cells at single cell level and 3) Chromatin immuno precipitation (ChIP), a technique to study the binding of proteins to specific DNA fragments in multiple cells.

In FRAP fluorescent proteins within a small volume are bleached by a short exposure to intense light. By studying the redistribution of (bleached and) fluorescent molecules/proteins at low light intensities that do not induce bleaching, dynamics of the protein can be measured that give insight into the functioning of proteins in the living cell. FRAP has revealed unexpectedly high nuclear dynamics of steroid receptors as well as their coactivators, suggesting that receptors and coactivators continuously exchange at promoters. On the other hand, ChIP has also revealed cyclic, and ordered binding of steroid receptors, as well as their coactivators and general transcription factors to promoter regions. The main findings of these ChIP studies are described.

Although there is a lot of knowledge on the AR itself and the proteins that interact with it, knowledge on the functioning of the receptor in the living cell is limited. In Chapters 4 to 7 experimental studies on the behavior of the AR in living cells using a GFP-AR fusion protein are described.

In **Chapter 4** it is shown that GFP-AR is functional in spite of the large GFP-tag. Transcription activation and translocation to the nucleus and subnuclear distribution were comparable to untagged AR. Using two different FRAP methods and computer simulations it is shown that in the presence of the androgen R1881 a fraction of ARs (10-20%) is immobilized for approximately 1 minute. This immobilization was shown to be DNA-binding dependent, since an AR mutant with a mutation in the DBD (A573D) resulting in inability to bind DNA is not immobilized. Remarkably, an AR-mutant lacking the LBD constitutively and more potently activates transcription, although this mutant is immobilized to a much lesser extent than the full-length receptor. Moreover a focal intranuclear distribution is observed with the wild-type receptor, whereas the DBD and LBD-deletion mutants showed homogeneous distribution of fluorescence.

Antiandrogens like bicalutamide and hydroxyflutamide are used to treat patients with metastasized prostate cancer. To obtain insight into the mechanism by which these antagonists block AR function in living cells, in **Chapter 5** we studied nuclear mobility and localization of GFP-tagged AR in the presence of

agonist R1881 or the antagonists bicalutamide and hydroxyflutamide. As controls two mutants (W741C and T877A) with broadened ligand specificity and the non-DNA-binding AR mutant (A573D) were investigated. In presence of R1881 AR localized in numerous intranuclear foci. Using the complementary FRAP approaches and computer modeling, we confirmed the findings of Chapter 4 that a fraction of ARs is transiently immobilized for ~40-60s in a DNA-binding dependent manner. In contrast, antagonist-bound GFP-AR showed no detectable immobile fraction and the mobility was similar to that of the R1881-liganded non-DNA-binding mutant (A573D), indicating that antagonists do not induce the relatively stable DNA-binding dependent immobilization observed with agonist-bound AR. Moreover, in presence of bicalutamide and hydroxyflutamide GFP-AR was homogeneously distributed in the nucleus. Binding of bicalutamide and hydroxyflutamide to GFP-AR(W741C) and GFP-AR(T877A), respectively, resulted in similar mobility and heterogeneous nuclear distribution as observed for R1881-liganded GFP-AR. These results indicate that the antagonists interfere with events early in the transactivation function of AR.

In **Chapter 6** the role of molecular chaperone hsp90 on mobility of the AR is studied. Hsp90 inhibition by geldanamycin (GA) resulted in complete inhibition of nuclear import of AR. Exposure to GA of ARs that had been translocated into the nucleus by prior addition of hormone, resulted in the permanent immobilization of ~10% of receptors in a DNA-binding dependent manner. However, GA did not affect the agonist-dependent accumulation in foci or the ligand-dependent amino-carboxyl terminal interaction of the AR. Remarkably, GA also resulted in a permanent DNA-binding dependent immobile fraction in presence of the antagonist OH-flutamide. This suggests that hsp90, next to its well-known roles in the cytoplasm, has a role in dissociating agonist as well as antagonist liganded receptors from DNA and may therefore regulate transcription activation at multiple levels.

Chapter 7 is a preliminary study into the mechanisms of action underlying the translocation of hormone-bound AR from the cytoplasm to the nucleus. The role of microtubules (MTs) and proteins that bind to plus ends of MTs (+TIPs) in nuclear translocation of the AR was studied using chemical disruption of MTs by nocodazole and mouse embryonic fibroblast cell lines defective for the +TIPs cytoplasmic linker protein (CLIP) 115 and 170 or CLIP associated protein 2 (CLASP2). The preliminary results presented in this chapter indicate that MTs and CLIP115 and/or CLIP170 are involved in efficient nuclear

translocation of the AR. The results suggest that similar to vesicle transport, the action of CLIPs is required to load AR on MTs to facilitate nucleus directed transport of the receptor in the cytoplasm.

Chapter 8 is the general discussion of this thesis and puts the findings of the studies (Chapters 4 to 7) into a broader perspective and their possible implications for disease are discussed.

The studies described in this thesis provide fundamental knowledge on the working mechanism of ARs in the living cell and may be useful for the development of new therapeutic strategies for diseases in which the AR is involved.

Samenvatting

De geslachtsontwikkeling van de man vereist een goede hormonale signaaltransductie door de androgenen testosteron en dihydrotestosteron. Deze androgenen oefenen hun werking uit via de androgeenreceptor (AR), een lid van de familie van steroïdreceptoren, dit zijn kernreceptoren die functioneren als ligand (hormoon) geactiveerde transcriptiefactoren.

Hoofdstuk 1 geeft een introductie in de moleculaire structuur van de AR en de ziektes die geassocieerd worden met het disfunctioneren van de receptor. Net als alle andere steroïdreceptoren heeft de AR een modulaire structuur en is opgebouwd uit een amino-terminaal transactivatiedomein (TAD), een centraal DNA-bindend domein (DBD) en een carboxyl-terminaal ligand bindend domein (LBD). De drie ziektes waarin de AR een belangrijke rol speelt zijn: androgeen-ongevoeligheidssyndroom, de ziekte van Kennedy en prostaatkanker.

In de cel wordt door het binden van hormoon aan de AR een cascade van gebeurtenissen geactiveerd die uiteindelijk resulteert in de transcriptie van androgeen gereguleerde genen. In **Hoofdstuk 2** worden de gebeurtenissen die plaatsvinden voor de activering van transcriptie beschreven. In de afwezigheid van hormoon bevindt de AR zich in een complex met chaperonne-eiwitten in het cytoplasma van de cel. Activering van de receptor door androgenen leidt tot transport de receptor naar de kern, waarbij waarschijnlijk de chaperonne-eiwitten en het celskelet betrokken zijn. In de celkern wordt de toegang tot de promoters van genen geblokkeerd door compact chromatine. Om deze blokkering ongedaan te maken, binden de ARs als dimeren op specifieke bindingsplaatsen in het DNA, de zogenaamde androgeen responsieve elementen, en rekruteren eiwitten, die als coactivator functioneren. Deze eiwitten vervormen de structuur van chromatine door de positie en structuur van nucleosomen, de structurele eiwitcomponent van chromatine, te verdanderen en door chemische modificatie van de staarten van de histon eiwitten, waaruit de nucleosomen zijn opgebouwd. Vervorming van de locale chromatinestructuur maakt het binden van andere transcriptiefactoren mogelijk. Deze factoren kunnen samen met de AR, algemene transcriptiefactoren en RNA-polymerase II (RNAP2) een transcriptie initiatie complex vormen op de promoter en het transcriptieproces initiëren.

In **Hoofdstuk 3** wordt een overzicht gegeven van de huidige kennis over de dynamiek van eiwitten die de chromatine structuren vervormen en de histonen modificeren, met een nadruk op de activering van transcriptie door

steroïdreceptoren. Drie technieken hebben ervoor gezorgd dat de bestudering van de dynamiek van steroïdreceptoren en hun coactivatoren een grote vlucht heeft genomen: 1) De ontdekking van genetisch gecodeerde eiwitten zoals groen fluorescerend eiwit/proteïne (GFP) stelt onderzoekers in staat om de dynamiek van GFP-gemerkte fusie-eiwitten te bestuderen in de levende cel. 2) Fluorescence recovery after photobleaching (FRAP), een methode om de beweeglijkheid van fluorescente moleculen/eiwitten in levende cellen op het niveau van een enkele cel te bestuderen en 3) Chromatine immunoprecipitatie (ChIP), een techniek die gebruikt kan worden om het binden van eiwitten aan specifieke DNA-fragmenten in een celpopulatie te bestuderen.

FRAP maakt gebruik van de eigenschap van fluorescerende moleculen dat ze gebleekt worden als ze worden aangestraald met licht met een hoge intensiteit. In FRAP worden fluorescerende eiwitten in een klein volume gebleekt door een korte blootstelling aan een hoge intensiteit licht. Door de herdistributie van (gebleekte en) fluorescerende moleculen/eiwitten te volgen door excitatie met een lage lichtintensiteit, die niet resulteert in uitdoving van de fluorescentie, kan de dynamiek van het eiwit worden gemeten. Het meten van deze dynamiek geeft inzicht in het functioneren van eiwitten in de levende cel. FRAP heeft geleid tot het inzicht dat steroïdreceptoren en hun coactivatoren een hoge dynamiek vertonen, wat suggereert dat receptoren en coactivatoren continu uitwisselen op promoters. Anderzijds, werd met ChIP ook een cyclisch en geordende binden van steroïdreceptoren en hun coactivatoren en algemene transcriptiefactoren waargenomen. De belangrijkste bevindingen van deze ChIP studies worden beschreven.

Ondanks dat er reeds veel bekend is over de AR zelf en de factoren die interacteren met de AR, is er weinig bekend over het gedrag van de receptor in de levende cel. In de hoofdstukken 4 tot en met 7 worden studies naar het gedrag van de AR in levende cellen beschreven, waarbij gebruik gemaakt wordt van een met GFP gemerkte AR.

In **Hoofdstuk 4** wordt aangetoond dat het GFP-AR eiwit, ondanks het GFP-label, functioneel is. Het transport naar de kern, de lokalisatie in de kern en de activering van transcriptie zijn min of meer vergelijkbaar met die van de ongemerkte AR. Door gebruik te maken van twee verschillende FRAP-methoden en computersimulaties wordt aangetoond dat in de aanwezigheid van het androgeen R1881 een fractie van de ARs (10-20%) gedurende ongeveer 1 minuut geïmmobiliseerd wordt. Deze immobilisatie is afhankelijk van de mogelijkheid om aan DNA te binden, want ARs met een mutatie in het DBD

(A573D), die resulteert in het verstoren van het binden aan DNA, ondergaan geen immobilisatie.Het is opmerkelijk, dat een AR-mutant die het LBD mist en daardoor niet meer afhankelijk is van hormoon voor activering van transcriptie veel korter geïmmobiliseerd wordt dan de volledige receptor. Bovendien worden met de volledige receptor ophopingen (foci) gezien in bepaalde gebieden van de kern. ARs met een mutatie van het DBD of een deletie van het LBD vertonen echter een homogene distributie van de fluorescentie in de kern.

Anti-androgenen (of antagonisten), zoals bicalutamide en hydroxyflutamide worden gebruikt bij de behandeling van uitgezaaide prostaat tumoren. Om inzicht te krijgen in het werkingsmechanisme van deze antagonisten hebben we in **Hoofdstuk 5** de lokalisatie en mobiliteit van de AR in de kern van levende cellen in de aanwezigheid van de agonist R1881 of en de antagonisten bicalutamide en hydroxyflutamide bestudeerd. Ter controle werden twee AR-LBD mutanten (W741C en T877A), die een verbrede ligandspecificiteit hebben en een AR-mutant (A573D), die niet aan DNA bindt, bestudeerd. Door gebruik te maken van de complementaire FRAP benaderingen en computer modellering, bevestigden we de bevindingen van Hoofdstuk 4, dat een fractie van de door agonist bezette ARs geïmmobiliseerd wordt gedurende circa 40-60 seconden, afhankelijk van de mogelijkheid om te binden aan DNA. Daarentegen liet een met een antagonist bezette GFP-AR geen aantoonbare gebonden fractie zien en de mobiliteit hiervan was gelijk aan die van de AR-mutant die niet aan DNA kan binden. Dit impliceert dat antagonisten niet de relatief stabiele immobilisatie laten zien, die afhankelijk is van de mogelijkheid om aan DNA te binden, die we waarnamen bij een door een agonist bezette AR. Bovendien was GFP-AR in aanwezigheid van bicalutamide en hydroxyflutamide homogeen verdeeld over de kern. Het bezetten van GFP-AR(W741C) en GFP-AR(T877A) met respectievelijk bicalutamide en hydroxyflutamide resulteerde in dynamiek en een verdeling van de receptor die gelijk was aan die van de ongemuteerde receptor in de aanwezigheid van de agonist R1881. De resultaten van deze studie suggereren dat antagonisten interfereren met vroege gebeurtenissen bij de activering van transcriptie door de AR.

In **Hoofdstuk 6** wordt de rol van het chaperonne-eiwit hsp90 op de dynamiek van de AR bestudeerd. Het remmen van de activiteit van hsp90 met geldanamycine (GA) resulteerde in een complete blokkade van de nucleaire import van de AR naar de kern. Toevoegen van GA nadat de receptoren eerst naar de kern waren getransloceerd door toedienen van hormoon, resulteerde in de permanente immobilisatie van circa 10% van de receptoren die afhankelijk is

van het binden aan DNA. GA had daarentegen geen invloed op de agonist-afhankelijke accumulatie in foci of de ligand-afhankelijke interactie van de amino- en carboxyl-termini van de AR. Het is opmerkelijk dat incubatie met GA in aanwezigheid van de antagonist hydroxy-flutamide ook resulteerde in een permanente immobilisatie van de AR, die afhankelijk was van de mogelijkheid om aan DNA te kunnen binden. Deze observaties suggereren dat hsp90, naast de alom bekende rol in het cytoplasma, ook een rol speelt in de dissociatie van zowel door agonisten als door antagonisten gebonden AR en dat dit eiwit daarom de activering van transcriptie mogelijk op meerdere niveaus reguleert.

Hoofdstuk 7 beschrijft de eerste resultaten van een studie naar het mechanisme dat ten grondslag ligt aan de translocatie van hormoongebonden ARs naar de celkern. We hebben de rol van microtubuli (MTs) en eiwitten die binden aan de plus einden van MTs (+TIPs) bestudeerd door gebruik te maken van chemische destabilisatie van MTs door nocodazole en muis embryonale fibroblasten, die deficiënt zijn voor de +TIPs cytoplasmatisch linker eiwitten (CLIP) 115 en 170 of voor het CLIP-geassocieerd eiwit 2 (CLASP2). De eerste resultaten suggereren dat, net als bij transport van blaasjes (vesicles), de activiteit van CLIPs vereist is om de AR te laden op het MT transport systeem, dat assisteert bij transport van de AR van het cytoplasma naar de kern.

Hoofdstuk 8 vormt de algemene discussie van het proefschrift en plaatst de bevindingen van de studies beschreven in Hoofdstuk 4 t/m 7 in een breder perspectief. Daarnaast worden de mogelijke implicaties voor ziekten waarbij de AR een rol speelt besproken.

De studies beschreven in dit proefschrift leveren een bijdrage aan de fundamentele kennis van het werkingsmechanisme van de AR in de levende cel. Deze kennis kan mogelijk gebruikt worden bij de ontwikkeling van nieuwe therapeutische strategieën voor ziektes waarin de AR een rol speelt.

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van onze discussies zowel binnen het JNI als tijdens onze ontdekkingstochten van de omgeving van Erasmus MC. Leuk dat je mijn paranimf wilt zijn. Karin, ik ben erg benieuwd naar toekomstige bevindingen, houd me op de hoogte! Sónia, we maar kort samen op het lab hebben gezeten, maar je was een gezellige collega die altijd te vinden was voor een terrasje.

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Pascal

Curriculum Vitae

Pascal Farla werd op 20 juli 1976 geboren te Geldrop. In 1994 behaalde hij zijn VWO-diploma aan het Lorentz Lyceum te Eindhoven, waarna hij in september van dat jaar startte met de studie Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen (KUN). Na het behalen van de propedeuse in 1995 volgde hij de doctoraalfase van deze opleiding. In 1997 deed hij een korte stage bij de afdeling Farmacologie van de KUN onder leiding van dr. Frans Russel en Leni Siero. Begin 1998 startte hij zijn hoofdvakstage bij de Universitaire Transfusie Dienst van het Academisch Ziekenhuis Nijmegen St. Radboud in de groep van dr. Irma Joosten, onder leiding van Hans Koenen. In deze stage bestudeerde hij de effecten van cyclosporine A, dat gebruikt wordt bij transplantaties om afstotingsreacties te onderdrukken, op een celkweekmodel voor afstoting. Van september 1998 tot maart 1999 deed hij onderzoek naar de effecten van kwikchloride op intracellulaire signaaltransductieroutes in de groep van dr. David Barnes in het Center of Excellence for Poultry Science van de University of Arkansas te Fayetteville. Na zijn terugkeer uit de V.S. behaalde hij in april 1999 zijn doctoraal diploma Biomedische Gezondheidswetenschappen, met als afstudeerrichting Toxicologie. In december 1999 begon hij als Assistent in Opleiding in de groep van dr. Adriaan Houtsmuller in samenwerking met de groep van prof. dr. Jan Trapman aan het promotieonderzoek dat beschreven is in dit proefschrift. Vanaf september 2005 is hij als post-doc werkzaam in de groep van dr. Bob van de Water bij de afdeling Toxicologie van de Universiteit Leiden.

List of publications

Farla, P., Hersmus, R., Geverts, B., Mari, P. O., Nigg, A. L., Dubbink, H. J., Trapman, J. and Houtsmuller, A. B. (2004). The androgen receptor ligand-binding domain stabilizes DNA binding in living cells.
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Submitted.

List of abbreviations

+TIP	MT plus-end interacting protein
AD1/2	Activation domain 1/2
ADP	Adenosine biphosphate
AF-1/2	Activation function-1/2
AIS	Androgen insensitivity syndrome
AR	Androgen receptor
ARA	Androgen receptor activator
ARE	Androgen response element
ATP	Adenosine trisphosphate
BAF	BRM/BRG associated factor
BRG	Brahma related gene
BRM	Brahma
cAMP	Cyclic adenosine monophosphate
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
cdk	Cyclin dependent kinase
CFP	Cyan fluorescent protein
CHD	Chromodomain
ChIP	Chromatin immuno precipitation
CREB	cAMP response element binding protein
CTD	C-terminal domain
Da	Dalton
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
DNA	Deoxyribonucleic acid
E2	Estradiol
ER	Estrogen receptor
ERCC	Excision repair cross complementation
FKBP	FK506 binding protein
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GRIP1	Glucocorticoid receptor interacting protein 1
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyl transferase
Hsp	Heat shock protein
ISWI	imitation switch
LBD	Ligand-binding domain
MAPK	Mitogen activated protein kinase
MED	Mediator
MMTV	Mouse mammary tumor virus
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MT	Microtubule
N-CoR	Nuclear receptor corepressor
NER	Nucleotide excission repair
NLS	Nuclear localization signal
NR	Nuclear receptor
NTD	Amino (NH_2)-terminal domain
NuRD	Nucleosome remodeling and deacetylation
P/CAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PMRT1	protein arginine methyltransferase 1
PPAR	Peroxisome prolifererator-activated receptor
PR	Progesterone receptor
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RNAP	RNA-polymerase
RSC (-complex)	(Complex with capacity to) remodel the structure of chromatin
RXR	Retinoic X receptor
SBMA	Spinal bulbar muscular atrophy

SMRT	Silencing mediator of retinoid and thyroid receptors
SNF	Sucrose non-fermenting
SRC	Steroid receptor coactivator
SWI	Switch
TAF	TBP associated factor
TBP	TATA-box binding protein
TF	Transcription factor (as in TFIIH)
TIF2	Transcription intermediary factor 2
TR	Thyroid hormone receptor
VDR	Vitamin D receptor
XP	Xeroderma Pigmentosum (as in XPA)
YFP	Yellow fluorescent protein