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THE ROLE OF LIGAND IN THE INTERACTION OF ANDROGEN RECEPTOR WITH DNA AND COACTIVATORS

Ulla Karvonen

Institute of Biomedicine/Physiology
University of Helsinki
Finland

Academic Dissertation

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Supervised by

Docent Jorma J. Palvimo

and

Professor Olli A. Jänne

Reviewed by

Professor Anitta Mahonen

University of Kuopio

and

Docent Veli Isomaa

University of Oulu

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CONTENTS

SUMMARY

Androgen receptor (AR) belongs to the family of nuclear receptors which are ligand-regulated transcription factors. Testosterone and dihydrotestosterone are the principal physiological agonistic ligands for AR. Androgen antagonists (antiandrogens) can be divided into full and mixed antiandrogens based on their ability to block AR-dependent transcription. In addition to the ligand, the transcriptional activity of AR and other steroid receptors is regulated by coregulators. Mechanisms applied by coregulators in governing steroid receptor activity include covalent modifications, such as phosphorylation, acetylation, ubiquitinylation and sumoylation. Sumoylation, conjugation of SUMO-1 (small ubiquitin-related modifier 1) to proteins, regulates several cellular events: transcriptional activity, targeting and turnover of proteins.

Microscopy of living cells transfected with enhanced green fluorescent protein (EGFP)-tagged ARs was used to examine trafficking of the receptor and to study the effect of coactivator SNURF on cellular movement kinetics and nuclear localization of the receptor. Androgen exposure led to a rapid and complete nuclear import of the receptor, whereas the pure antiandrogen casodex elicited a much slower and incomplete transfer. Mutations in the basic amino acids within the second zinc finger and the hinge region severely compromised the nuclear import. The ligand-binding domain was found to contain another nuclear localization signal. AR coactivator SNURF was able to facilitate AR nuclear import even in the absence of ligand, and it is the only known AR coactivator to support the trafficking of apo-AR. SNURF also tethered AR to the nuclear matrix, which may be important in the regulation of the transcriptional activity of AR.

Pure antiandrogens, hydroxyflutamide (OH-Flu) and casodex (BCA), blocked several events mandatory for AR-dependent transcription. These compounds inhibited DNA binding of AR in intact cells as assessed by promoter interference assay, whereas a mixed antiandrogen, cyproterone acetate (CPA), enhanced DNA binding of AR. Confocal laser microscopy was used to study the effect of agonistic and antagonistic ligands on the localization pattern of AR and its coactivator GRIP1. GRIP1 colocalized with AR in nuclei in an agonist-dependent manner and enhanced transcriptional activity of AR, indicating that agonist-induced conformational change in AR is needed for the receptor to recruit GRIP1. Pure antiandrogens, OH-Flu and BCA, but not the partial antagonist CPA, blocked this recruitment.

GRIP1 was shown to be covalently modified by SUMO-1 at lysine residues 239, 731, 788 and 1452. Sumoylation-deficient mutants of GRIP1 were studied to clarify the effect of SUMO-1 modification on the interaction of GRIP1 with AR. Destruction of the principal sumoylation sites in GRIP1 impaired AR-GRIP1 colocalization in about half of unsynchronized cells, which was reflected by the reduced ability of the sumoylation-deficient GRIP1 to enhance AR-dependent transcription. This suggests that sumoylation of GRIP1 may be regulated in a cell cycle-dependent fashion. Thus, in addition to hormonal ligand, covalent modifications, such as sumoylation, regulate the AR-GRIP1 interaction.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Karvonen U, Kallio PJ, Jänne OA, Palvimo JJ (1997) Interaction of androgen receptors with androgen response element in intact cells. Roles of amino- and carboxyl-terminal regions and the ligand. J Biol Chem 272:15973-15979.

II Poukka H, Karvonen U, Yoshikawa N, Tanaka H, Palvimo JJ, Jänne OA (2000) The RING finger protein SNURF modulates nuclear trafficking of the androgen receptor. J Cell Sci 113:2991-3001.

III Karvonen U, Jänne OA, Palvimo JJ (2002) Pure antiandrogens disrupt the recruitment of coactivator GRIP1 to colocalize with androgen receptor in nuclei. FEBS Lett 523:43-47.

IV Kotaja N, Karvonen U, Jänne OA, Palvimo JJ (2002) The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1. J Biol Chem 277:30283-30288.

In addition, some unpublished results are presented.

ABBREVIATIONS

AF-1	activation domain 1
AF-2	activation domain 2
AR	androgen receptor
ARE	androgen response element
BCA	casodex
CARM1	protein arginine methyltransferase I
CaP	prostate carcinoma
CBP/p300	CREB-binding protein
CPA	cyproterone acetate
DBD	DNA-binding domain
EGFP	enhanced green fluorescent protein
ER	estrogen receptor
FHL2	four and a half LIM domain protein 2
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein 1
HAT	histone acetylase
HDAC	histone deacetylase
HRE	hormone response element
hsp	heat shock protein
LBD	ligand-binding domain
MAPK	mitogen-activated protein kinase
MAR	matrix attachment region
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
NcoR	nuclear receptor corepressor
ND10	PML nuclear domain
NLS	nuclear localization signal
non-FAT	non-factor acetyltransferase
NPC	nuclear pore complex
NR	nuclear receptor
NTD	N-terminal domain
OH-Flu	hydroxyflutamide
p160	p160 family of NR coactivators
PIA	promoter interference assay
PIAS	protein inhibitor of activated STAT
PML	promyelocytic leukemia protein
PR	progesterone receptor
RanGAP1	RanGTPase-activating protein 1
RAR	retinoid acid receptor
RNA pol II	RNA polymerase II
SMRT	silencing mediator of RAR and TR
SNURF	small nuclear RING finger protein
SR	steroid receptor
Src	tyrosine kinase-c
SRC-1	steroid receptor coactivator 1
SUMO-1	small ubiquitin-related modifier 1
Ubc9	ubiquitin-conjugating enzyme 9 (SUMO-conjugating E2 enzyme)
VDR	vitamin D_3 receptor

REVIEW OF THE LITERATURE

1. NUCLEAR RECEPTORS WITH A SPECIAL REFERENCE TO ANDROGEN RECEPTOR (AR)

1.1. Introduction

Androgens are needed for the development and maintenance of male reproductive system and their action is mediated through the androgen receptor (AR) (Quickley et al., 1995). Appropriate regulation by androgen is necessary for a range of developmental and physiological processes, particularly male sexual development and maturation as well as for the maintenance of male reproductive organs and that of spermatogenesis (McLachlan et al., 1996; Roy et al., 1999). AR is expressed in cells of a wide range of tissues far beyond primary and secondary sexual organs (Lindzey et al., 1994; Quickley et al., 1995). The principal physiological androgens, testosterone (T) and its metabolite 5α-dihydrotestosterone (DHT), predominantly mediate their biological effects through binding to the androgen receptor. Antiandrogens are androgen antagonists that block AR transactivation.

AR belongs to the superfamily of nuclear receptors (NR) which are transcription factors that activate transcription of their target genes in response to specific ligands. NRs can be divided into three classes (Beato et al., 1996). Type I receptors include the classical steroid receptors AR, estrogen receptor α and β (ER), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). The type II nuclear receptors dimerize with the 9-cis retinoic acid receptor (RXR) and include the receptors for vitamin D_3 (VDR), thyroid hormone (TR), retinoid acid (RAR), and the peroxisome proliferator-activated receptors (PPAR). Orphan NRs (ONRs) are categorized in the type III class within the NR superfamily (Giguère, 1999; Mangelsdorf and Evans, 1995).

NRs consist of three principal domains: highly variable amino-terminal (N) transactivation domain (TAD), conserved DNA-binding domain (DBD) and somewhat variable carboxy-terminal (C) ligand binding-domain (LBD) (Mangelsdorf et al., 1995). The transactivation functions of NRs reside in N-terminal activation function (AF-1) and in C-terminal AF-2 (Figure 1a). The AR AF-2 is weak compared to that of other NRs. In the case of AR, AF-1 consists of two short adjacent amino acids stretches, and its activity displays promoter and cell-line specificity (Ikonen et al., 1997). To activate transcription, AR triggers the assembly of transcriptional complexes at an androgen response element (ARE) in response to androgen binding. AR action is regulated to some degree by a negative feedback to transcription of the AR gene itself, since AR mRNA levels are regulated by androgen and by other steroid hormones.

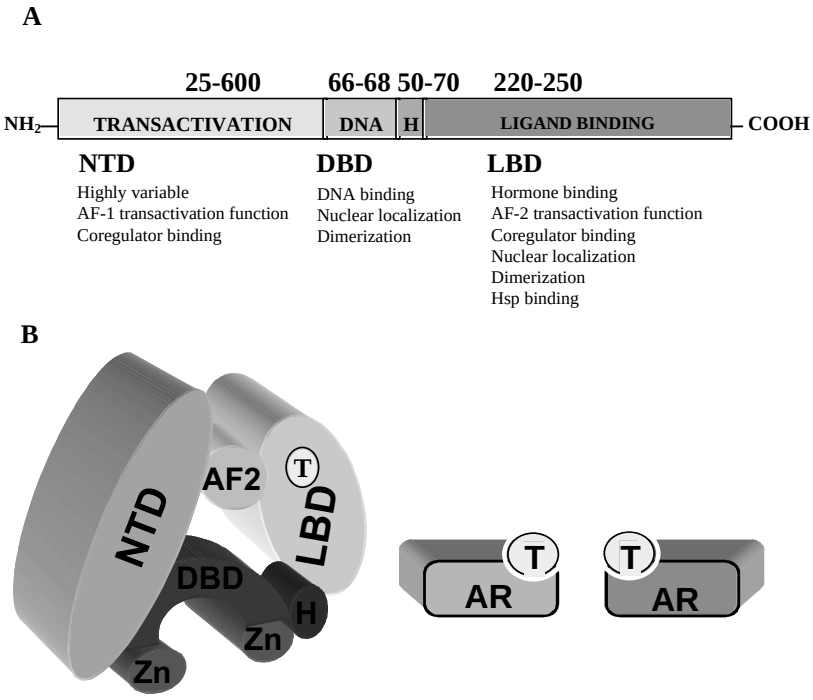


Fig. 1. Panel A: modular structure of nuclear receptors. The main functions of each domain are shown. The numbers depict the number of amino acids in each domain. Panel B, on the left: the N-C-terminal interaction and the conformational change of the LBD of AR induced by agonistic ligand binding, on the right: ligand binding induced dimerization of AR. Abbreviations: NTD, N-terminal domain; DBD, DNA-binding domain, LBD, ligand-binding domain; T, testosterone, AF2, activation function 2 of LBD, Zn, zinc finger.

1.2. Hormone-induced effects

To activate transcription, the steroid receptors (SRs) have to enter the nucleus, dimerize and bind to DNA. Ligand binding triggers this chain of events. In the absence of hormone, SRs are bound by heat-shock protein (hsp)/immunophilin complexes (Pratt and Toft, 1997) that act as chaperones. Subcellular localization of unliganded receptors is variable. One critical function of the hsp heterocomplex is to facilitate folding of the LBD into a high-affinity steroid-binding conformation. Hsp90 regulates hormone binding affinity *in vivo* (Fang et al., 1996), and hsp90s are required for the acquisition of active conformation in agonist-bound AR to regulate nuclear transfer, nuclear matrix binding, and transcriptional activity (Georget et al., 2002). In the case of GR, the switch between immunophilins FKBP51 and FKBP52 in the heterocomplexes controls the subcellular localization and nuclear transport of GR (Davies et al., 2002). Chaperones p23 and hsps selectively reduce NR transactivation by disabling transcriptional regulatory complexes by dissociating receptors from DNA and coactivators from receptors (Freeman and Yamamoto 2002).

1.2.1. Hormone-induced conformational change

Crystal structures of RXRα (Bourguet et al., 1995), of RARγ (Renaud et al., 1995) and TRα (Wagner et al., 1995) LBDs show a high degree of conservation. The apo-LBDs consist almost entirely of helices arranged into three layers to form an anti-parallel α-helical sandwich. LBD contains ligand-dependent activation function AF-2 (Webster et al., 1988; Danielian et al., 1992; Barrettino et al., 1994). Agonist binding induces a conformational change especially in the C-terminal AF-2, which exposes an amphipathic α-helix for interaction with coactivator proteins. Many coactivators bind to a surface formed by helices 3, 4, and 12, and the repositioning of helix 12 is crucial for the interaction (Figure 2). Upon binding of an agonist, the NR LBDs adopt an active conformation which allows the presentation of a coactivator binding pocket, permitting the docking of coactivator proteins via a helical LXXLL motif (Shiau et al., 1998; Nolte et al., 1998; Heery et al., 1997).

AR appears to be one of the most selective NR, as it interacts with only a limited subset of LXXLL sequences. AR is also unique among SRs, since its N-terminal AF-1 is critical in transcriptional activation and an LBD-deficient AR is constitutively active (Jenster et al., 1991; Zhan et al., 1994; Ikonen et al., 1997). In the case of holo AR, the agonist-induced conformational change of the LBD leads to amino-carboxyterminal interaction which is a prerequisite for AR transactivation (Ikonen et al., 1997; He et al., 2002) (Figure 1b). The N/C-terminal interaction creates a coregulator binding pocket in the LBD that differs from that of GR (Sack et al., 2001). AR LBD has a C-terminal extension that is essential for hormone binding (Jenster et al., 1991; Klotzbucher et al., 1997).

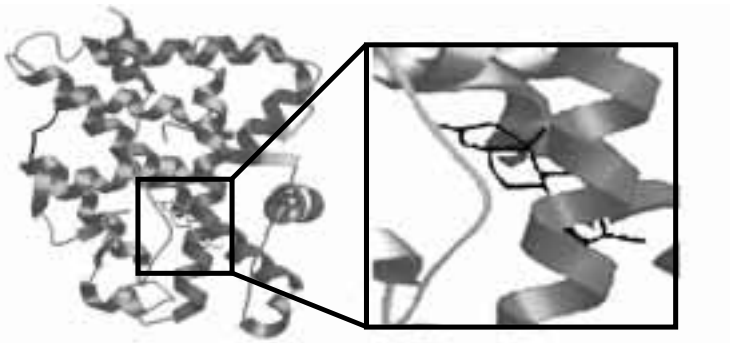


Fig. 2. Ligand bound to the LBD of AR. On the left, LBD of AR with bound 5α-dihydrotestosterone. On the right, magnified view of 5α-dihydrotestosterone bound to the ligand binding pocket of AR LBD. (Adapted from Sack et al., 2001.)

1.2.2. Trafficking and nuclear localization

Most NRs are constitutively nuclear. Hormone binding induces transformation, which leads to transport of cytoplasmic receptors to the nucleus (Ylikomi et al., 1992; Jenster et al., 1993; Zhou et al., 1994; Sackey et al., 1996; Carey et al., 1996). This requires, at least in the case of GR, both the presence of hsp90 and intact cytoskeleton (Galigniana et al., 1999) (Figure 3). Within the nucleus, the receptors are further compartmentalized to subnuclear domains associating and dissociating with chromatin and the nuclear matrix (van Steesel et al., 1995; Yong et al., 1997; Tang et al., 1996; 1998). Also ERα that is constitutively nuclear, changes in its nuclear distribution pattern in response to ligand (Htun et al., 1999; Stenoien et al., 2000); agonists, but not antagonists, induce redistribution of ERα together with the coactivator SRC-1 to nuclear matrix-associated speckles (Stenoien et al., 2000).

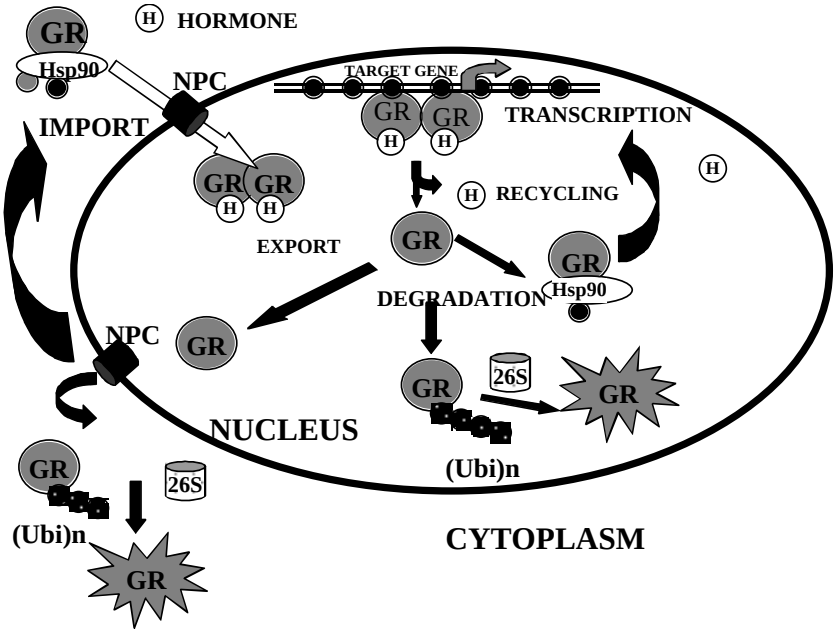


Fig. 3. Recycling of GR. The aporeceptor resides in the cytoplasm complexed with hsp90 (heat shock protein 90) and immunophilin proteins. Upon ligand binding receptor dimerizes and translocates to the nucleus through nuclear pore complex (NPC). Agonist-occupied receptor (GR, glucocorticoid receptor) binds to recognition sites on specific promoters on target genes and triggers transcription. Receptor binds to and detaches from chromatin in a cyclic fashion. Ligand removal results in either receptor recycling or degradation. Recycling is achieved either through renewed ligand binding already inside the nucleus or after export to the cytoplasm. Ubiquitinylated (ubiquitin, Ubi) receptors are degraded in proteasomes (26S) either in the nucleus or in the cytoplasm. (Adapted from DeFranco, 2002.)

SRs shuttle between the cytoplasm and the nucleus and the balance between export and import defines their localization (Madan and DeFranco, 1993; Dauvois et al., 1993; Guichon-Mantel et al., 1994; Hachè, 1999). Nuclear localization signal (NLS) is needed for nuclear targeting of

proteins. In SRs, the DBD and the flanking hinge region contain a bipartite NLS (NL1) (Ylikomi et al., 1992) which acts constitutively. Export mechanisms are not well understood, but often nuclear export is mediated through Leu-rich sequences (Gerace et al., 1995; Corbelt and Silver, 1997). After hormone withdrawal, the export of SRs from the nucleus usually takes several hours, even though the release of hormone from receptor occurs very quickly (Hachè, 1999; Madan and DeFranco, 1993; Tyagi et al., 1998). At least GR detached from chromatin resides in a distinct subnuclear compartment, and it is still able to bind hormone (Yong et al., 1997).

1.2.3. DNA binding

Members of the nuclear receptor superfamily directly activate or repress target genes by binding to hormone response elements (HREs) in promoter or enhancer regions of the genes (Tsai and O'Malley, 1994; Mangelsdorf et al., 1995; McKenna et al., 1999). Recently, it has become evident that transcriptionally active NRs bind to and detach from DNA continuously in a cyclic fashion (Shang et al., 2000; 2002; Kang et al., 2002).

SRs bind as ligand-induced receptor dimers to HREs consisting in many cases of two inverted 6 bp half sites separated by three nucleotides. HREs provide specificity to receptor dimer binding (Bourguet et al., 2000), and the spacer nucleotides and the regions flanking the half sites play an important role in establishing receptor binding specificity (Nelson et al., 1999). ER has an unique HRE (ERE) whereas AR, GR, PR and MR share a common consensus response element, although in natural promoters the HREs most likely differ from the consensus sequence thereby creating receptor specificity (reviewed by Freedman, 1992).

The interaction of AR with specific AREs is required for androgen-dependent transcriptional activation, whereas DNA binding is not usually necessary for transrepression by AR (Kallio et al., 1995; Palvimo et al., 1996; Schneiker et al., 1996). In addition to palindromic ARE, also AREs composed of direct repeats exist (Zhou et al., 1997; Shoenmakers et al., 2000; Verrijdt et al., 2000). The probasin promoter harbors selectively androgen-regulated complex response elements (Rennie et al., 1993; Kasper et al., 1994; Greenberg et al., 1994; 1995). Next level of SR selectivity is provided by the highly variable N-terminal regions of SRs that are responsible for steroid-specific regulation of target genes (Adler et al., 1992). Also regions near the AR DBD are critical for the DNA-binding specificity (Kallio et al., 1994; Schoenmakers et al., 1999; 2000). The most common AREs on natural AR-regulated promoters contain the 5´-A/GAACAnnnA/TGT/GG/A/TC/T/AT-3´ sequence (Claessens et al., 2001).

1.2.4. Nongenomic androgen action: AR-independent regulation by androgens

Androgens have been reported to induce rapid activation of protein kinase signaling cascades and modulate intracellular calcium levels (reviewed by Heinlein and Chang, 2002**)**. These rapid effects are considered to be nongenomic, because they occur in cell types that lack a functional AR or they are thought to be mediated through AR functioning on cell surface or in the cytoplasm to induce the mitogen activated protein kinase (MAPK) signal cascade (Benten et al., 2002). In addition, androgens may function through the sex hormone-binding globulin receptor and possibly a distinct G protein-coupled receptor to activate second messenger signaling mechanisms (reviewed by Heinlein and Chang, 2002**)**. These second messenger cascades may ultimately serve to modulate the transcriptional activity of AR or other transcription factors. AR, progesterone receptor (PR) and the estrogen receptor (ER) are able to activate the MAPK through a nongenomic mechanism independent of their transcriptional activity (Migliaccio et al., 2000; Kousteni et al., 2001; Boonyaratanakornkit et al., 2001). In addition to the classical AR, androgens can also stimulate second messenger cascades through at least one plasma membrane receptor. Membrane receptor-mediated events are typically not blocked by antagonists of the classical AR, and they can be observed in cells devoid of AR (Konoplya et al., 1992; Benten et al., 1999a; 1999b). AR, PR, and ER have been found to interact with the intracellular tyrosine kinase c-Src, triggering c-Src activation (Migliaccio et al., 2000; Kousteni et al., 2001; Boonyaratanakornkit et al., 2001). In LNCaP cells, inhibition of c-Src kinase or MAPK activity prevents androgen-induced cell cycle progression (Migliaccio et al., 2000).

1.3. Interactions with coregulators

Regulation of gene transcription by nuclear receptors requires the recruitment of proteins characterized as coregulators (Rosenfeld and Glass, 2001). Coregulators modulate the transcriptional activity of nuclear receptors; coactivators enhance and corepressors repress nuclear receptor-dependent transcription. Coregulatory complexes provide means to regulate gene expression in a cell- and promoter-specific fashion. These coregulatory components may, in turn, be targets of diverse intracellular signaling pathways. Coactivators themselves may also function to facilitate ligand binding, promote receptor nuclear translocation or mediate signal transduction.

A diverse group of proteins have emerged as potential NR coactivators. Coregulators function primarily to facilitate DNA occupancy, chromatin remodeling, or recruitment of general transcription factors associated with the RNA polymerase II holocomplex (Figure 4). These coactivators are often components of large multiprotein complexes. Many of these factors are capable of potenting nuclear receptor activity in transient cotransfection assays. These factors act

in a sequential and/or combinatorial manner to reorganize chromatin templates and to modify and recruit basal transcription factors and RNA polymerase II (Wu et al., 1997; Wade et al., 1999).

Ligand-dependent recruitment of coactivators is most often dependent on NR AF-2 (Bourguet et al., 2000), although some of coactivators interact with multiple sites on the receptor. For example, steroid receptor coactivator-1 (SRC-1) and glucocorticoid receptor-interacting protein-1 (GRIP-1) may interact with both the AF-1 and AF-2 of PR and ER (McInernet et al., 1996; Webb et al., 1998; Onate et al., 1998). AR is again an exception, in that it is primarily dependent on AF-1 in its interactions with coactivators.

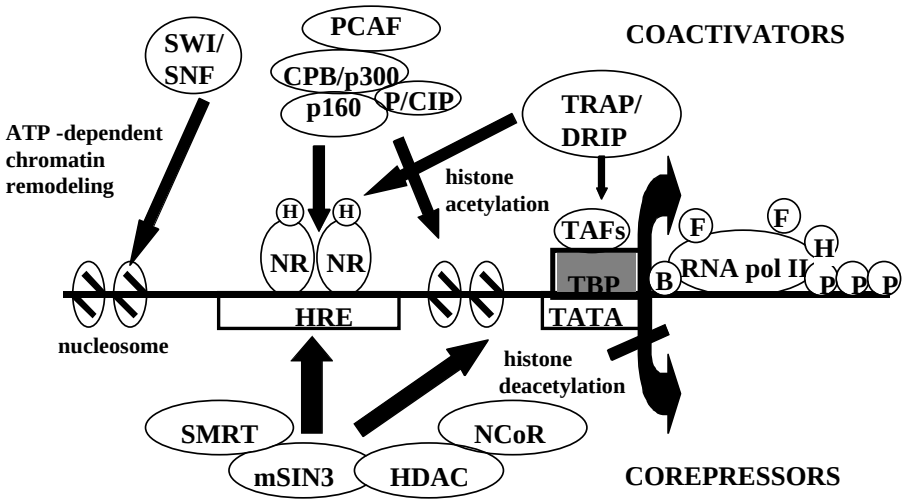


Fig. 4. Coactivator and corepressor complexes modulate the activity of the nuclear receptors (NR). Chromatin is remodeled by ATP-dependent complexes. For transcriptional activation, histones are acetylated by several histone acetylase (HAT)-activity containing factors, including CBP/p300 (p300/CREB binding protein) and PCAF (p300/CREB binding protein (CBP)-associated factor), P/CIP (p300/CBP-interacting protein) and p160 (NR coregulator protein family), are necessary coactivators. NR interaction with basic transcription machinery is mediated by the TRAP/DRIP complex. Transcriptional repression is achieved by interaction of apo-receptors or antagonist-occupied receptors with repressors SMRT (silencing mediator for RAR and TR) or NCoR (nuclear receptor corepressor) thereby recruiting histone deacetylase (HDAC) complexes. B, F, E and H depict TFIIB, TFIIF, TFIIE and TFIIH. HRE (hormone response element) on target promoter. (Adapted from Rosenfeld and Glass, 2001.)

1.3.1. ATP-dependent chromatin remodeling complexes and the TRAP-DRIP-ARC complex

Transcription by nuclear receptors is thought to be a multistep process wherein the agonist-loaded receptor binds to the target DNA recognition sequence, and coactivators assist in establishing or maintaining an open chromatin structure either through direct modification of nucleosomes or by recruiting chromatin modifying complexes (Jenster et al., 1997; Wong et al., 1997) (Figure 4).

Critical aspect of gene activation involves nucleosomal remodeling (reviewed by Wu et al., 1997; Wade et al., 1999; Struhl et al., 1999). Two general classes of chromatin remodeling factors appear to play important roles in transcriptional activation by nuclear receptors. These are ATP-dependent nucleosome remodeling complexes and factors that contain histone acetyltransferase (HAT) activity. The yeast SWI/SNF complex facilitates the binding of sequence-specific transcription factors to nucleosomal DNA by causing local changes in chromatin structure in an ATP-dependent manner. Components of the SWI/SNF complex have been shown to interact with ER and GR, and mutations in the SWI/SNF genes in yeast prevent transcriptional activation by GR (Ichinose et al., 1997). A novel ATPase, ARIP4, that belongs to the SNF2-like family of proteins, interacts with AR and modulates androgen-dependent transcription (Rouleau et al., 2002). Transfection of ATPase-defective alleles of either Brg1 or hBrm into mammalian cell lines leads to a significant decrease in the ability of several nuclear receptors to activate transcription (Pazin and Kadonaga, 1997a; Wu et al., 1997; Wade et al., 1999; Struhl et al., 1999).

The thyroid hormone receptor associated protein-vitamin D interacting protein-ARC (TRAP-DRIP-ARC) complex is recruited to nuclear receptors in a ligand-dependent manner via a 220-kDa component referred to as PBP/TRAP220/DRIP205 (Fondell et al, 1999 Rachez et al., 1998; Naar et al., 1999). The TRAP-DRIP-ARC complex consists of more than a dozen polypeptides and mediates interactions of NRs with RNA polymerase II complexes.

1.3. 2. Acetyltransferases

Rates of gene transcription correlate roughly with the degree of histone acetylation, with hyperacetylated regions of the genome appearing to be more actively transcribed than hypoacetylated regions (reviewed by Pazin and Kadonaga, 1997b). The recruitment of a protein complex with HAT activity to a promoter may play a critical role in overcoming repressive effects of chromatin structure on transcription (Wade at al., 1999; Struhl et al., 1999; Pazin and Kadonaga, 1997a). Regulated activation events might involve the exchange of complexes containing histone deacetylase functions with those containing HAT activity. It appears that in most cases the acetyltransferases are not directly recruited to nuclear receptors but they associate with other coactivators that exhibit higher affinity for the liganded receptor. The acetyltransferase functions of factors such as CREB-binding protein (CBP/p300) are directly required for enhanced transcription on chromatin templates (Kraus et al., 1999).

Acetylation facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of a target gene (Kouzarides et al., 2000; Ogryzko et al., 1996). Furthermore, coregulators may directly acetylate nonhistone proteins, including transcription factors (reviewed by Tatham et al., 2001 and Wang et al., 2001). Direct

coactivator-mediated acetylation of numerous transcription factors has emerged as a major determinant in regulating transcriptional activity, akin to the role of phosphorylation in signal transduction cascades. The finding that several transcription factors, such as p53 (Gu et al., 1997; Juan et al., 2000) and MyoD (Mal et al., 2001), are targets for direct acetylation and deacetylation, imply that these events play an active role in the regulation of transcription factor function and that the status of acetylation of both histones and transcription factors heavily influences gene expression profiles.

1.3.3. SRC/p160 coactivators

Several insights into the mechanisms by which coactivator complexes are recruited to nuclear receptors in a ligand-dependent manner have been provided by the identification of the p160 family of nuclear receptor coactivator (reviewed by McKenna et al., 1999) (Table 1). The p160 coactivators exhibit a common domain structure; the central conserved domain mediates ligand-dependent interactions with the nuclear receptor AF-2, whereas the conserved C-terminal transcriptional activation domain mediates interactions with either CBP/p300 or protein-arginine methyltransferase 1, CARM1 (Chen at al., 1999; Koh et al., 2001) (Figure 5). The p160 family members function as coactivators, at least in part, by serving as adapter molecules that recruit CBP and/or p300 complexes to promoter-bound nuclear receptors in a ligand-dependent manner (Torchia et al., 1997; Kurokawa et al., 1998). At least GRIP1 can associate with CARM1, which potentiates ligand-dependent transcription by several nuclear receptors (Chen et al., 1999).

Table 1. The three steroid receptor coactivator SRC/p160 family members. Abbreviations: SRC-1, steroid receptor-coactivator-1, NCoA1, mouse homolog of SRC-1; TIF2, transcription intermediary factor 2; GRIP1, mouse homolog of TIF2 glucocorticoid receptor-interacting protein-1; p/CIP, p300/CBP cointegrator-associated protein; ACTR, activator of thyroid hormone receptor; RAC, receptor-associated coactivator; AIB1, amplified in breast cancer; TRAM-1 thyroid hormonereceptor activator molecule 1.

Coactivator	Species	Reference
SRC-1	human	Onate et al., 1995
NCoA-1	mouse	Kamei et al., 1996
TIF2	human	Voegel et al., 1996
GRIP1	mouse	Hong et al., 1996
NCoA-2	mouse	Torchia et al., 1997
p/CIP	mouse	Torchia et al., 1997
ACTR	human	Chen et al., 1997
AIB1	human	Anzic et al., 1997
RAC3	human	Li et al., 1997
TRAM-1	human	Takeshita et al., 1997

The p160 coactivator LXXLL motif has been found to be necessary and sufficient for ligand-dependent interactions with the nuclear receptor LBD (Torchia et al., 1997; Heery et al., 1997; Nolte et al., 1998; Feng et al., 1998; Darimont et al., 1998; Shiau et al., 1998). There are multiple LXXLL motifs within a single coactivator. Co-operative interactions with nuclear receptor dimers or heterodimers and additional residues contribute to binding specificity (McInerney et al., 1998; Heery et al., 2001). Furthermore, these contacts are sensitive to conformational changes induced by structurally distinct ligands. Corepressor-binding site partly overlaps with the coactivator binding site in nuclear receptors (Xu et al., 2002). Binding of the corepressor motif is reinforced by antagonist binding that blocks the AF-2 helix from adopting the active position. According one model, binding of corepressors to nuclear receptors occurs in the unliganded state and can be stabilized by antagonists, but this concept has been challenged lately **(**Dotzlaw al., 2002).

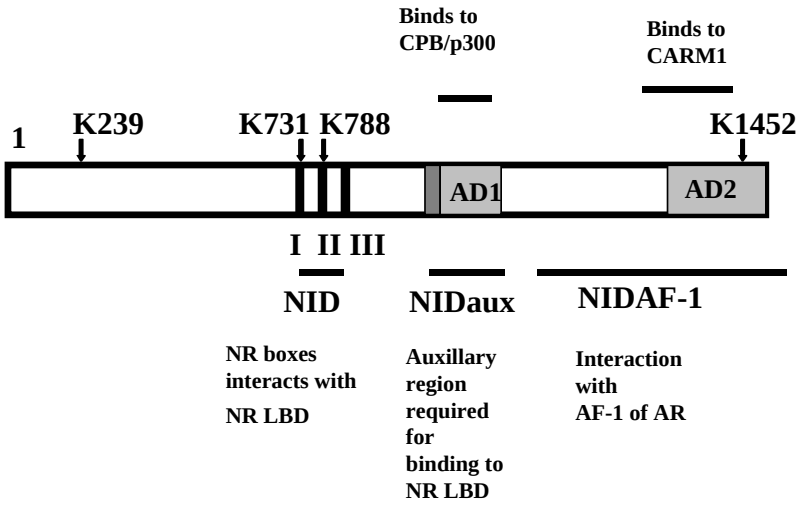


Fig. 5. Schematic structure of a p160 coactivator. NID; nuclear receptor interaction domain; AD1 and AD2; activation domains 1 and 2. The principal functions for different domains are shown. K239, K731, K788 and K1452 indicate the sumoylated lysine residues of GRIP1. (Adapted from Ma et al., 1999.)

AR is unique among the SRs, since its N-terminal AF-1 is critical in transcriptional activation, and the LBD-deficient AR is constitutively active (Jenster et al., 1991; Zhan et al., 1994). The primary site for interaction with coactivator molecules in AR is the N-terminal domain (NTD). The NTD is also the region of AR with the least evolutionary conservation. While SRC-1 and TIF-2 interact with the AR AF-2, this interaction is not essential for coactivation (He et al., 1999; Bevan et al., 1999), instead, SRC-1 and TIF-2 primarily interact with the AR N terminus and possibly the DBD. Contrary to other nuclear receptors, AR-SRC1/TIF2 interactions are independent of LXXLL motifs (He et al., 1999; Bevan et al., 1999). AR N-terminal WXXLF sequence competes with LXXLL containing coactivators for binding to AF-2 (He et al., 2000: 2001; 2002). SRC-1 and TIF-2 do not stabilize the N/C-terminal interaction of AR (He et al., 1999). Instead, this stabilization may be mediated by CBP (Ikonen et al., 1997). In any event, AR

N/C-terminal interaction creates a coregulator binding pocket in the LBD that differs from other SRs (Sack et al., 2001).

Mice lacking any of the p160 factors are viable, and only subtle defects are shown for specific receptor functions (Xu et al., 1998; Qi et al., 1999; Xu et al., 2000; Wang et al., 2000). Targeted disruption of SRC-1 in mice does not cause a significant androgen-insensitive phenotype (Xu et al., 1998), suggesting that other SRC coactivators are able to compensate for the loss of SRC-1. TIF-2 mRNA level is indeed increased in the testis of SRC-1 knockout mice (Xu et al., 1998). One GR mutation in familiar glucocorticoid resistance affecting cofactor interactions is known to result in a disease; mutated GR causes SRC-1 to sequester in coarse nuclear speckles and fails to interact with GRIP1 properly (Vottero et al., 2002).

1.3.4. Nuclear receptor- and promoter-specific coregulators

In addition to coactivators that are shared by many NRs, a number of factors have been isolated that can act in a receptor- or promoter-specific fashion. The latter coregulator proteins potentially add important enzymatic activities or protein-protein interactions and act synergistically or antagonistically with other complexes. More than 30 additional putative coactivators have been identified, including proteins with protease activity and an RNA molecule that appears to function as a coactivator (reviewed by McKenna et al., 1999). Different protein complexes can act either sequentially, combinatorially, or in parallel (McNally et al., 2000; Shang et al., 2000). These actions may contribute to receptor stability in the presence of an agonist or influence subcellular distribution of receptor. For AR, this category includes coregulators that stabilize the ligand-bound receptor, such as ARA70 (Yeh et al., 1996; 1997; Zhou et al., 2002) and coregulators such as filamin that facilitate the translocation of the ligand-bound receptor to the nucleus (Ozanne et al., 2000).

Because a growing number of SR coactivators and corepressors appear to function widely across the SR family with conserved AF-2 regions, it is unlikely that these factors alone determine specificity of receptor transcriptional regulation. Especially in the case of heavily AF-1-dependent AR, the potential existence of multiplicity of AR-specific AF-1-interacting coactivator proteins suggests that receptor- and tissue-specific coactivators are essential for selectivity and fine tuning in AR-dependent transcriptional regulation. Several AR-specific or -selective coactivators have been reported: ARA70, ARA55, and TIP60 (Yeh et al., 1996; Alen et al., 1999; Brady et al., 1999) and the AR AF-1 is often the binding target. ARA24, ARN-27, ARA160, ART-27, BRCA1, SRA, cyclin E and ANT-1 all bind to AR AF1 (Yamamoto et al., 2000; Markus et al., 2002; reviewed by Heinlein and Chang, 2002). ANT-1, a U5 small ribonucleoprotein particle-binding protein, enhances the ligand-independent autonomous transactivation function AF-1 of AR, and ANT-1 may recruit AR into the transcription-splicing-

coupling machinery (Zhao et al., 2002). The FXXLF motif in the AR N terminus is necessary for the N/C-terminal interaction of AR (He et al., 2000). This motif is also present in several AR coregulators, including ARA70, ARA55, ARA54, and FHL2.

A component of the hsp90 chaperone heterocomplex, BAG-1L, enhances transcriptional activity of AR in the presence of androgen, presumably by promoting the appropriate folding of AR (Froesch et al., 1998; Zhou et al., 2000). The AR coactivator ARA70 may play a role in AR ligand binding (Zhou et al., 2002). In addition to enhancing AR transactivation in response to normally weak agonists, ARA70 has also been postulated to enable the AR antagonists hydroxyflutamide and casodex to behave as AR agonists (Yeh et al., 1997; 1999; Miyamoto et al., 1998). Also ARA55 enhances transcriptional activity of AR in the presence of testosterone, estrogen and hydroxyflutamide (Fujimoto et al., 1999), suggesting that the specificity of sex hormones can be modulated by selective AR coactivators.

Interestingly, some of cytoskeletal acting-linking proteins interact with AR. β-Catenin plays an important role in cell-cell adhesion by linking the actin cytoskeleton to adherens junctions formed by cadherin and β-catenin. β-Catenin has also been shown to function as a transcriptional coactivator of AR in prostate cancer cells (Trucia et al., 2000). Agonist-, but not antagonist-, occupied AR shuttles β-catenin to the nucleus and nuclear interaction of AR with β-catenin may modulate transcriptional activity, since AR and β-catenin antagonize each other's effects on transcription (Pawlowski et al., 2002). In another study, β-catenin augmented ligand-dependent activity of AR in prostate cancer cells, and it was suggested to be involved in prostate tumorigenesis (Yang et al., 2002). In LNCaP cells, β-catenin enhanced AR-dependent reporter activity and sensitized AR to weak AR agonists, including androstenedione, dehydroepiandrosterone, and 17-estradiol (Trucia et al., 2000). CARM1 binds to β-catenin and can coactivate AR in synergistic fashion with β-catenin and p300 (Koh et al., 2002).

FHL2 selectively increases transcriptional activity of AR in an agonist- and AF-2-dependent manner (Müller et al., 2000). FHL2 colocalizes with AR in prostate epithelial cells. FHL2 is suggested to act as a tissue- and receptor-specific coactivator of AR. The LIM domain of FHL2 is a cysteine-rich motif that coordinately binds two zinc atoms and mediates protein-protein interactions (Bach et al., 2000). Stimulation of Rho signaling pathway appears to induce translocation of FLH2 to the nucleus and transcriptional activation of FLH2 and AR target genes (Müller et al., 2002). Prostate tumors that overexpress RhoGTPases display altered FHL2 localization concomitant with tumor dedifferentiation (Müller et al., 2002).

Nuclear translocation of a subset of nuclear receptors is mediated partly by a cytoskeleton-associated network. While disruption of microtubules or actin-containing microfilaments does not influence the ability of PR to translocate to the nucleus (Perrot-Applanat et al., 1992), disruption of the cytoskeleton blocks okadaic acid inhibition of GR nuclear localization in

response to dexamethasone (Caligniana et al., 1999). Recently, the f-actin cross-linking protein filamin has been shown to interact with the AR hinge domain and to be essential for AR nucleocytoplasmic trafficking (Ozanne et al., 2000). Filamin may be acting as a mediator between receptor and the molecular chaperone hsp90, controlling the release of activated receptor after ligand binding. After the release of hsp90 from AR, filamin may act as a molecular chaperone, maintaining the active hAR in a stable conformation (Ozanne et al., 2000).

SNURF (RNF4) is a 21-kDa AR coactivator protein that contains a RING finger motif, and it is also expressed in prostate epithelial cells (Moilanen et al., 1998). SNURF interacts with several proteins and DNA (Häkli et al., 2001). SNURF modulates transcriptional activities of AR and Sp1 via different domains, and it may act as a functional link between steroid- and Sp1-regulated transcription (Poukka et al., 2000). The POZ-AT hook-zinc finger protein (PATZ), in turn, is a SNURF-interacting protein that can attenuate SNURF/RNF4-mediated enhancement of AR transcription (Pero et al., 2002).

1.3.5. Nuclear receptor corepressors

Several members of the nuclear receptor family appear to exert critical physiological roles by actively repressing transcription. According to the classical view, NRs other than SRs, including thyroid hormone receptor isoforms, retinoic acid receptor isoforms, and vitamin D receptor, bind DNA and function as transcriptional repressors in the absence of ligand (Tsai and O'Malley, 1997). This repression is mediated in part by two related corepressor proteins, nuclear receptor corepressor (NCoR) and silencing mediator for RAR and TR (SMRT). NCoR and SMRT harbor several repressor domains and they are components of several distinct corepressor complexes (Figure 4). NCoR-null mice die in midgestation (Jepsen et al., 2000). Two sequences in the C-terminal regions of NCoR and SMRT function co-operatively to mediate interactions with DNA-bound thyroid hormone receptor/RXR heterodimers (Hu et al., 1999; Perissi et al., 1999; Nagy et al., 1997). The N-terminal repression domain of corepressors interacts with histone deacetylase (HDAC) complexes (Nagy et al., 1997). HDACs remove acetyl groups from lysine residues of histone tails, resulting in a more compact chromatin structure and decreasing the accessibility of chromatin for transcription factors, as exemplified by the ability of HDAC fused to Gal4-DNA-binding motif protein to repress transcription by 60% on chromatin templates but not on naked DNA (Huang and Kadonaga, 2001). Also cellular localization of target proteins and HDAC proteins themselves is an important regulatory mechanism (McKinsley et al., 2000a; b).

The NR corepressors NCoR and SMRT interact with NRs through a binding site that overlaps with the SRC-binding site in the LBD. The ligand-induced movement of helix 12 in the LBD of NRs that generates the SRC-binding site concomitantly occludes the NCoR- and SMRT-binding site, thereby acting as a corepressor-coactivator switch (Chen and Evans, 1995; Ordentlich et al.,

1999; Park et al., 1999). Lately, it has become clear that corepressors also regulate liganded SRs. Both NCoR and SMRT interact with ER when bound to the mixed agonist tamoxifen, an ER ligand that acts as an agonist or antagonist in a tissue-specific manner, and overexpression of either corepressor abolishes agonist activity of tamoxifen (Jackson et al., 1997). NCoR also appears to interact directly with AR and represses its dihydrotestosterone-stimulated activity (Cheng et al., 2002). Agonist-bound AR has been suggested to readily flip between coactivator and corepressor binding conformations. The relative concentration of NCoR and other corepressors vs. that of coactivators may be more important for the regulation of AR function than the function of other SRs, since androgen levels do not fluctuate markedly in adult males. At least two corepressors of androgen-bound AR have been identified to date, cyclin D and calreticulin. Cyclin D1 reduces AR transcription in the presence of the synthetic androgen R1881 (Knudsen et al., 1999). Calreticulin, a calcium binding chaperone protein, may inhibit AR-dependent transcription in response to R1881 and prevent AR binding to its response element (Dedhar et al., 1994).

Coregulators are organized *in vivo* into complexes (Fondell et al., 1996; McKenna et al., 1998; Rachez et al., 1998) many of which share subunits (reviewed in Rosenfeld and Glass, 2001), suggesting that these biochemical complexes trade and switch subunits contributing to cyclical assembly of coregulator complexes (Freeman and Yamamoto, 2001; Shang et al., 2000). Coregulators may play an important role in interpreting the tissue specificity of many NR ligands and selective receptor modulators, such as tamoxifen and raloxifene for ER (Smith et al., 1997; Graham et al., 2000).

Existence of promoter-specific coregulator requirements (Korzus et al., 1998; Puigserver et al., 1998) and promoter-specific usage of coregulatory factors leads to complicated nature of transcriptional regulation: contrary to expectations, the expression of only eight genes of 340 studied responded to histone deacetylase inhibitor treatment in a differential display analysis (Van Lint et al., 1996). More recently, promoter identity has been shown to effect functional inversion among coregulators, such that corepressors can become coactivators (Jepsen et al., 2000) and vice versa (Xu et al., 2002). In addition, variations in ligand-specified recruitment of coactivators (Katzenellenbogen et al., 2001) and/or dissociation of corepressors may be a supplementary source of signaling flexibility at NR-regulated promoters. To further complicate the picture, corepressors may be capable of binding to liganded receptor to sensitize the transcriptional response to ligand (Montano et al., 1999).

1.3.6. Coactivators and corepressors as targets of signal transduction pathways

Coactivators and corepressors are themselves targets of multiple signal transduction pathways. Regulation of coactivator and corepressor function potentially provides means for integration of

responses to specific signals across families of sequence-specific transcription factors. For example, the histone acetyltransferase activity of CBP has been suggested to be regulated by cyclin-dependent kinases, which presumably alter its coactivator activities during the cell cycle (Ait-Si-Ali et al., 1998). The p160 nuclear coactivators can be phosphorylated in response to several signaling events (Wang et al., 2000). Acetylation of p160 coactivators may facilitate transcriptional repression by dissociating coactivators from DNA-bound receptors (Chen et al., 1999). Likewise, corepressors are apparent targets of signal transduction pathways; activation of MAP kinase cascades is linked to redistribution of SMRT from a predominantly nuclear location to a predominantly perinuclear or cytoplasmic compartment (Hong et al., 2000). ER phosphorylation resulting from MAPK activation leads to detachment of NCoR from antagonist tamoxifen-occupied ER (Lavinsky et al., 1998).

1.4. Posttranslational modifications of AR

1.4.1. Phosphorylation

Posttranslational modifications have emerged as versatile and quick switches in the regulation of NRs. Transcriptional activity of AR has been found to be influenced by growth factors and cytokines through stimulation of multiple signal transduction cascades (reviewed by Russell et al., 1998: Djakiew, 2000). Insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) and keratinocyte growth factor (KGF) were able to stimulate AR-dependent reporter gene expression (Culig et al., 1995). Protein kinase A (PKA) activator was shown to activate AR in the absence of androgen (Nazareth and Weigel, 1996). This activation can be blocked by the AR antagonists casodex and flutamide, indicating that the activation effect was dependent on AR. Furthermore, treatment of LNCaP cells with protein kinase A (PKA) activators resulted in a dose- and time-dependent increase in prostate specific antigen (PSA) mRNA levels (Sadar et al., 1999). Moreover, the AR antagonist casodex blocked the PKA-dependent increase in PSA mRNA.

Kinase cascades may regulate AR function by activating the receptor in the absence of ligand or sensitizing it to reduced levels of ligand. EGF, insulin-like growth factor 1, and PKA agonist increased stimulation of the AR and sensitization of the AR to low levels of androgen (Ikonen et al., 1994; Reinikainen et al., 1996). Direct phosphorylation of AR has been shown to influence its ability to interact with coregulators. There are seven phosphorylation sites in AR (Gioeli et al., 2002). AR is postulated to be a target of the kinase Akt, one of the kinases of the phosphatidylinositol 3-kinase (PIK3) signal transduction pathway. MAPK and Akt phosphorylate AR serines 213 and 791 (Yeh et al., 1999; Lin et al., 2001; Wen et al., 2000). Phosphorylation of AR by Akt results in a decrease in the transcriptional activity of AR, and it is associated with a decrease in the ability of AR to interact with ARA70 (Lin et al., 2001). In contrast, stimulation of

MAPK by overexpression of ErB2/Her2/Neu proto-oncogene, a member of the epidermal growth factor family, is postulated to enhance AR-dependent transcription through phosphorylation of AR (Yeh et al., 1999).

Forskolin, EGF, and phorbol 12-myristate 13-acetate differentially regulate the phosphorylation state of endogenous AR in LNCaP prostate cancer cells (Gioeli et al., 2002). At least phosphorylation of GR appears to be cell cycle-regulated (Hu et al., 1994; Hsu et al., 1995). Additionally, the phosphorylation status of GR plays a prominent role in the receptor turnover (Webster et al., 1997). This appears to apply to AR as well. Akt and Mdm2, an ubiquitin ligase, may form a complex with AR and promote phosphorylation-dependent AR ubiquitinylation, which results in AR degradation by the 26S proteasome (Lin et al., 2002).

The steroid receptor coactivator (SRC) family of transcriptional coactivators are targets of MAPK and phosphorylation of SRCs by MAPK results in an increased ability of these coactivators to recruit additional coactivator complexes to the DNA-bound receptor (Font de Mora et al., 2000; Lopez et al., 2001; Rowan et al., 2000). Interleukin-6 enhances both ligand-dependent and -independent transactivation by AR to a similar extent by a mechanism that appears to be dependent MAPK and phosphorylation of SRC-1 (Ueda et al., 2002).

1.4.2. Acetylation

Recently, also AR has been found to be a substrate for p300- and PCAF-mediated acetyltransferase activity (Fu et al., 2002). Acetylation of three lysine residues in the KLKK sequences flanking the DBD increases the transcriptional activity of AR, implicating direct acetylation as a mechanism for regulating AR activity. AR activity is repressed at G1/S transition in a histone deacetylase-dependent fashion (Martinez and Danielsen, 2002). Tip60 (Tat-interacting protein, 60 kDa) is an AR coactivator protein that also acetylates the receptor (Gaughan et al., 2002). AR, Tip60, and HDAC1 form a trimeric complex on PSA promoter as judged by chromatin immunoprecipitation assays (Gaughan et al., 2002). Thus, both an acetylase and a deacetylase appear to concomitantly occupy the promoter.

1.4.3. Ubiquitinylation

In the ubiquitinylation, one or several 76-amino acid ubiquitin proteins are covalently attached to the lysines of target proteins (reviewed by Weissman, 2001). Three types of enzymes participate in ubiquitinylation. E1 ubiquitin-activating enzyme forms a thioester bond with ubiquitin and transfers it to E2 ubiquitin-conjugating enzyme. E3 ubiquitin ligase transfers ubiquitin to the target protein. Both the ubiquitinylating enzymes and the formed ubiquitin conjugates determine

specificity in ubiquitinylation (Weissman, 2001). Ubiquitin-mediated degradation of regulatory proteins is critical to several cellular processes, such as cell-cycle progression, signal transduction, transcriptional regulation, steroid receptor down-regulation and endocytosis (Hershko and Chechanoor, 1998; Hochstrasser, 2002). Ubiquitinylation can act as a signal for protein trafficking, activation of transcription factors and kinases and other non-proteolytic processes (Hicke et al., 2001; Pickart, 2001). Targeting for proteasomal degradation by 26S proteasome is the best characterized function mediated by polyubiquitinylation (Coux et al., 1996; Baumeister et al., 1998). Interestingly, an ATPase subunit of the 26S proteasome complex SUG1/TRIP1 associates with NRs and modulates their function (von Baur et al., 1996). Monoubiquitinylation has been shown to act as a signal for regulation of protein trafficking (Hicke, 2001).

The ubiquitin proteasome pathway is responsible for selective degradation of several transcription factors, including nuclear receptors, often in a ligand-dependent manner (Lonard et al., 2000) (Figure 3). ERα (Lonard et al., 2000) and PR (Lange et al., 2000) are degradated in a hormone-dependent fashion, but VDR (Nomura et al., 1999) and AR (Yeap et al., 1999) are stabilized by the cognate ligand. In the case of ER and PR, ligand-dependent proteasomal degradation has been linked to increased transcriptional activity (Lonard et al., 2000). Proteasome pathway regulates GR turnover and proteasome inhibition increases GR-mediated transactivation (Wallace and Cidlowski, 2001; Deroo et al., 2002). Blocking of GR turnover by proteasome inhibition also reduces the mobility of GR in the nucleus and increases GR attachment to the nuclear matrix (Deroo et al., 2002). Also in the case of ER, proteasomal inhibition traps ERα to the nuclear matrix (Stenoien et al., 2001). Coactivators that are targets for ubiquitin-proteasome degradation include GRIP1 (Bauman et al., 2001), CBP/p300, SRC-1 and PIC/RAC. The level of NCoR is also regulated by ubiquitin proteasome degradation (Zhang et al., 1998).

Degradation and transcriptional activation function of SRs are often interrelated. The activation domains AF-1 and AF-2 cooperate in the retinoid acid-induced RARγ2 degradation (Gianni et al., 2002). The AF-1 domain signals degradation through a marked increase in its phosphorylation that is secondary to a RA-induced activation of p38MAPK (Gianni et al., 2002). The RA-liganded AF-2 domain acts through the recruitment of the proteasomal SUG-1 subunit (Gianni et al., 2002). Thus, degradation is needed for transcriptional activation by RARγ2 (Gianni et al., 2002). SUG-1 may displace coactivators through its ATP-dependent chaperone activity (Hochstrasser, 1995). This exchange may funnel RARγ2 into the proteasome and subsequently result in its degradation (Gianni et al., 2002). Recently, proteasomal activity has been also implicated to be required for the transcriptional activity of AR (Chang et al., 2002; Kang et al., 2002; Lin et al., 2002).

1.4.4. Sumoylation and PIAS protein family

Small ubiquitin-related modifier proteins, SUMO-1 and its family members SUMO-2 and SUMO-3, are 10-11 kDa proteins with homology to ubiquitin (Melchior et al., 2000; Muller et al., 2001). SUMOs can be conjugated to a large number of cellular proteins at specific lysine residues embedded in the consensus sequence ψKxE (Melchior, 2000; Yeh et al., 2000).

The conjugation involves an enzymatic cascade analogous to ubiquitinylation and the modification can be reverted by isopeptidases (Figure 6). Heterodimeric E1 enzyme (Sua1/Uba2) and E2 enzyme (Ubc9) have been characterized (Johnson et al., 1997; Okuma et al., 1999; Desterro et al., 1999; Gong et al., 2000; Johnson et al., 1997; Schwarz et al., 1998). Recently, two types of E3 ligases, enzymes that conjugate SUMO to substrates, have been discovered: the PIAS protein family and the nucleoporin RanB2/Nup358 (Takahashi et al., 2001; Kahyo et al., 2001; Johnson et al., 2001; Kotaja et al., 2002). Vertebrates have a large family of Ulp2-related isopeptidases.

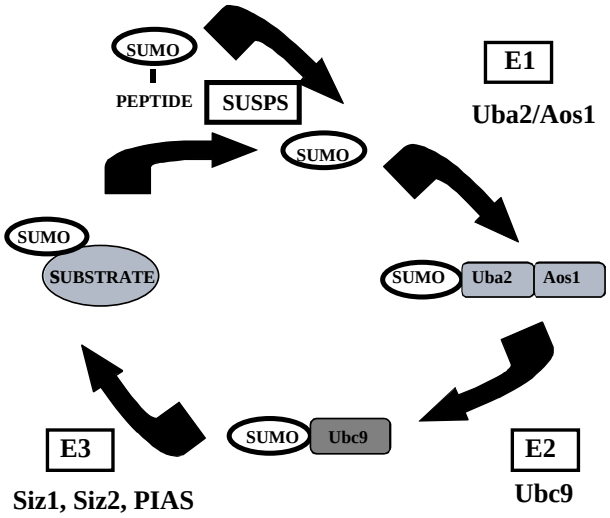


Fig. 6. Sumoylation and desumoylation cycle. E1, E2 and E3-like enzymes are involved in sumoylation of target proteins and SUSPs catalyze desumoylation and precursor processing. SUSPs are desumolating enzymes; E1s are SUMO-activating enzymes; E2s SUMO-conjugating enzymes, and E3s SUMO-protein ligases. (Adapted from Kim et al., 2002.)

Sumoylation appears to have multiple functions, including protein targeting, stabilization, and transcriptional regulation (Pichler and Melchior, 2002). Sumoylation of specific targets can be constitutive, cell-cycle regulated and induced by stress or DNA damage (Pilcher and Melchior, 2002). The RanGTPase-activating protein, RanGAP1, associates with the nucleoporin RanBP2 in a sumoylation-dependent fashion (Matunis 1996; Mahajan et al., 1997; Pichler et al., 2002), and sumoylation of RanGAP1 results in movement of the protein from the cytoplasm to the nuclear

pore complex (Saitoh et al., 1998; Mahajan et al., 1998; Matunis et al., 1998). Ulp-1 SUMO isopeptidase is tethered to the nuclear pores by interaction with karyopherins which are associated with nucleoporins. This location could allow Ulp-1 to remove SUMO-1 from sumoylated cargo proteins during their passage through the NPC in yeast (Panse et al., 2003). In the case of PML (promyelocytic leukemia) proteins, sumoylation regulates PML subnuclear localization to structures termed PML nuclear bodies (ND10s) (Sternsdorf et al., 1997; Müller et al., 1998; Kamitani et al., 1998; Duprez et al., 1999). Sumoylation appears to play a key role in the formation of subnuclear structures, at least speckles of transcription factors HIPK2 and TEL2 (Fogal et al., 2000; Jackson, 2001; Hofman et al., 2002).

One third of currently known sumoylation substrates are transcription factors, but depending on the factor, the effects of SUMO-1 attachment are diverse. Sumoylation of p53 has been proposed to regulate the transcriptional activity of p53 (Rodriguez et al., 1999; Gostissa et al., 1999; Muller et al., 2000). However, contradictory results exist for effects of sumoylation on p53 transcriptional activity (reviewed by Kim et al., 2002). Transcriptional activity of c-Jun is negatively regulated by sumoylation (Müller et al., 2000). Heat shock transcription factors 1 and 2 (HSF1 and HSF2) are sumoylated, and their activity may be regulated by sumoylation (reviewed by Kim et al., 2002). Sumoylation represses Sp3 transcriptional activation and regulates subnuclear localization of the Sp3 protein (Ross et al., 2002).

Sumoylation may antagonize ubiquitin-mediated protein degradation. Sumoylation of IκBα leads to stabilization of the NF-κB-IκBα complex thereby preventing NF-κB-dependent transcription (Desterro et al, 1998). Even though sumoylation has been shown to induce global stabilization of IκB, the cellular fraction of SUMO-1 modified IκB is still barely detectable (Desterro et al., 1998).

AR has been shown to be modified by SUMO-1 in intact cells (Poukka et al., 2000). Mutation of the sumoylation lysine residues 386 and 520 blocks the sumoylation and increases the transactivation ability of AR, suggesting that SUMO modification attenuates AR activity (Poukka et al., 2000; Nishida and Yasuda, 2002). In addition to AR, the activity of other steroid receptors is regulated by sumoylation. GR is sumoylated (Tian et al., 2002). PR autoinhibition and transrepression, exerted by PR-A on PR-B, involve sumoylation (Abdel-Hafiz et al., 2002). Sumoylation of a nuclear receptor coregulator, SRC-1, increases interaction of PR and SRC-1 (Chaucherean et al., 2003). GRIP1 sumoylation is needed for its interaction with AR (Kotaja et al., 2002). Among the sumoylated proteins involved in SR-mediated gene regulation are also histone deacetylases. For HDAC4, sumoylation and nuclear import seem to be coupled (Kirsch et al., 2002).

SUMO modification is reversible, and SUMO-1 proteases are involved in regulation of transcription. SuPr-1 is a protease that can induce c-Jun-dependent transcription (Best et al.,

2002) and alleviate SUMO modification-dependent repression of Sp3 (Ross et al., 2002). Most likely SuPr-1 enhances transcription indirectly by altering the properties and/or localization of other coactivators and/or repressors. PML concentrates coactivators and other factors to the ND10s in a SUMO-modification dependent manner, and the action of SuPr-1 may result in their release (Best et al., 2002). One possible function for SUMO-1 proteases, such as SuPr-1, could be the disruption of the nuclear ND10 structure during the cell cycle.

PIAS1 (protein inhibitor of activated STAT1) and PIAS3 were originally discovered as transcriptional coregulators of the JAK-STAT pathway (Liu et al., 1998; Chung et al., 1997). The human PIAS family consists of five homologous proteins: PIAS1, PIAS3, PIASxα, PIASxβ, PIASy. The RING finger-like domain of each PIAS protein is necessary for the SUMO E3 ligase activity (Kahyo et al. 2001; Takahashi et al., 2001; Hochstrasser et al., 2001; Kotaja et al., 2002). PIAS proteins have been shown to interact with AR (Tan et al., 2002; Kotaja et al., 2002). Rat ARIP3 (androgen receptor interacting protein 3) (Moilanen et al., 1999) is an ortholog of human PIASxα. PIAS1 and PIASxα function as SUMO E3 ligases toward AR and modulate AR-dependent transcription (Kotaja et al., 20002; Nishida and Yasuda, 2002). PIAS1 and ARIP3/PIASxα are highly expressed in the testis including cell types that express AR (Tan et al., 2000; Moilanen et al., 1999). Interestingly, the coregulatory effects of PIAS proteins are influenced by cell type and the gene enhancer/promoter in transient cotransfection assays (Kotaja et al., 2000).

2. ANDROGEN ANTAGONISTS

2.1. Effects of antiandrogens on transactivation

Androgen antagonists have been divided into two major classes; pure and mixed antiandrogens (Figure 7). Hydroxyflutamide (OH-Flu) and casodex (bicalutamide, BCA) are pure antiandrogens that exhibit solely antagonist activity, whereas cyproterone acetate (CPA) is a partial agonist that induces transcriptional activity at high concentrations (Terouanne et al., 2000; Kemppainen et al., 1992; 1999).

One difference between the pure antiandrogens hydroxyflutamide and casodex has been previously detected in their ability to stabilize AR protein, since only casodex has been reported to induce quick turnover of AR protein (Waller et al., 2000). Several of the steps in the activation of AR are interfered by antiandrogens. Induction of AR phosphorylation has been used as proof of agonistic activity of hydroxyflutamide and antagonistic nature of non-AR-phosphorylating casodex (Wang et al., 1999).

Occupation of AR with an antagonist may interfere with the first step in the AR activation chain: AR-chaperone protein interaction. Hsp90s are required for the acquisition of the active

conformation in the agonist-bound AR, and they regulate nuclear transfer, nuclear matrix binding, and transcriptional activity of AR. Pure antiandrogens may freeze AR into an inactive complex by blocking the conformational change of LBD (Georget et al., 2002). However, all antiandrogens, both pure and partial antagonists, induce AR translocation into the nucleus, although at a slower rate than agonists (Kemppainen et al., 1991; Jenster et al., 1993).

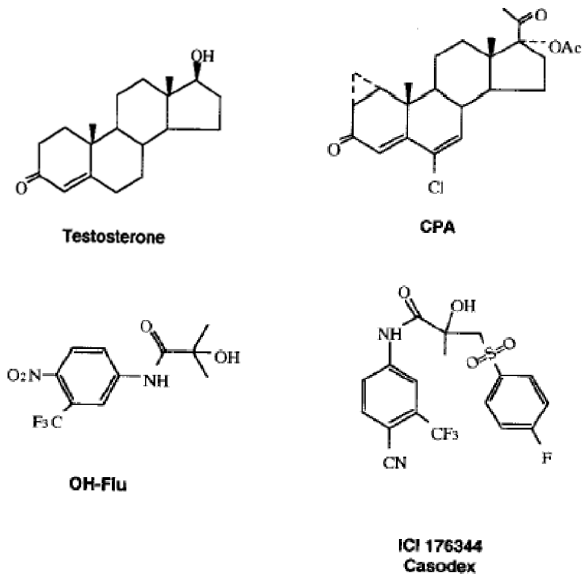


Fig. 7. Antiandrogens. Cyproterone acetate, CPA, is steroidal antiandrogen of mixed agonist/antagonist-type. Hydroxyflutamide (OH-Flu) and casodex are nonsteroidal antiandrogens which are classified as pure androgen antagonists.

Chang's group has postulated that there is a link between AR antagonists and ARA coregulators in the modulation of transactivation function of AR. They reported that hydroxyflutamide, casodex and cyproterone acetate can promote interaction of AR with ARA70 and that ARA70 enables AR antagonists hydroxyflutamide and casodex to behave as AR agonists (Yeh et al., 1997; Miyamoto et al., 1998). Also ARA55 has been shown to be able to increase the androgenic activity of hydroxyflutamide (Yeh et al., 1999; Fujimoto et al., 1999).

At least one of the antagonists, CPA, has been postulated to induce the association of the corepressor SMRT with AR. SMRT may bind to the N terminus of AR and inhibit the transactivation function of the receptor (Shang et al., 2002). SMRT does not interact with AR in the presence of the antiandrogens OH-Flu and BCA, indicating that their regulatory activities are based on other mechanisms (Dotzlaw et al., 2002).

Nonsteroidal nuclear receptors bind NCoR in the absence of ligand, but steroid receptors have been found to bind NCoR only in the presence of partial agonists such as tamoxifen and

raloxifene in the case of ER (Shang et al., 2000). NCoR has been suggested to interact directly with AR and repress 5α-dihydrotestosterone-stimulated activity of AR (Chang et al., 2002) and the antiandrogen casodex appears to induce NCoR binding to AR (Chang et al., 2002). Thus holo-AR, in contrast to other steroid receptors, may be regulated by NCoR.

2.2. Prostate cancer and antiandrogens

The effect of antiandrogens on AR-dependent transcription has been a target of intense investigation over the past ten years. The reason for this is the all more frequent occurrence of prostate cancer (CaP). Normal prostate epithelial cells are dependent on androgen for growth and differentiation (Craft et al., 1998; Kokontis et al., 1998). Prostate cancer cells initially grow in an androgen-dependent fashion, but later undergo a transition to an androgen-independent state (Sadar et al., 1999; Pilat et al., 1998; Klotz et al., 2000). AR function contributes to CaP tumor cell survival after androgen ablation and to the growth of androgen-independent prostate cancer (van der Kwast et al., 1991; Ruizeveld de Winter et al., 1994; Taplin et al., 1995; Hobish et al., 1995). In cancer patients encountered phenomena are AR amplification, AR mutations, altered expression of AR coactivator and corepressor proteins, and activation of other pathways that can modify AR function (van der Kwast et al., 1991; Hobish et al., 1995; Balk et al., 2002 and reviewed by Gelmann, 2002). AR mutations have been found in ~40% of patients who relapsed after initial combined therapy with the AR antagonist flutamide (Taplin et al., 1999).

In the beginning, hormone ablation therapy and antiandrogen treatment are effective, but, with time, the cancer cells become hormone-independent or some antiandrogens turn into agonists for AR. This is also seen in the prostate cancer-derived cell line LNCaP in which AR (T877A) has undergone mutation in the LBD, enabling it to use antiandrogens BCA (Hobish et al., 2000) and OH-Flu as agonists (Langley et al., 1998). The mutation found most frequently in the flutamide-treated patients (T877A) is the same as the mutation identified initially in the LNCaP cells (Veldscholte et al., 1992). Importantly, the mutant AR is strongly activated by 4-hydroxyflutamide, estradiol and progesterone (Veldscholte et al., 1992). Significantly, casodex is still an antagonist of this mutant AR *in vitro*, and clinical responses to casodex has been observed in patients bearing these mutations (Joyce et al., 1998; Taplin et al., 1999).

Increased coactivator expression could be a mechanism contributing to AR activity in androgen-independent prostate cancer, since SRC-1 and SRC-2 expression is increased in androgen-independent prostate cancer (Gregory et al., 2001; Fujimoto et al., 2001; Li et al., 2001). The therapeutic activities of antiestrogens tamoxifen and raloxifene in breast cancer reflect their ability to induce alternative conformations in ER to favor NCoR binding (Smith et al., 1997; Brozozowski et al., 1997; Shiau et al., 1998). Likewise, AR may be regulated by corepressors (Yu et al., 2001; Yuan et al., 2001), including NCoR (Cheng et al., 2002), and development of

compounds promoting AR-corepressor interaction could be beneficial for blocking AR activation in prostate cancer.

3. FUNCTIONAL ORGANIZATION OF THE NUCLEUS AND TRANSCRIPTIONAL REGULATION

Nuclear organization contributes significantly to the control of transcription. Macromolecular complexes assemble on chromatin and provide architectural framework for ensuing functional interactions that mediate gene expression, i.e., nuclear organization is activity-driven (Misteli, 2001). Nuclear domain proteins dissociate and associate with domains, suggesting dynamic movement (Kruhlak et al., 2000). Also NRs are dynamic: GR is in continuous movement (McNally et al., 2000), and ERα rapidly circulates by attaching to and detaching from chromatin (Schaufele et al., 2000).

The cascades of cellular responses that are triggered by endocrine signals require the formation of specific protein-protein partnerships and these protein-protein interactions must be coordinated both in space and time. High mobility is a general feature of proteins in the mammalian nucleus. Chromatin is probably the major immobile component in the mammalian nucleus, but proteins may also be slowed down by their transient interactions with the nuclear matrix. A main characteristic of the cell nucleus is the presence of distinct compartments and domains (Hodges et al., 1998, Matera et al., 1999, Maul et al., 2000; Stein et al., 2000; Dundr and Misteli, 2001; Spector, 2001) (Figure 8). Among traditional nuclear compartments are nucleoli, typically one to five per nucleus, which are sites of rRNA synthesis, rRNA processing and assembly of ribosomal proteins. Pre-mRNA splicing factors are localized in a pattern of 25-50 nuclear splicing speckles, but they are also found diffusely distributed throughout the nucleus. The splicing factors are recruited from the speckles to thousands of transcription sites scattered in nucleoplasm. Cajal bodies, previously called Coiled bodies, typically one to ten per nucleus, also contain pre-mRNA splicing factors. The most studied nuclear domain-type is the PML body, ND10, also known as ND19, POD or Krüppel body.

Cellular structures are self-organizing and unstable, and they persist by exchanging subunits from their environment. Although subnuclear domains are morphologically stable, their content is steadily and rapidly exchanged. Of nuclear proteins, only the core histones are immobile, whereas linker histones are mobile. Linker histone H1 binds transiently to chromatin for one to two minutes (Misteli et al., 2000). HMG proteins have a shorter residence time on chromatin and steroid receptors attach only for seconds to chromatin (Stenoien et al., 2000). Dynamic structures are highly responsive to change: the build-in dissociation property of H1 periodically creates a window of opportunity for other factors to bind. H1 detachment may enable chromatin remodeling activity to bind to open chromatin permitting transcription factor binding. Competition for binding sites can occur at each step of gene activation. Also differently modified

forms of the same protein may compete for binding. Collectively, there is a high degree of multifunctionality in the organization of nucleus: multiple steps and individual proteins participate in multiple processes in several subnuclear sites (Andersen et al., 2002).

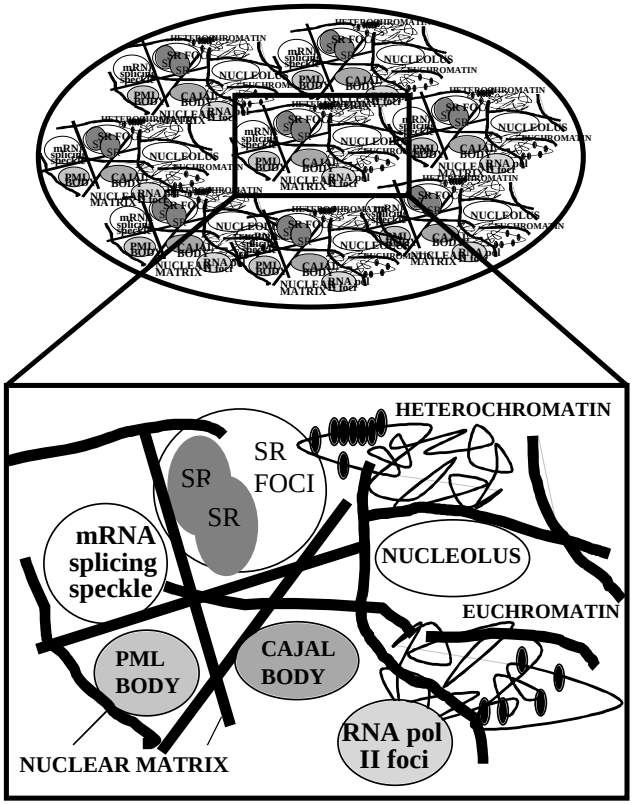


Fig. 8. Main nuclear compartments. Holo-SRs reside in nuclear speckles that are specific nuclear domains. PML bodies are the best characterized nuclear domains. Nuclear proteins including SRs and PML move inside nucleus. Chromatin is the most static nuclear compartment, although also it decondenses and condenses into chromosomes cell cycle-dependently, and the transcribed chromatin decondenses into euchromatin. (Adapted from Stein et al., 2000.)

3.1. Transcription sites

A mammalian cell contains approximately 2 m of DNA in the 10-μm diameter nucleus. In interphase cells, a single chromosome is concentrated within a distinct subvolume of the nucleus, termed chromosomal territory (Cremer and Cremer, 2001). Chromosomal territories are three-dimensional networks wherein transcription factors move. Periodic and specific attachments of chromatin fibers to the nuclear matrix create the chromatin loop domains (Figure 9). Genes contain repeated motifs, scaffold attachment regions (SARs), also called matrix attachment regions (MARs), which bind DNA to the nuclear matrix. Transcribing RNA polymerase II (RNA pol II) localizes to the bases of the loops and attaches to the nuclear matrix (Cook, 2002; Wei et al., 1999) (Figure 9). Silent DNA is out in the loop and becomes attached in the core of

chromatin when transcribed (Mintz and Spector, 2000). Pre-mRNA splicing factors are recruited from nuclear speckles, in which they are concentrated, to sites of transcription in a phosphorylation-dependent fashion (Misteli et al., 1998). This coordinates spatially transcription and mRNA splicing. Transcriptional regulation by NRs and regulation of splicing seem to be closely connected through specific cofactors (Zhao et al., 2002).

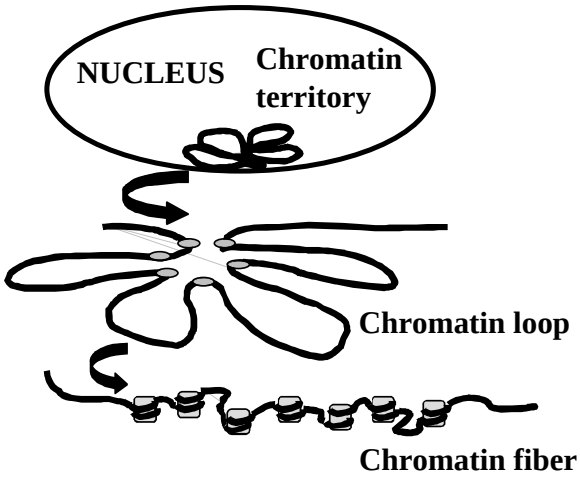


Fig 9. Model for chromatin organization in the nucleus. DNA is coiled around histone octamer to form a chromatin fiber, which organizes into loops (86-200 kb) through transcription factors and transcribing RNA polymerases. Ten to twenty of loops form chromatin cloud around the transcription factory. Fifty to hundred successive clouds form a chromatin territory. (Adapted from Cook, 2002).

Transcription and DNA replication occur at numerous spatially distinct foci that are associated with the nuclear matrix intermediate filament scaffold (NM-IF). HeLa cells have been reported to contain 75 000 nascent transcripts concentrated in about 2400 nuclear sites that serve as transcription factories (Jackson et al., 1998). According to this study, each transcription factory contains 30 active RNA polymerases transcribing templates arranged as clouds of loops around the site (Jackson et al., 1998).

3.2. Nuclear matrix

Nuclear matrix is composed of nucleoprotein network (Pederson, 1998; 2000; Nickerson, 2001) (Figure 8). Nuclear matrix, nucleoskeleton, consisting of internal lamin structures extending from nuclear lamina, contains sites involved in transcription, RNA processing and in DNA replication (Hozak et al., 1994a; 1994b, Gant and Wilson, 1997). The inner network of nuclear matrix consists of core filaments and diffuse nucleoskeleton (Philimonenko et al., 2001). In addition to nuclear lamina, lamins form a variety of nucleoplasmic assemblies, distinct foci and nucleoplasmic veil (Moir et al., 1994; Kennedy et al., 2000). Nuclear lamins and lamin-associated proteins contain chromatin-binding domains, suggesting that lamins may also be involved in the organization of chromatin (Moir et al., 1995; Wilson et al., 2000). Interestingly,

disruption of nuclear lamin organization inhibits RNA pol II activity (Spann et al., 2002). Nuclear lamins are also necessary for DNA replication and may serve as a scaffold upon which replication complexes assemble (Moir et al., 1994). Nuclear matrix is present already in transcriptionally inactive mouse 2-cell stage embryos. Nuclear architecture regulates gene expression in embryos, and specific anchorage to the nuclear matrix occurs when chromatin domains become active (Vassetzky et al., 2000).

PIAS proteins have characteristics of a scaffold/matrix attachment region-binding (SAF/SAP) proteins (Kipp et al., 2000; Bode et al., 2000). Like other S/MAR-binding proteins, such as SAF-A and SAF-B, PIAS proteins localize in nuclear clusters that form a speckled pattern. PIAS proteins contain a RING finger like domain, and RING domains can self-assemble into macromolecular scaffolds that attach other regulatory molecules (Kentsis et al., 2002). SAF-A and SAF-B (Renz et al., 1996; Oesterreich et al., 2000) have been reported to interact with steroid receptors (Oesterreich et al., 2000; Eggert et al., 1997; Tang et al., 1998). SAF-A also binds to RNA pol II and may serve as an assembly point for the formation of a "transcriptosomal" complex (Nayler et al., 1998).

3.3. PML bodies

3.3.1. Components of PML bodies

Nuclear dots (ND10) containing PML and SP100 proteins represent a specific nuclear domain (Dyck et al., 1994; Weis et al., 1994; Szostecki et al., 1987). The composition of ND10s is heterogeneous and ND10s are modified in spatial-, temporal- and cell type-specific fashion (Bloch et al., 1999). Cells contain on average 10 to 30 PML bodies measuring from 0.2 to 1 µm in size. PML is present throughout the cell cycle, but most bodies are observed during G1. PML bodies are associated with the nuclear matrix (Dyck et al., 1994). Viruses and environmental stress alter the morphology of ND10s and type I and II interferons dramatically increase the number of ND10s. PML bodies may act as sensors for foreign or inappropriately expressed proteins acting as a subcellular immune system, since, for example, polyglutamine repeat disorder proteins accumulate in PML bodies.

ND10 components, especially SP100 proteins, can contact chromatin by interacting with heterochromatin protein 1 (HP1) providing a link between ND10s and chromatin (Seeler et al., 1998). ND10 can be a scaffold onto which coregulator complexes are assembled to be utilized by transcription factors. PML may thus act as a scaffold protein to maintain the correct architecture for constructing coactivator and corepressor complexes and machinery for apoptosis.

The involvement of ND10s in transcription is still a controversial issue. Lack of RNA pol II, TFIIF and DNA in ND10s suggests that they are not transcription sites (Boisvert et al., 2000). On the other hand, CBP and mRNAs have been found in ND10s (LaMorte et al., 1998), and PML can regulate CBP movement (Boisvert et al., 2001). PML bodies may also act as storage sites for transcriptional regulators, thereby titrating the levels of proteins in the nucleoplasm.

3.3.2. PML

PML, promyelocytic leukemia protein, belongs to a family of proteins characterized by the presence of the RBCC (RING B-box-coiled coil motif). RBCC consists of a C_3HC_4 zinc finger motif and one or two additional cysteine-rich zinc-binding regions (B-boxes) followed by a leucine coiled coil. Alternative splicing results in several PML isoforms with different carboxy-termini (reviewed by Borden, 2002) (Figure 10). Unfortunately there is some confusion about the nomenclature of PML isoforms (Jensen et al., 2001). PML is ubiquitously expressed in adult tissues. The majority of PML resides in nuclear bodies, but part of it is also diffusely distributed in the nucleoplasm (Bauman et al., 2001). PML bodies move in the nucleus metabolic energy dependently (Muratani et al., 2002).

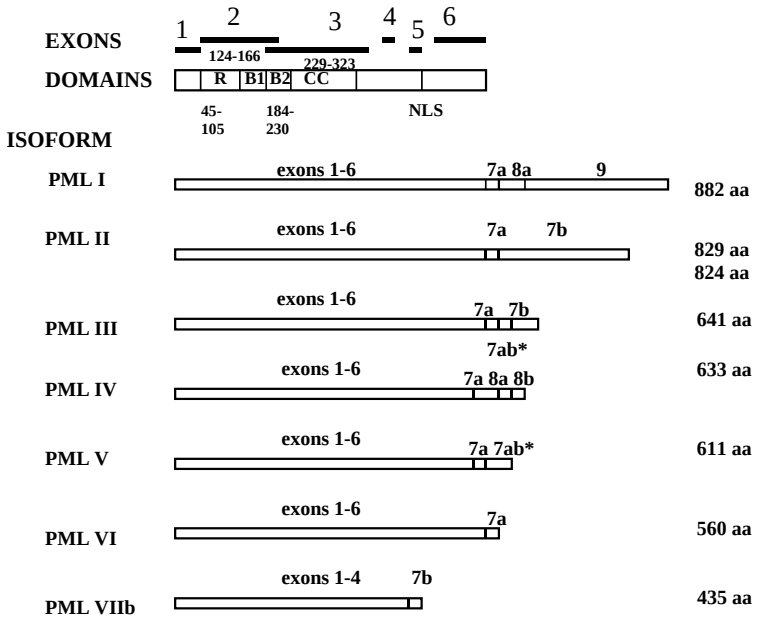


Fig 10. PML isoforms. All PML isoforms share identical N terminus. N terminus of PML harbors a RING finger, two B-boxes and a predicted coiled-coil domain, that together form the RBCC/TRIM motif found in many proteins. In PML this RBCC/TRIM domain is essential for PML body formation and in interactions with other proteins in growth suppression, apoptotic and antiviral activities of PML. (Adapted from Jensen et al., 2001).

Expression of the PML gene is not required for viability, but homozygous disruption of the *pml* gene in mice enhances tumorigenesis and inhibits the differentiation of myeloid precursor cells

(Wang et al., 1998). PML is a tumor suppressor protein (Pearson et al., 2001; Salomoni and Pandolfi, 2002), especially PML IV is able to cause premature senescence through p53 activation (Bischof et al., 2002). In PML -/- cells, ND10 proteins SP100, Daxx, CBP and SUMO-1 fail to accumulate in ND10s, which leads to disaggregation of the ND10s (Ishov et al., 1999; Zhang et al., 2000). PML can act as either a transcriptional repressor or activator, depending on the promoter and system used. Daxx is another transcriptional regulator that directly interacts with PML. Daxx represses transcription and its sequestration in ND10 blocks this activity (Zhong et al., 2000; Li et al., 2000) thereby promoting sensitivity to Fas-induced apoptosis (Torii et al., 1999). Another mechanism by which PML represses transcription is the functional and physical interaction with histone deacetylases (Wu et al., 2001). Interestingly, PML is required for transcriptional repression by Mad with synergistically acting corepressors c-ski, NCoR and mSIN3A (Khan et al., 2001). Mad is a helix-loop-helix transcription factor that acts as a negative regulator for multiple target genes.

Altogether, more than 50 PML-interacting proteins have been reported, including nuclear receptors (Guichon-Mantel et al., 1995). In valuating the relevance of interactions, care has to be taken since overexpression of PML results in G1 arrest and apoptosis (reviewed by Borden et al., 2002). PML interacts with CBP and has been suggested to be required for the ability of CBP to act as a transcriptional coactivator of RAR (Doucas et al., 1999; Zhong et al., 2000). Moreover, PML has been shown to enhance NR transcriptional activity through its association with CBP (Doucas et al., 1999).

3.3.3. SP100

Alternatively spliced SP100 proteins vary in respect to subcellular and ND10 localization. There are two major classes of SP100 splice variants (Guldner et al., 1999). SP100-HMG contains HMG1/2 domain and binds to DNA. SP100C has a PHD finger and a bromodomain which can influence chromatin structure and interact with acetylated lysines. SP100 interacts with HP1 and appears to recruit this protein into ND10. HP1, in turn, interacts with the lamin B receptor which is an integral component of the nuclear envelope (Ye et al., 1996). HP1 is found to be associated with a number of transcription repressors (Jones et. al., 2000; Nielsen et al., 2001). Interestingly, the C-terminal region of SP110 is homologous to the transcription intermediary factor (TIF1) family of proteins. SP110 is a leukocyte-specific ATRA- and interferon-inducible ND10 component that enhances signal transduction mediated by RAR (Bloch et al., 2000).

3.3.4. Regulation of PML body proteins by sumoylation

There are three sumoylation sites in PML at residues 65, 160 and 490 (Kamitani et al., 1998). Sumoylation is required for the association of some PML partner proteins, such as SP100 and Daxx, with the PML body (Zhong et al., 2000). SUMO modification is needed for nuclear transport of SP100, but not for its localization in ND10 (Sternsdorf et al., 1999). Sumoylation of PML also recruits p53 to ND10s and leads to stimulation of transcriptional activity of p53 (Fogal et al., 2001). The splice variant PML-3 recruits p53 into ND10s sumoylation dependently which enhances p53 transcription in a promoter-specific manner and affects cell survival (Fogal et al., 2001). On the other hand, HIPK2, which colocalizes with p53 and CBP in PML bodies (Hofman et al., 2001), phosphorylates p53 facilitating CBP-mediated acetylation of p53 and promoting p53-dependent gene expression (Diorazi et al., 2002). PML-3, but not PML-L, recruits HIPK2 to PML ND10s (Hofman et al., 2001). Sumoylation also changes the stability of SP100-HP1 complexes (Seeler et al., 2001). Phosphorylation is important for the regulation of cell cycle-dependent sumoylation of PML (Everett et al., 1999). The nuclear matrix targeting of PML is regulated by phosphorylation; dephosphorylation triggers PML multimerization and formation of ND10s, but maturation of ND10 is sumoylation-dependent (Müller et al., 1998). Sumoylation of PML also appears to control proteasome recruitment to PML bodies (Lallenmand-Braitenbach et al., 2001). Interestingly, proteasome inhibition has been reported to result in the movement of PML body proteins to the nucleolus (Mattson et al., 2001). Thus, sumoylation directly or indirectly promotes PML degradation.

AIMS OF THE STUDY

The main aim of the study was to investigate the role of ligands, especially antiandrogens, on different aspects of AR function, with a special emphasis on the following topics:

1. Factors influencing DNA binding of AR in living cells.
2. The role of ligands and coactivator SNURF in trafficking and nuclear localization of AR.
3. The pattern of nuclear AR in relation to distribution of coactivator GRIP1 and the effect of antiandrogens on AR-GRIP1 localization and interaction.
4. The role of GRIP1 sumoylation on the colocalization of the AR and GRIP1 and AR-dependent transcription.

METHODS

Detailed descriptions of the methods and materials used in this study are found in the original publications (I-IV) as specified in the table below.

Table 2. Methods used in this study

Method	Original publication
Plasmid construction and recombinant DNA technology	I, II, IV
Cell culture	I, II, III, IV
Transfections and reporter gene assays	I, II, III, IV
SDS-PAGE and immunoblotting	I, IV
Immunocytochemistry	I, II, III, IV
Confocal microscopy	II, III, IV
Microscopy of living cells	II
Promoter interference assay	I
Nuclear matrix extraction for microscopy	II
Preparation of nuclear matrix from cultured cells	II

In addition, PML expression plasmids pSG5-PML-L (de Thè et al., 1991) coding for 641-amino acid PMLIII and pCDNA-CMV-flag-PML-3 (Fagioli et al., 1992) encoding 633-amino acid PML IV were used in transient transfections and confocal microscopic experiments. In double labeling, 0.75 μg, and in triple labeling experiments, 0.5 μg, of PML expression plasmid was cotransfected with 0.5 μg of EGFP-AR and pSG5-GRIP1 expression plasmids, described in articles III and IV, into COS-1 cells. Transfection, fixation of cells and immunocytochemical labeling were carried out as described in articles III and IV. PML was detected with polyclonal goat anti-PML antibody (Santa Cruz Biotechnology Inc) at 1:100 dilution. Rhodamine-conjugated donkey anti-goat at 1:300 dilution was used to detect the primary antibody. For triple labeling experiments, monoclonal antibody (against GRIP1) was detected with Cy5-labeled secondary antibody, sheep anti-mouse, which in turn was exited at 660 nm in confocal microscopy. In double detection of PML and GRIP1, GRIP1 was detected with FITC-labeled sheep anti-mouse secondary antibody. Otherwise confocal microscopic detection was performed as described in the articles III and IV. All secondary antibodies were from Jackson ImmunoResearch and they were used at 1:300 dilution.

RESULTS AND DISCUSSION

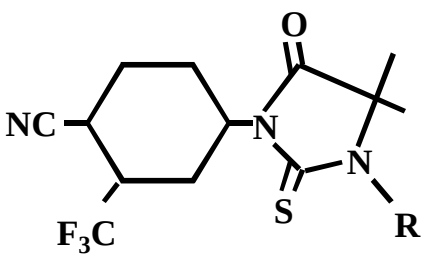
1. FACTORS INFLUENCING DNA BINDING OF AR IN LIVING CELLS (I)

Promoter interference assay (PIA) in green monkey kidney (CV-1) cells was employed to examine the roles of the functional domains of AR and the ligand for specific DNA interactions in intact cells. Promoter interference assay is based on competition of DNA-binding proteins for binding with essential transcription factors driving a constitutively active heterologous promoter (Hu and Davidson, 1987). The interference was achieved by inserting two AREs between the TATA box and the start site of transcription in the CMV promoter.

It was important to examine AR DNA binding in living cells, since observations based on *in vitro* binding assays have yielded controversial results. Even apo-AR binds to DNA in electrophoretic mobility shift assay (EMSA) (Palvimo et al., 1993). With the promoter interference assay, we showed that AR DNA binding in living cells is strictly agonist-dependent and that mutant ARM807R associated with androgen insensitivity does not bind DNA in intact cells, although it binds to DNA *in vitro* as assayed by EMSA (Kallio et al., 1994).

Our PIA results with AR deletion mutants indicate that AR binding to ARE is not dependent on amino or carboxy terminus of the receptor or their interaction. The inability of the ligand-binding deficient mutant hARM807R to bind and the ability of pure antiandrogens to inhibit DNA binding of AR in promoter interference assay prove that, in the context of native AR, the androgen-induced conformational change in the LBD is mandatory for the generation of a transcriptionally competent receptor that binds to DNA in intact cells. Taken together, the LBD provides the means to regulate the function of AR in an androgen-dependent fashion. Based on our results with PIA, it seems that agonist-induced conformational change in the LBD is primarily required for the release of AR from associated inhibitory protein complexes rather than for generation of a receptor form capable of ARE recognition.

Three N-substituted steroidal arylthiohydantoin antiandrogens, RU56187, RU57073 and RU 59063 (Figure 11), were tested in PIA. These compounds bind to AR *in vitro* as well as or even more effectively than testosterone, which is about hundred times better than nonsteroidal antiandrogens casodex and hydroxyflutamide (Teutsch et al., 1994). In reporter assays in transfected cells, these RU compounds exhibit considerable agonistic capacity that is 30-50% of that of testosterone (our unpublished results). On the contrary, they act as effective androgen antagonists in rats, with RU56187 being three times more potent antiandrogen than casodex (Teutsch et al., 1994).



R=

CH_3	**RU 56187**
CH_2CH_2OH	**RU 57073**
$(CH_2)_4OH$	**RU 59063**

Fig 11. Antiandrogenic RU compounds tested in promoter interference assay.

In the *in vivo* DNA binding assay of the antiandrogens studied, only hydroxyflutamide showed pure antagonistic activity, since it blocked agonist-induced binding of AR to DNA totally. Also casodex reduced DNA binding of AR efficiently, whereas the partial antagonist cyproterone acetate proved to be even more efficient than testosterone in inducing DNA binding of AR. In this respect PIA results parallel our transactivation results, proving hydroxyflutamide as the most efficient antiandrogen; casodex an efficient antagonist, and cyproterone acetate as only a partial antagonist. Taken together, promoter interference assay showed that mere DNA binding is not enough to trigger transcription, since antiandrogenic RU compounds were as efficient agonist as testosterone in inducing DNA binding of AR. Moreover, conformation requirements of LBD are different for DNA binding and transactivation, since antagonistic RU compounds induced full AR DNA binding without leading to full transactivation capacity.

Other investigators have also shown, by using either *in vitro* electrophoretic mobility shift assay or *in vivo* promoter interference assay, that AR binding to DNA is affected by antiandrogens. In EMSA, 1 μM hydroxyflutamide has been shown to inhibit AR DNA binding (Wong et al., 1993; Kemppainen et al., 1999). In promoter interference experiments by another group, both hydroxyflutamide and casodex antagonized agonist-dependent DNA binding (Kuil and Mulder, 1996) and cyproterone acetate behaved as a weaker antagonist being able to inhibit agonist-induced DNA binding only by 50% (Kuil and Mulder, 1996). Also in this study transfected LNCaP AR mutant (T877A) responded divergently to hydroxyflutamide by binding to DNA (Kuil and Mulder, 1996).

Agonist-induced conformational change in AR LBD is different from that induced by antiandrogens, in that several studies have shown that antiandrogens fail to bring about correct conformational change. Trypsin digestion sensitivity of agonist- and antagonist-occupied ARs is different (Kallio et al., 1994). Also structural modeling studies of the LBDs of mutated ARs have predicted that hydroxyflutamide makes contacts with different amino acids than the agonists (Poujol et al., 2002).

Additionally, our PIA results suggested that AR LBD has an intrinsic transcriptional inhibitory activity. The ligand-independent repression activity of LBD was proven by the fact that LBD repressed transcription in a heterologous context. A reporter pSV40CAT, containing five AREs upstream of the SV40 promoter, was repressed by GAL4-LBD (LBD fused to GAL4 DNA-binding domain) more efficiently in the absence than the presence of the ligand. This may derive from the interaction of apo-AR with transcriptional repressors SMRT and NCoR (Chang et al., 2002; Shang et al., 2002).

Promoter interference assay has previously shown that PR binds to DNA in the absence of ligand (Mymryk et al., 1995). The PIA results regarding the role of ligand on ER DNA binding have been contradictory; ER has been shown to bind DNA both in an agonist-dependent (Xing and Shapiro, 1993) or -independent fashion (Reese and Katzenellenbogen, 1992). However, in the latter case, ligand appeared to stabilize receptor-DNA-complexes. Our results with AR agree with the findings of Kuil and Mulder (1996) obtained with the PIA in a different cell line. Even though PIA measures protein-DNA interaction in an intact cell environment, there are several shortcomings in this assay. First, the AR recognition sequences were not in normal chromatin context, although PR and stably integrated interference construct has yielded results similar to those with transient templates (Mymryk et al., 1995). Second, in transient transfections proteins are often overexpressed, which may lead to squelching artifacts. However, CV-1 cells that were employed in our studies do not readily overexpress proteins from transfected templates. The assay should have preferentially been performed in a more natural cellular environment. The lack of proper androgen-responsive prostatic cell line is a major obstacle in all androgen research. Available cell lines either lack AR or express mutated AR with changed ligand-binding and transactivation characteristics.

This work was done before more powerful *in vivo* techniques for assessment of DNA binding were developed. At that time, *in vivo* genomic footprinting assay would have been the only alternative method to study DNA binding in intact cells. However, also footprinting assay has weaknesses, in that these assays often rely on stable cell lines with multiple copies of reporter promoter integrated to poorly controlled locations in the genome. This is a major fault, since the activity of the promoter may vary according to the integration site. *In vivo* footprints derived from cells from animal tissues have turned out to be difficult to interpret. Results from androgen-regulated mouse Slp (sex-limited protein) enhancer are confusing, in that AR DNA binding does not seem to correlate with target gene expression (Nelson and Robins, 1997).

The dynamic nature of DNA binding of SRs was revealed by more recent methods. Microscopic bleaching experiments with fluorescently-tagged receptors in living cells have demonstrated the rapid movement of GR. Hormone-bound GR undergoes continuous exchange between nucleoplasmic compartment and its binding sites in chromatin (McNally et al., 2000). Chromatin immunoprecipitation assays (ChIP) have shown that AR rapidly and repeatedly attach to and

detach from promoters (Chang et al., 2002; Kang et al., 2002; Shang et al., 2002). ChIP technique is a step further from DNA binding to transactivation assays, since it can also detect binding of endogenous coregulators needed for the assembly of AR-dependent transcription complex (Figure 12). Chromatin immunoprecipitation of the PSA promoter in LNCaP cells has shown that AR binds to two AREs in the promoter and one ARE in the enhancer in response to agonist, and the receptor recruits coactivators in a cyclic fashion (Shang et al., 2002; Kang et al., 2002). Casodex-bound AR was also able to recruit corepressors, suggesting that antagonism not only operates by blocking coactivator binding, but also includes an actively repressive activity (Shang et al., 2002). In chromatin immunoprecipitation assay casodex-occupied AR binds to PSA promoter in LNCaP cells (Kang et al., 2002; Shang et al., 2002; Masiello et al., 2002). Casodex exposure leads to mere promoter occupancy and association of corepressors NcoR and SMRT and HDAC1 and 2 with AR (Shang et al., 2002).

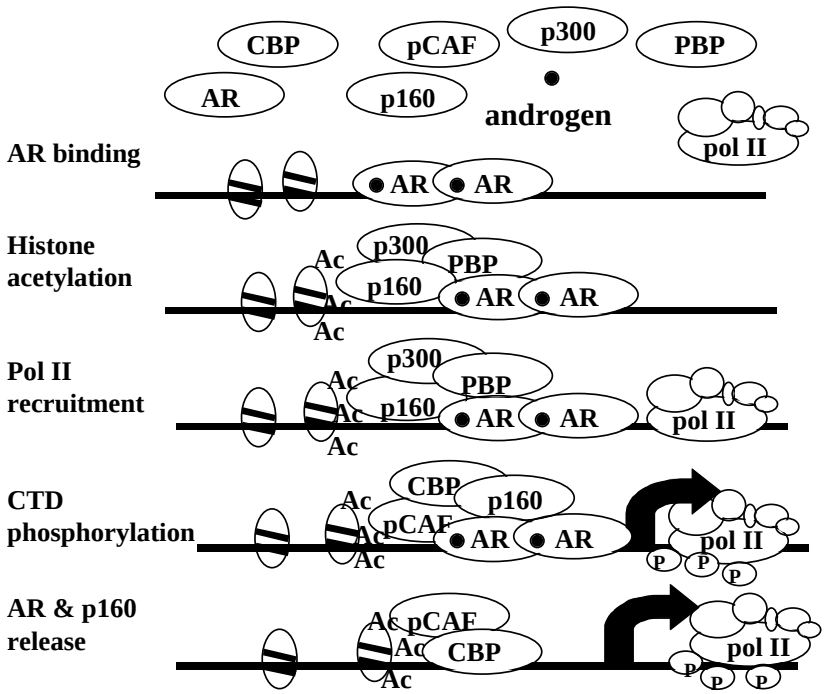


Fig. 12. Cyclic binding and detachment of androgen receptors and transcriptional coregulators to AR regulated promoter. Coregulators are marked as in Figure 4. CTD: the RNA polymerase C-terminal domain that has to be phosphorylated in order to initiate transcription. Ac: acetylated histone. (Adapted from Shang et al., 2002.)

2. THE CELLULAR LOCALIZATION OF APO- AND HOLO-AR (I, II, III, IV)

Subcellular localization of unliganded SRs is variable. In fixed tissue sections AR is predominantly nuclear as assessed by immunohistochemical methods (Sar et al., 1990; Husmann et al., 1990; Takeda et al., 1990; Young et al., 1991). ERα and PR are exclusively nuclear

(Ylikomi et al., 1992; Tun et al., 1999; Lim et al., 2000), whereas apo-GR resides mostly in the cytoplasm (Picard and Yamamoto, 1987; Sackey et al., 1996). Apo-AR shows predominantly cytoplasmic or nuclear localization depending on the cell-line and experimental method used (Simenthal et al., 1991; Jenster et al., 1993; Zhou et al., 1994; Tyagi et al., 2000). In transient transfections, cellular localization of unliganded AR has varied according to transfection method and cell type used (Simenthal et al., 1991; Jenster et al., 1993). Also we got conflicting results of apo-AR localization. Immunocytochemical analysis of CV-1 cells transfected with very toxic and inefficient calcium phosphate transfection method showed that apo-AR was predominantly nuclear. In contrast, EGFP-tagged apo-AR transfected with nontoxic and efficient DOTAP or FuGene reagents in CV-1 and COS-1 cells was predominantly cytoplasmic. In the latter case, also detection of AR was based on different methods. Immunocytochemistry may create artifacts because of fixation or antibody recognition characteristics. In retrospect, it is possible that the first fixation method used in study I, involving methanol and acetone, was perhaps too harsh and may have caused cytoplasmic components including AR to be rinsed from the cells. Another explanation lies in the very different detection levels reached with normal fluorescent microscopic inspection as compared to that obtained with a very sensitive CDD video camera. At least 75% of cells observed with CDD camera in AR trafficking study (II) would have been below the detection level in immunocytochemical analysis. Therefore, the reason that mainly nuclear AR distribution was seen in the first study may well be that only a subpopulation of AR-expressing cells was detected, since in our later experiments with more gentle paraformaldehyde fixation method apo-AR has constantly been predominantly cytoplasmic. EGFP tag does not affect AR distribution, since we have shown that transfected AR detected with antibodies resides in similar granular pattern in nuclei. Also endogenous AR forms hundreds of small granules in LNCaP cells.

Transcriptionally active, agonist-occupied EGFP-AR produces typically 250-400 fluorescence foci on the boundary region between euchromatin and heterochromatin, but antagonist hydroxyflutamide- or casodex-occupied AR is spread throughout the nucleus (Tomura et al., 2001; Tyagi et al., 2000; Saitoh et al., 2002). We have seen casodex-occupied AR always to form speckles (III), but the sharpness and form of the speckles vary from cell to cell, some casodex-occupied AR granules are fuzzier and others more distinct. However, the same cellular diversity in AR speckle forms is also seen in the presence of testosterone and the antiandrogen cyproterone acetate and even with estrogen. However, on average, testosterone-occupied AR forms the most clear-cut speckled pattern with least diffuse staining. All these compounds are able to induce AR nuclear transport and cause AR to form speckles, even though estrogen- and casodex-occupied AR is transcriptionally inert.

3. SNURF AND NUCLEAR TRANSPORT OF AR (II)

3.1. Nuclear transport

Jellyfish green fluorescent protein (GFP) is a 25 kDa-protein that absorbs near UV-light and emits green light. GFP fusion proteins have been used in trafficking studies of SRs, for example GR (Htun et al., 1996). Enhanced GFP was fused to the N terminus of human AR and several AR forms that contain point mutations or deletions in the bipartite nuclear localization signal (NLS) or adjacent leucine residues. These EGFP-AR fusion proteins are functional in transactivation and ligand-binding assays and their movement can be inspected by fluorescence microscopy in living cells. Apo-EGFP-AR was predominantly cytoplasmic in CV-1 cells and even more so in COS-1 cells (study II). Upon addition of ligand, EGFP-AR moved rapidly in 30 minutes into the nucleus so that AR was totally nuclear in 90% of COS-1 cells. This finding is comparable to previous results with GFP-AR in COS-7 cells (Georget et al., 1997). The antiandrogen casodex slowed nuclear transport considerably, by at least four fold.

3.2. Nuclear localization signal of AR

Large proteins have to be imported into the nucleus and nuclear localization signal (NLS) is mediating the process. In SRs the DBD and the flanking hinge region contain a bipartite-type NLS (NL1) (Ylikomi et al., 1992) which acts constitutively. At least in GR, NL1 is responsible for rapid nuclear import and is related to importin binding (Guichon-Mantel et al., 1994; Tyagi et al., 1998; Savory et al., 1999). AR has a bipartite NLS in the DBD and hinge region comprising amino acids 617-633 (Jenster et al., 1993; Zhang et al., 1994). Another NLS, NL2, exists in the LBD of GR. NL2 is hormone-inducible and masks the constitutive activity of NL1 in apo-GR (Savory et al., 1999). To determine the role of bipartite NLS in the trafficking of AR, several receptor mutants were tested. In the EGFP-GA mutant, codons 617R and 618K in the first part of NL1 were change to G and A, respectively. In the EFGP-LA, there was a leucine to alanine substitution at codons 634, 637 and 639. EGFP-GA and EGFP-Δ629-633 were permanently cytoplasmic in 50% of cells. Unliganded NLS mutants were predominantly cytoplasmic in 75% of cells, and nuclear translocation upon agonist exposure was slow: AR was found exclusively in the nucleus only in 65% cells after 4 h. For EGFP-GAΔ629-633 with both parts of NLS destroyed, nuclear transport was nearly nonexistent in the presence of testosterone. For EGFP-LA nuclear import was total but slower, indicating that in addition to the basic amino acids in the bipartite NLS also the flanking C-terminal leucine residues are involved in hormone-induced nuclear transport of AR.

The AR LBD was shown to contain another NLS, NL2, which was initially suggested to be an agonist-specific (II). However, in later experiments with longer detection times, casodex was able to induce nuclear transport of EGFP-LBD. This parallels the slowness of the nuclear trafficking of casodex-occupied AR.

3.3. Binding of AR to the nuclear matrix

SNURF is among a number of coregulators that interact with the NL1 region of steroid receptors (Powers et al., 1998; Jackson et al., 1997; Poukka et al., 1999; McKenna et al., 1999). At least in prostate epithelium, SNURF and AR are expressed in the same cells (Moilanen et al., 1998). Interestingly, we found AR to reside in nuclei in 30% of COS-1 cells even in the absence of ligand in cells cotransfected with SNURF as opposed to 1% of nuclear AR positive cells without cotransfected SNURF. In addition, nuclear export of AR appeared to be delayed when the receptor was coexpressed with SNURF. SNURF interacts with the DBD/hinge of AR (Moilanen et al., 1998), and ectopic SNURF failed to influence nuclear trafficking of ARΔ629-633. SNURF mutant Δ31-65CS1 was incapable of influencing nuclear trafficking of EGFP-AR, indicating that the AR interaction region (amino acids 31-65) and an intact RING finger structure of SNURF are needed for modulation of AR trafficking. One mechanism by which SNURF may tether AR to nucleus is that cotransfected SNURF increases the amount of nuclear matrix-associated AR in the presence of androgen. A subpopulation of nuclear AR and GR have been previously shown to be tightly attached to the nuclear matrix (van Steesel, 1995; Tang et al., 1996; 1998).

Mechanisms by which SNURF enhances AR nuclear transport and association to nuclear matrix or decreases nuclear export are not known. One possibility is that SNURF could increase the activity of chaperone proteins towards AR. Chaperone proteins, such as hsp90, regulate several aspects of AR function. Hsp90 regulates the nuclear translocation of AR (Georget et al., 2002) and GR (Czar et al., 1997; Galigniana et al., 1998). AR may be still associated with hsp90s in the nucleus, and these proteins may be required for induction of an active AR conformation that is able to interact with the nuclear matrix. SNURF may influence AR-hsp90 interaction, thereby inducing apo-AR-hsp90 trafficking to the nucleus. Additionally, SNURF may be able to unmask the constitutively active NL1 thereby causing apo-AR transport to the nucleus. Alternately, newly-translated SNURF by itself could carry AR to the nucleus by a piggy-back mechanism similar to that suggested for AR-mediated shuttling of β-catenin to the nucleus (Pawlowski et al., 2002).

Translocation into a different cellular compartment is emerging as an important control mechanism in the regulation of gene expression. FHL2, an AR coactivator that selectively increases the transcriptional activity of AR (Müller et al., 2000), resides normally mainly in the cytoplasm, but stimulation of Rho signaling pathway induces translocation of FHL2 to the

nucleus thereby stimulating the transcriptional activity of AR on its target genes (Müller et al., 2002). SNURF may regulate AR function by bypassing the need for hormone by transporting apo-AR to the nucleus. Since SNURF is coexpressed with AR in prostate epithelial cells, SNURF may contribute to the hormone-independent activation of AR in prostate cancer (Moilanen et al., 1998). SNURF is a RING finger protein and control of cellular targeting is one of RING finger domain proteins' functions (Schedev et al., 2000). ANT-1, U5 small ribonucleoprotein particle binding protein, enhances the ligand-independent autonomous transactivation function AF-1 of AR (Zhao et al., 2002). ANT-1 recruits AR into the splicing speckles, a specific nuclear compartment different from AR speckles (Zhao et al., 2002).

Another RING finger protein, ARA54, is also a ligand-dependent AR coactivator (Kang et al., 1999). ARA54 promotes ubiquitinylation and is itself a target of ubiquitinylation (Ito et al., 2001). Also SNURF appears to act as a ubiquitin ligase (Häkli, manuscript in preparation). Interestingly, ubiquitinylation and proteasomal activity have recently been shown to be critically involved in NR-dependent transcription (Lonard et al., 2000), also in the case of AR (Kang et al., 2002).

4. NUCLEAR DISTRIBUTION OF AR AND GRIP1 (III)

4.1. AR-GRIP1 interaction and transactivation

The p160 family member GRIP1 and its human homolog TIF2 (Vogal et al., 1998) bind to AR (Ding et al., 1998) and potentiate AR transactivation (Bevan et al., 1999) up to 10-fold in reporter gene assays (Koh et al., 2001). According to our results, agonist-occupied AR modifies the distribution pattern of GRIP1 from tens of large round foci to conform to AR distribution pattern of hundreds of small granules, indicating that AR and GRIP1 colocalize and interact in a testosterone-dependent fashion. In the context of full-length AR, the apo-LBD seems to keep the scarce nucleolar AR population separate from GRIP1. GRIP1 by itself always localizes to nucleus and resides in round granules which are both larger and fewer in number than AR granules. The distribution pattern of transfected GFP-tagged GRIP1 has shown to be complex varying from diffuse nucleoplasmic to discrete intranucleolar foci (Bauman et al. 2001). Coexpression of GRIP1 with AR increased testosterone-induced and AR-dependent reporter gene activity by three-fold which is in agreement with previous results (Slagsvold et al., 2000). Ectopic expression of GRIP1 had no effect on the activity of probasin promoter in the absence of androgen. Surprisingly, also estradiol- and partial agonists cyproterone acetate-occupied AR is able to transform GRIP1 distribution pattern to conform to AR distribution pattern (II and data not shown).

Dissociation of ligand after androgen removal and thorough washes resulted in the return of AR to the cytoplasm. Upon disruption of AR-GRIP1 interaction, GRIP1 regains its original kind of

distribution in tens of big round granules. Since synthesis of new proteins was not blocked, we cannot rule out the possibility that part of originally observed colocalized AR and GRIP1 molecules were degraded, and among the molecules that adopted the characteristic cellular localization of these proteins were also newly synthesized ones.

In transient transfection experiments, usage of correct amount of transfected expression plasmid is crucial, since the dosage of NR expression vector strongly influences the degree of cooperation observed among multiple coactivators (Lee et al., 2002). At concentrations high enough to force almost constant occupancy of enhancer elements, the NRs may recruit and activate the transcription initiation complex without assistance from coactivators. However, our results were obtained at expression plasmid levels where coactivators GRIP1 clearly enhanced AR-dependent transactivation.

4.2. AR domains in interaction with GRIP1

To study the function of individual AR domains in the AR-GRIP1 interaction several AR mutants were tested. The amino-terminal fragment of AR, amino acids 1-575 (NTD), distributes evenly throughout the cell (Figure 13, panel A). Interestingly, coexpression of GRIP1 induces AR NTD to enter nucleus and interact and colocalize with GRIP1 (Figure 13, panel B). AR NTD and GRIP1 form giant colocalization granules, that exhibit sizes comparable to nucleoli. Previously AR amino acids 1-503 have been shown to be sufficient for AR binding to GRIP1 by *in vitro* pull-down experiments (Slagsvold et al., 2000). More specifically, amino acids 351-537 on AR N terminus form the interaction surface with the C terminus of GRIP1 (Irvine et al., 2000). In addition, the AF-1 of AR N terminus has also been shown to be activated by GRIP1 and SRC-1 (Alen et al., 1999). Interestingly, in addition to p160 proteins, several AR coactivators bind to AF-1: ARA24, ARA160, cdk-activating kinase, ART-27, BRCA1, SRA, cyclin E and ANT-1 (reviewed by Heinlein and Chang, 2002).

In the absence of ligand, AR LBD, amino acids 658-919, is localized mainly in the cytoplasm, and it often tends to form cytoplasmic granules (Figure 13, panel C). Testosterone exposure triggers nuclear transport of this mutant. In the presence of testosterone, AR LBD and GRIP1 colocalize in a pattern closely resembling that of NTD of AR with GRIP1 in giant cogranules (Figure 13, panel D). In mammalian two-hybrid system GRIP1 has been also shown to interact with AR LBD in isolation and activate AF-2 (Slagsvold et al., 2000). In addition, we found that DNA binding is not a requirement for AR GRIP1 interaction, since the DBD-deleted AR Δ578-657 can colocalize with GRIP1 in granules in the nucleus (data not shown).

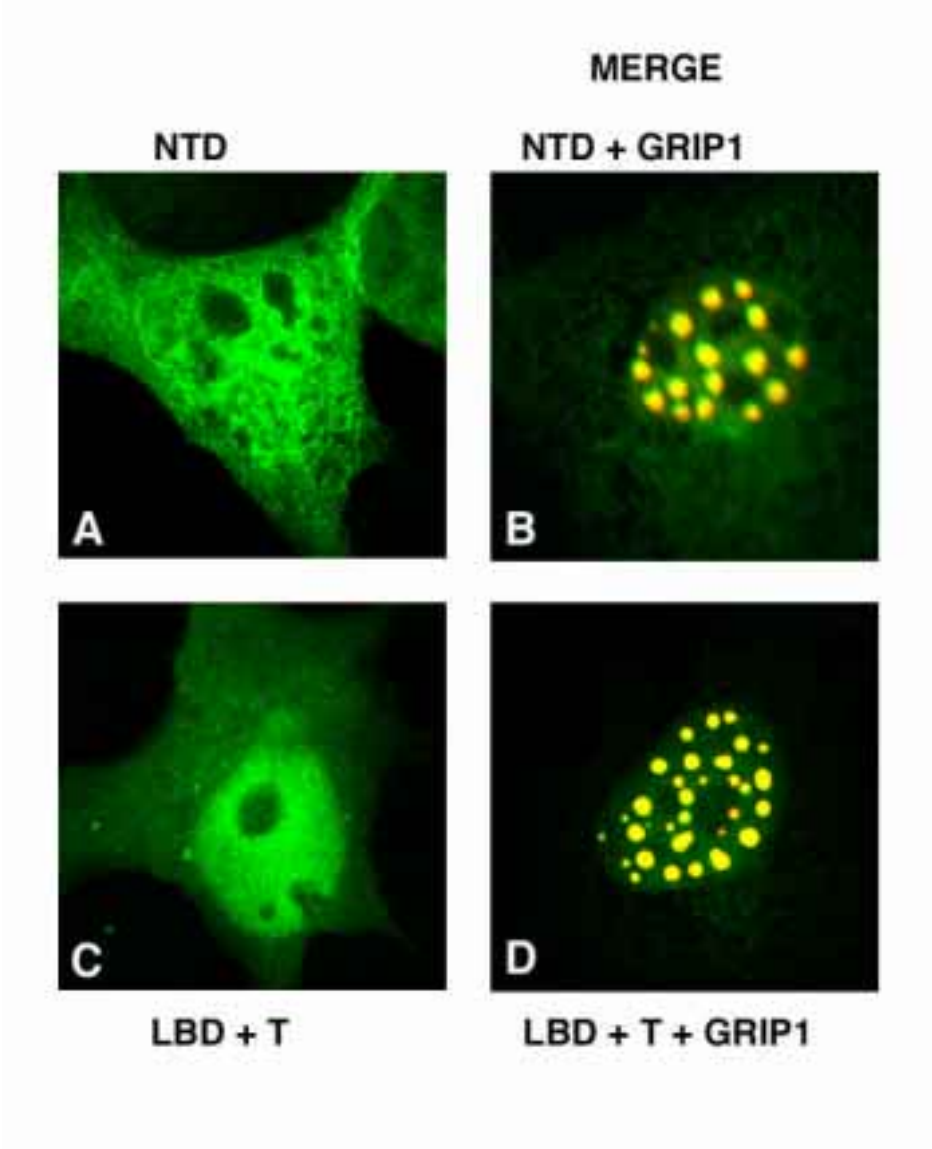


Fig. 13. Role of AR domains for AR-GRIP1 interaction. Isolated N- and C-terminal domains of AR are recruited by GRIP1. Panel A: pEGFP-NTD, panel B: pEGFP-NTD with cotransfected pSG5-GRIP1, panel C: pEGFP-LBD, panel D: pEGFP-LBD cotransfected with pSG5-GRIP1. Both N- and C-terminal fragments of AR colocalize perfectly with GRIP1. Confocal microscopic experiments from transfected cells. GRIP1 was detected from fixed cells with monoclonal antibody and successively with rhodamine-labeled secondary antibody detected at 568 nm excitation, and EGFP-tagged AR fragments were detected with 488 nm excitation.

4.3. Effects of antiandrogens on AR-GRIP1 colocalization and transactivation

Antiandrogens have been previously classified in several studies based on the use of androgen-responsive, but not androgen-specific, MMTV promoter in reporter gene assays (Kemppainen et al., 1991; 1999; Simenthal et al., 1992; Veldscholte et al., 1992; Kuil and Mulder, 1996). To obtain physiologically more reliable information, our transactivation experiments were performed with the natural androgen-responsive probasin promoter.

In accordance with the effects on colocalization of AR and GRIP1, hydroxyflutamide and casodex also inhibited the ability of GRIP1 to coactivate AR-dependent transcription. Partial antagonist cyproterone acetate activated AR transactivation only slightly, but it was able to support GRIP1 coactivation of AR-dependent transcription to 50% of that induced by testosterone.

Complete antagonists hydroxyflutamide and casodex were the only ligands tested which kept AR and GRIP1 separate although they induced AR transport to the nucleus. In competition experiments with testosterone at 100:1 molar ratio, hydroxyflutamide was somewhat more potent than casodex in preventing colocalization of AR with GRIP1. This is in line with our results from promoter interference studies, where we found difference between these two antagonists; hydroxyflutamide totally abolished AR DNA-binding *in vivo*, whereas casodex was less efficient in inhibiting AR DNA binding. The failure of pure antiandrogens to facilitate AR-GRIP1 colocalization could result from the fact that antagonists fail to induce a correct conformational change in the LBD (Kallio et al., 1994). These *in vitro* trypsin digestion experiments could not differentiate between the effects of antagonist casodex and cyproterone acetate (Kallio et al., 1994). However, our *in vivo* AR GRIP1 colocalization studies distinguish the effects of casodex from those of cyproterone acetate.

In conclusion, hydroxyflutamide and casodex are pure androgen antagonists that inhibit both DNA binding of AR and the interaction of the receptor with the coactivator GRIP1. In contrast, antiandrogen cyproterone acetate does not block DNA binding of AR, and it in fact supports efficiently coactivation of AR-dependent transcription.

In summary, antiandrogens are able to interfere with multiple steps of AR activation, ranging from nuclear trafficking and DNA binding to coactivator interactions (Figure 14).

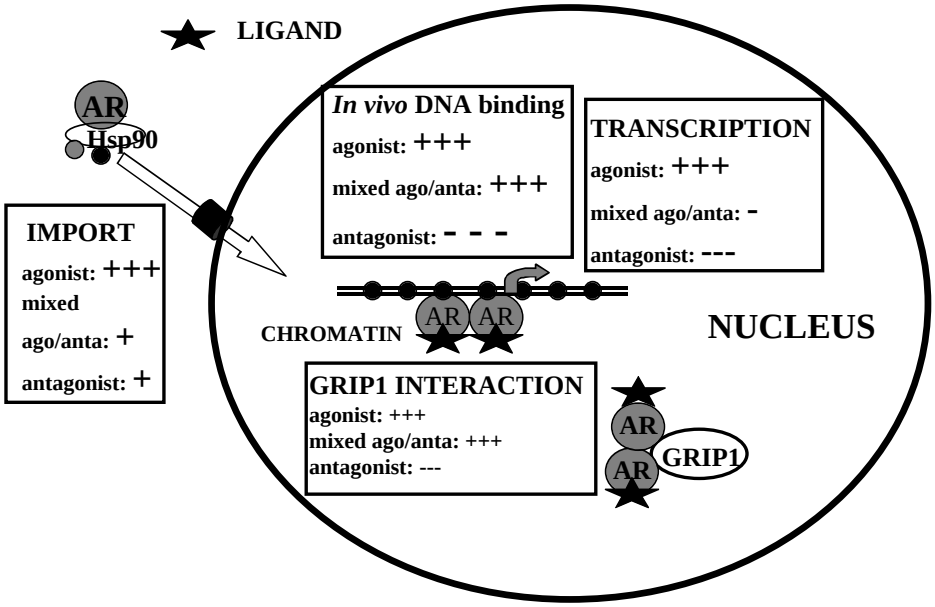


Fig. 14. Effects of partial agonists and pure antiandrogens on the function of AR. All antiandrogens support nuclear import of AR. Pure antagonists inhibit AR DNA binding. Mixed antagonists support the transactivation function somewhat but pure antagonists block AR-dependent transcription. Partial antagonists are as good as full agonist in supporting colocalization of AR with coactivator GRIP1 and in enhancing GRIP1 potentiation of AR transactivation. Partial antagonist tested: cyproterone acetate. Pure antagonists tested: hydroxyflutamide and casodex. Symbols: +++ : strong positive effect, + : weak positive effect, -: weak negative effect, ---: strong negative effect.

5. NUCLEAR DOMAINS AND THE EFFECT OF SUMOYLATION ON AR-GRIP1 INTERACTION (IV)

GRIP1 interacts with the PIAS proteins that act as SUMO E3 ligases (Kotaja et al., 2002a). We showed that GRIP1 is sumoylated and that lysine residues 239, 731 and 788 of GRIP1 serve as principal attachment sites for SUMO-1. We substituted lysines 731 and 788 to arginines to examine the effect of sumoylation on the ability of GRIP1 to colocalize with AR and the ability of GRIP1 to enhance AR-dependent transcription. Lysines 731 and 788 reside in the NR interaction domain of GRIP1 and this domain is crucial for GRIP1 to interact with several proteins. Our results show, that mutating these lysines impaired AR-GRIP1 colocalization, since in half of the observed cells AR failed to recruit the mutated GRIP1 to colocalize with it. Mutation of sumoylation target lysines 731 and 788 in GRIP1 also weakened the ability of GRIP1 to potentiate the transcriptional activity of AR. Substitution of less important sumoylation target lysines at amino acids 239 and 1452 inhibited GRIP1 from colocalizing with AR in only 8% of cells. The overall distribution pattern of GRIP1(K731,788R) was similar to that of wild-type GRIP1 in COS-1 cells. However, marker proteins to construct a detailed subnuclear

structure map as a reference for GRIP1 distribution were not available and therefore, we cannot state with 100% certainty that the distribution of the sumoylation mutant is identical with that of wild-type GRIP1 protein. In addition, this experiment was performed in fixed cells, thereby lacking information about the possibly changed movement characteristics of sumoylation-deficient GRIP1. Namely, sumoylation has been implicated to influence the mobility of proteins (reviewed by Pilcher and Melchior, 2002 and Freiman and Tjian, 2003).

Recently, a subpopulation of GRIP1 has been localized to the PML bodies (Baumann et al., 2001), which suggests that sumoylation may be involved in regulating the interaction of GRIP1 with PML bodies. In cotransfection experiments, we have found GRIP1 to colocalize with the PML splice variant PML-3 (Figure15, panels D to F) , but found it to be totally separate from another PML variant, PML-L (Figure 15, panels G to I). GRIP1 and PML-3 were often localized next to each other as subdomains of larger domain structures. This is in agreement with the known doughnut-like structure of PML-domain with non-homogenous distribution of ND10 proteins (Müller et al., 1998). PML-L is also known as PML III and it encodes a 641-amino acid protein (de Thè et al., 1991) (Figure 15, panels C, F, L). PML-3 is also known as PML IV and it encodes a 633-amino acid protein (Fagioli et al., 1992) (Fig. 15, panel I).

PML sumoylation is regulated in a cell cycle-dependent manner (Everett et al., 1999). Interestingly, AR colocalized with GRIP1(K731, 788R) in about half of non-synchronized cells, which suggests that nuclear localization of GRIP1 and interaction with AR is regulated by sumoylation in a cell cycle-dependent manner. Furthermore, sumoylation of PML and other ND10 resident proteins has been suggested to regulate targeting of NR coregulators to different subnuclear locations and functions cell cycle-dependently (reviewed by Pilcher and Melchior, 2002). Intriguingly, sumoylation has been shown to regulate the activity of transcription factors, such as p53 (Gostissa et al., 1999). Only the splice variant PML-3 recruits p53 into PML bodies in a sumoylation-dependent fashion, and this enhances p53-dependent transcription (Fogal et al., 2001). This same PML variant colocalized with GRIP1 in our experiments (Figure 15, panels D to F).

We have also examined nuclear distribution of the PML variants PML-L and PML-3 with respect to localization of AR, SUMO-1 and GRIP1. Neither PML variant, PML-3 or PML-L, colocalized with AR, emphasizing the fact that AR speckles and PML bodies are two separate entities (Figure 15, panels A to C). In spite of ectopic PML-3, which in the absence of AR induces GRIP1 to redistribute with it into same domain structures (Figure 15, panel D), agonist-occupied AR was able to induce GRIP1 to conform to the AR distribution pattern (Figure 15, panels J to M). In our experiments, both PML variants either colocalized with SUMO-1 perfectly or they were totally separated from SUMO-1 in confocal microscopy in non-synchronized cell population (data not shown) therein suggesting that this phenomenon may be cell-cycle dependent.

The NR speckles are clearly different from the PML nuclear domains, in that SRs reside in hundreds of speckles, whereas PMLs are localized to only tens of dots. However, PML bodies may at least indirectly be involved in SR-regulated transcription, since p160 coactivators appear to reside part of the time in PML bodies (Baumann et al., 2002). Besides the recruitment of CBP and p300 to NRs occurs through p160 proteins and the association of latter proteins with AR triggers AR transcription complex formation (Shang et al., 2002; Kang et al., 2002). In summary, sumoylation is emerging as an important control mechanism in the regulation of GRIP1 subcellular targeting and interactions with other proteins.

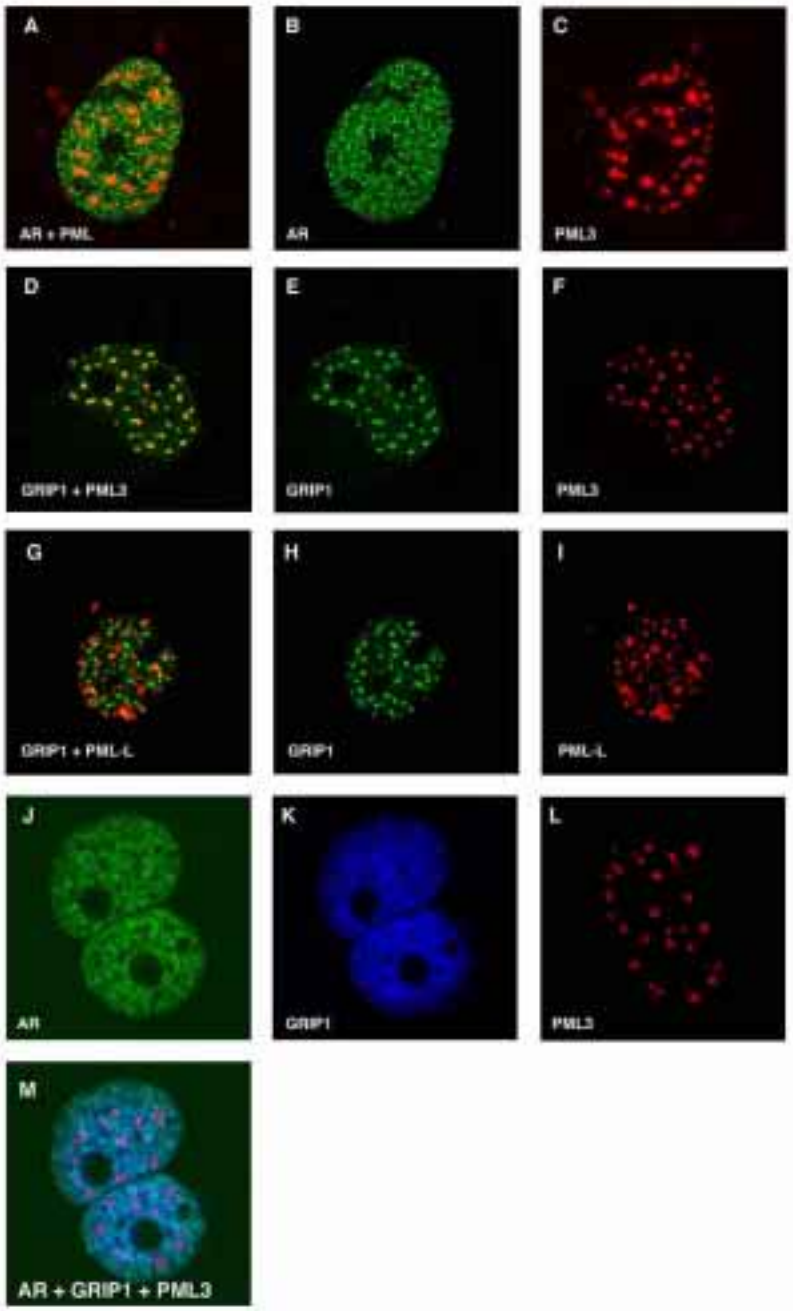


Fig 15. Confocal microscopy of PML-variants PML-3 and PML-L with AR and GRIP1 in COS-1 cell nuclei. AR does not colocalize with PML-3 (panels A-C) or PML-L (data not shown). GRIP1 colocalizes with PML-3, i.e. proteins reside next to each other in same domains as separate subdomain structures (panels D to F). GRIP1 and PML-L reside in totally separate nuclear domains (panels G to I). AR recruits GRIP1 to AR speckles to colocalize with AR in spite of coexpressed PML-3 (panels J to M). Transfections and fixation were done as described in III and IV. AR was fused to EGFP, GRIP1 and PML were detected with antibodies. In triple labeling experiments, fluorochromes of secondary antibodies (described in materials and methods) were exited with 488, 568 and 660 nm wavelengths. Otherwise confocal microscopy was done as described in III and IV.

In summary, in addition to the ligand, sumoylation is emerging as an important control mechanism in the regulation of subcellular targeting and interactions of steroid receptors and their coregulators (Figure 16).

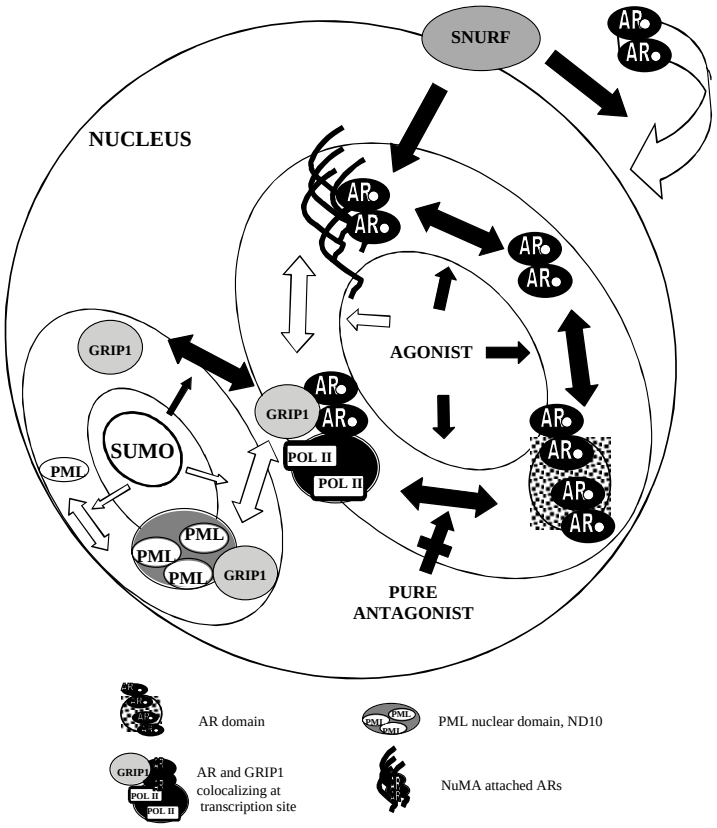


Fig. 16. Schematic summary of AR and coregulator trafficking. In principle, the ligand regulates the following AR functions: AR trafficking to the nucleus, DNA binding, transcription activation function, interaction with coregulators and the residence of AR in "AR speckles". Agonistic ligands support all the aforementioned functions, but pure antagonist, hydroxyflutamide and casodex, block DNA binding, transactivation and interaction with coactivator GRIP1. GRIP1 is distributed either diffusely in the nucleoplasm, in granular domains, in ND10s with PML or in SR domains. Sumoylation of GRIP1 may be an important element for both modulation of AR-dependent transcription and colocalization with AR. Agonist-occupied AR colocalizes with correctly sumoylated GRIP1 at transcription sites in a speckled pattern typical of AR domains. Sumoylation regulates the formation and composition of ND10s and interactions of PML body proteins. PML bodies may be involved in the regulation of SR activity through their ability to regulate p160 proteins. Another coregulator, SNURF, is able to facilitate AR trafficking into the nucleus and association of AR with the nuclear matrix (NuMa). The functions that we have examined in this study are indicated with black arrows, other interactions that are known to be important for the regulation of AR and GRIP1 function and the residence in nuclear domains are depicted with white arrows. In addition to ligand- and sumoylation-dependent regulation, SR activity is also regulated by ubiquitinylation. Degradation of SRs is ubiquitinylation-dependently regulated, and sumoylation has been shown to antagonize ubiquitinylation-dependent degradation of some proteins. Interestingly, SNURF is an ubiquitin ligase.

6. FUTURE DIRECTIONS

Characterization of factors regulating the residence and movement of steroid receptors and their coregulators in different nuclear domains is of major importance for understanding the transcriptional regulation.

We used confocal laser microscopy with optional sectioning to enhance resolution from that obtained with conventional fluorescence microscopy. With laser microscopy, the quality of three dimensional data obtained about protein distribution is vastly improved as compared to traditional fluorescence microscopy where diffuse distribution may hide especially subtle granular distribution patterns. The experimental setting was limited by that it was done in fixed cells, and monitoring of dynamic changes in the distributions of receptor and coactivator was not possible. Observation of the GFP-tagged molecules at single cell level in living cells as function of time in conjunction with fluorescence recovery after photobleaching techniques (FRAP) would certify the effects observed in fixed cells.

Next step in more detailed characterization of the functional nature of GRIP AR colocalization may also be microscopic. Fluorescence resonance electron transmission (FRET) microscopy applying spectral variants of GFP would allow monitoring of dynamic changes in protein-protein interactions in living cells (Day et al., 1999; 2001). Moreover, spatial resolution high enough for interpreting fluorescence data from thousands of nuclear domains, otherwise beyond fluorescence microscopy, can be achieved with FRET.

CONCLUSIONS

The results can be briefly summarized as follows:

- Agonist-induced conformational change is needed for AR DNA binding *in vivo*.

- SNURF facilitates AR import to nuclei and enhances association of AR to nuclear matrix.

- The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1, and sumoylation of GRIP1 is needed for complete nuclear colocalization of GRIP1 with AR.

- AR-GRIP1 colocalization is induced by agonists and a mixed agonist/antagonist, cyproterone acetate, and disrupted by the pure antagonists casodex and hydroxyflutamide.

- Pure androgen antagonists casodex and hydroxyflutamide inhibit DNA binding of AR and prevent AR from adopting its correct subnuclear distribution.

In conclusion, the ligand regulates DNA binding of AR and interaction of coregulatory proteins with AR. In addition to the ligand, SUMO-1 modification is a novel important mechanism controlling the ability of coactivator GRIP1 to interact and colocalize with AR.

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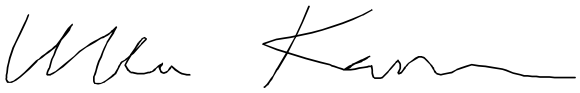
Lopuksi käsittääkseni viisas lause: “Ole tyytyväinen” (Olli L.)

Helsingissä toukokuussa 2003



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