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AWARD NUMBER: DAMD17-02-1-0082

TITLE: The hinge region as a key regulatory element of androgen receptor dimerization, DNA binding and transactivation

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REPORT DATE: May 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
May 2006	Final	1 Apr 02 – 31 Mar 06

4. TITLE AND SUBTITLE

The hinge region as a key regulatory element of androgen receptor dimerization, DNA binding and transactivation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0082

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Katholieke Universiteit Leuven
Research and Development
B-3000 Leuven Belgium

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The androgen receptor (AR) gene can undergo mutations during the development and treatment of prostate cancer. Two point mutations have been described in the hinge region, which is involved in DNA binding and nuclear translocation. These mutants display increased transactivating properties, despite a decreased nuclear translocation. We therefore performed a more detailed analysis of this part of the hinge region. Deletion of residues 629 to 636 (RKLKKLGN) resulted in a stronger androgen response. Truncation studies revealed that some hinge mutants are super active, in spite of their extremely low in vitro affinity for androgen response elements. Surprisingly, the activation functions AF1 and AF2 are not dramatically affected if the inhibitory 629-RKLKKLGN-636 motif is deleted. The ligand-dependent interaction between the amino-terminal domain and the ligand-binding domain plays an important role in transactivation by the AR. This interaction is strongly enhanced by mutation of the hinge region, but even in the absence of this N/C interaction, the hinge region mutants are more active compared to wild type AR. In conclusion, the AR hinge region is more than a flexible linker between the DNA-binding domain and the ligand-binding domain. Description of prostate cancer mutations revealed that the hinge co-ordinates the AR activity by interfering with several functions such as the nuclear localization, the DNA-binding, co-activator recruitment and the N/C interaction, and that the hinge might be involved in the development of prostate cancer.

15. SUBJECT TERMS
Androgen receptor, selectivity, DNA binding, transactivation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	91	**19b. TELEPHONE NUMBER** *(include area code)*

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THE HINGE REGION AS A KEY REGULATORY ELEMENT OF ANDROGEN RECEPTOR DIMERIZATION, DNA BINDING AND TRANSACTIVATION

INTRODUCTION

The androgen receptor (AR) mediates the biological effects of androgens, the male sex hormones. Androgens are responsible for the development of the male reproductive tissues and male secondary sex characteristics and are essential for spermatogenesis (De Gendt et al. 2004). The AR also plays a crucial role not only in the initiation and growth of prostate cancer, but also in the response to androgen-ablation and/or anti-androgen therapy. The benefits of the latter therapies are temporary since all patients eventually relapse, which coincides with an evolution from hormone-dependent to a hormone-independent tumour growth. The AR seems to be a key role player in this transition as well. It has been documented that the AR encoding-gene in these hormone independent cancers can have undergone alterations. Point mutations in and around the hinge region have been identified in prostate cancer patients.
In this study we describe the different functions of the AR which are affected by these mutations: they include DNA-binding, nuclear translocation and transactivation.

BODY

Part I. Co-Crystals of an AR-DBD fragment with a direct repeat ARE and translation of the structural data

I.A. DNA binding by the AR

Androgens enter the cell by passive diffusion through the cell membrane and bind to the AR which resides in the cytoplasm in an inactive form due to the association with multiprotein complexes of chaperones (reviewed in Claessens et al. 2001). After ligand binding, the receptor dissociates from the chaperones and rapidly converts to an active form in which the NTD interacts with the LBD. This is followed by nuclear translocation, dimerization and binding to androgen responsive elements (AREs) from which the receptor directs the transcription of androgen-regulated genes by the recruitment of co-activators (Claessens and Gewirth 2004).
The AR belongs to the super family of the nuclear receptors, which are characterized by a highly conserved DNA-binding domain (DBD). Nuclear receptors have a canonical structure consisting of a highly variable amino-terminal domain (NTD) containing a ligand-independent transcription activation function 1 (AF1), the DBD, and a carboxy-terminal ligand-binding domain (LBD) with a ligand-dependent transcription activation function 2 (AF2). The DBD and the LBD are separated by a non-conserved and flexible hinge region.
Initially, the hinge region of nuclear receptors was considered a flexible linker between the DBD and the LBD allowing a proper DNA-binding and dimerization. However, in case of the AR, the hinge region turned out to be a multifunctional domain, involved in DNA-binding and nuclear translocation (Schoenmakers et al. 1999; 2000). The androgen and glucocorticoid receptor have the same consensus sequence of DNA binding sites. In contrast to non-selective hormone response elements recognized by both receptors, there exist androgen-specific AREs, which are only recognized by the AR (Claessens et al. 1996; Verrijdt et al. 2000). The AR-DBD consists of two zinc coordinating modules. It requires a carboxy-terminal extension (CTE) of at least twelve residues (625-TLGARKLKKLGN-636) for optimal binding to androgen-selective AREs in band shift assays (Schoenmaekers et al. 1999).

I.B. Summary

The group of Dr. D. Gewirth, in close collaboration with our group has determined the structure of the AR-DBD bound to DNA (Figure 3 in Shaffer *et al.* 2004).
We have tried to confirm the structural data derived from the X-ray data (Shaffer *et al.* 2004) in functional analyses of mutant ARs.
We observed that a deletion of the hinge region results in an androgen receptor which is more active compared to the wild type AR cfr Moilanen et al. 1997, Wang et al. 2001). The same is true for two AR mutations in the hinge region.

I.C. Crystal data (cfr milestone I.A)

As described in Shaffer et al. 2004, the crystals were formed in a solution in which a direct repeat of 5'-TGTTCT-3' was mixed with AR-DBD fragments (residues 533 to 637) produced in *E. coli* (see also Figure 1 in Shaffer et al. 2004). Although earlier data indicated that the AR would dimerise on such sequences in a head-to-tail conformation, the crystal data clearly show AR-DBD dimers bound in a head-to-head conformation to the direct repeat element. This structure is highly similar to what has been observed for the GR-DBD bound to GRE4 and GRE3, and ER-DBD bound to an ERE. It was noted that for the AR, the dimerisation interface was larger and contained two additional H-bonds formed between a Threonine (an Isoleucine in the GR) of one monomer and a keto-function in the peptide backbone of the other DBD (and vice versa)(see fig. 4 of Shaffer et al. 2004). Another hydrogen bond is formed between the Serine at the same position in the two monomers. We hypothesised that this might explain why the AR is able to bind direct repeats. The GR, on the other hand, can not dimerise on such sequences. In this way, direct 5'-TGTTCT-3' repeats are androgen selective.

I.D. Translation of crystal data (cfr milestone I.B an II.B)

As described in the 2005 report, we have tested this hypothesis by exchanging the Threonine and Serine in the AR by Isoleucine and Glycine respectively. Much to our surprise, in transient transfection experiments, none of the mutations led to a change in specificity of the receptors (Figure 2 A, 2005 report). Hence, the reduction of the number of hydrogen bonds did not affect the transactivation by the AR via selective AREs. The establishment of these bonds in the GR, did not make it activate through the selective AREs, either.
These same mutations were introduced in isolated DNA-binding domains of AR and GR. In band shift assays, the selectivity was not changed (Figure 2B). Clearly, the stronger dimerization surface mediated by the two AR-specific residues in the so-called D-box is not sufficient to explain the changes in specificity between AR and GR.
It should be noted that, since the most important residues in the 5'-TGTTCT-3' hexamer recognition sequence are the G and C, and hence in direct as well as inverted repeats, these residues are present at the same positions. From very early swapping experiments we learned that, besides the second zinc coordinating module of the DBD, a short 12 amino acid long C-terminal extension of this fragment, called CTE, is involved in the recognition of the selective AREs (Schoenmakers et al. 1999). In this CTE, two residues were relevant to the binding of selective AREs, but not to classical AREs (Schoenmakers et al. 2000). We are now exchanging these residues between AR and GR, as well as between AR- and GR-DBDs. The effect of these mutations will be studied in transient transfections and band shift assays, respectively. Much to our surprise, the development of mutations in these residues (this fragment of the AR cDNA) is proving very difficult (cfr the hinge region of the PR, Dean Edwards, oral communication).

I.E. Selectivity of the androgen responses (milestone I.B)

A selective ARE was defined as such when the hormone responses obtained by co-transfection with an AR expression plasmid was much higher than that obtained by co-transfection with a GR

expression plasmid (reviewed in Claessens et al. 2001). This led first to the description of the PB-ARE-2 (Claessens et al. 1996), later of scARE and slpHRE2 as selective AREs (Verrijdt et al. 2000). The groups of Trapman and Haendler have added SARG-AR (Steketee et al. 2004) and the pem ARE (Barbelescu et al. 2001) to this list.
More recently, we have tested the responsiveness of these elements to progestagens. Since our mutation analyses showed that the D-box does not play a role, we turned our attention back to the CTE. The AR-specific Glycine (position 627) is conserved in the PR, while for the Leucine at position 634 in AR, a Phenylalanine is present in the PR (Figure 3A). The Glycine in the PRB has now been mutated to a Leucine (cfr GR). This mutation was very difficult to obtain because of technical PCR-related problems (see also elsewhere in this report). Functional assays of this mutant receptor are now being performed.
In transient co-transfection experiments, we observed that the unmutated PRB, like AR, is able to transactivate through the C3(1) ARE as well as through the PB-ARE-2 (Figure 3 of the 2005 report). The hormone concentration needed to observe a response is higher than the reported male serum concentration. Since progesterone is low in male serum, and since the PR will normally not be expressed and activated under conditions where the androgen-responsive genes are active, the AREs can still be called selective. The selectivity of the androgen response via selective AREs will be tested in ARE-based reporter mice. First transgenic mice have been obtained and are under investigation (other project)

I.F. Fluorescence Resonance Energy Transfer (FRET) (milestone I.B)

The aim of this experiment was to analyze conformational differences of the AR dimer binding to an AR-selective versus a classic ARE in solution. DNA oligonucleotides that contain the respective AR binding sites have been internally labeled with fluoresceine and can serve as FRET donors. The FRET-acceptor is the AR-DBD labeled with QSY 35 (Molecular Probes) at the sulfhydryl group of a specific cysteine (not involved in the coordination of zinc). Depending on the conformation of the proteins bound to the oligonucleotides, we expect differences in fluorescence-quenching. These differences can be used to calculate the distances between the donor-acceptor pair giving us important information about the orientation of the AR-dimer on the different types of binding sites. These experiments were performed in collaboration with the group of Dr. Engelborghs (Laboratory for Biomolecular Dynamics, KULeuven). Unfortunately, no clear signals could be read. Therefore, no conclusions could be drawn from these experiments.

I.G. Prostate cancer mutations in the hinge region (milestone III.B)

Two somatic point mutations in the hinge region of the human androgen receptor (AR) gene have been identified (www.**androgen**db.mcgill.ca).. A single G to A nucleotide change at position 2248 in exon 4 results in a R to Q amino acid substitution (R629Q). This mutation was isolated from a patient with androgen-independent prostate cancer after androgen ablation therapy. Another nucleotide change at position 2251 in exon 4, which gives rise to a K to T amino acid substitution (K630T), was identified in a prostate cancer patient before the onset of androgen ablation therapy. In this report, we aimed to determine the effect of the two identified point mutants on the roles of the hinge region. In the long run, this might give us valuable information for the development of new therapeutic strategies for the treatment of prostate cancer.
Because the two reported mutated residues are located within the earlier described CTE that is involved in DNA recognition by the AR-DBD, we evaluated their DNA-binding capacities (figure 1). No difference in binding was observed. These mutations will be discussed in further sections.

Part II. Structure-function relationships within the hinge region of the AR

II.A.Introduction

As for all nuclear receptors, the LBD of the AR has a very specific three-dimensional structure. Unique for the AR, however, is the fact that the activation function in the LBD (AF2) is very weak, probably because the AR AF2-coactivator interactions are weaker if compared to e.g. the estrogen receptor. Instead AF1 is the major transactivation function of the AR and the AR-NTD is the main recruiting surface for co-activators (Alen et al. 1999; Bevan et al. 1999, Christiaens et al. 2002). In addition, the AF2 co-activator recruitment surface of the AR is the primary interaction site for the AR-NTD. It is a 23-FQNLF-27 motif which occupies the hydrophobic cleft in the LBD (Dubbink et al. 2004, Callewaert et al. 2003) . This interaction is functionally important since deletion of the 23-FQNLF-27 motif diminishes AR activity on some reporters (21), and seems necessary for chromatin-based templates (Li et al. 2006).
What are the functions of the hinge region, i.e. the link between the DNA-binding domain and the ligand-binding domain? The hormone-dependent nuclear translocation is mainly mediated through a bipartite nuclear localization signal (NLS) consisting of two clusters of basic residues that are located in the DBD and the hinge region (Jenster et al. 1993). The AR can also be acetylated in its hinge region at residues K630, K632 and K633 (Fu et al. 2000). Acetylation of these residues has been reported to regulate transcriptional activity, subcellular distribution and folding of the AR (Fu et al. 2002, Gaughan et al. 2005), co-activator and co-repressor binding and the kinetics of this modification has been proposed to be altered in some forms of prostate cancer.

II.B. Studies in yeast (cfr milestone II.C)

It has been suggested that a deletion of part of the hinge region affects AR-AF-2 in yeast (Moilanen et al. 1997). However, we could not confirm this (see earlier reports).

II.C. The hinge region inhibits the AR activity (cfr milestones I.C., II.A)

The hinge region has been reported to have an inhibitory effect on the transcriptional activity of the human AR. The transactivation potential of Δ1, an AR deleted for residues 628 to 646 was compared to that of the wild type AR on four different androgen responsive reporter constructs (figure 2A). Indeed, Δ1 shows an increased induction of the MMTV promoter construct, as well as on reporter constructs based on the PB-ARE-2, the SC ARE and the C3(1) ARE.

II.D. Functional analysis of two prostate cancer mutations in the AR gene (alternative to a.o. milestone II.B)

The effect of the prostate cancer mutations (see section I.G) on the functionality of the AR was investigated (figure 2B). In transient transfection experiments both the R629Q and the K630T AR mutant have a small increase in transactivation potential, when compared to the normal AR. Expression was checked by immunoblotting (figure 2B).

II.E. Delineation of the inhibitory region (milestone I.C)

To delineate the inhibitory region, a truncation analysis was performed both at the N-terminal (Δ2-Δ5) and at the C-terminal (Δ6-Δ8) border of the region of interest (figure 2C). The resulting constructs are represented and their activity on the MMTV based reporter was compared. Expression was checked by immunoblotting. The Δ1 and Δ2 constructs show an increased potency with induction factors ranging from 21 to 14 in contrast to the wild type AR-mediated induction of 7.4-fold. Smaller deletions resulted in receptor constructs (Δ3 to Δ5) that acted

similar to the wild type AR, with induction factors ranging form 5.1 to 5.8. The constructs with larger deletions starting at residue 628 (Δ6 to Δ8), mediated higher induction factors.
A further analysis was performed by making three additional deletions (Δ9-Δ11). Although both Δ9 and Δ10 show increased transcriptional activity with induction factors of respectively 13 and 12, the minimal deletion which resulted in the most potent receptor (Δ11) was from position 629 to 636. In conclusion, the motif between position 629 and 636 (629-RKLKKLGN-636) in the hinge region of the human androgen receptor limits its transcriptional activity (figure 2C).

II.F DNA binding by the AR deletion mutants (cfr milestones II.A, IV.B)
Because the inhibitory region overlaps with the earlier described CTE that is involved in DNA recognition by the AR-DBD, we evaluated the DNA-binding capacities of the AR deletion mutants Δ1 to Δ11 (also described in earlier reports). The DNA binding was tested in band shift experiments using whole cell extracts of COS cells transfected with the expression plasmids of the different mutants, and the C3(1) and PB-ARE-2 as DNA probe (figure 3). The largest deletion in the hinge (Δ1) diminishes the DNA-binding to the C3(1) ARE, and binding to the PB-ARE2 was only detected after longer exposures of the gels. Increasing the length of the CTE (Δ2 to Δ5), improved DNA binding to both probes with the most pronounced effect for the C3(1) ARE. Stepwise deletions in the N-terminal part of the CTE (Δ6 to Δ8) destroy DNA-binding to the PB-ARE2, but not to the C3(1) ARE. Deletion of the inhibitory region of 8 amino acids (Δ11) gives the same results as Δ1. The Δ9 and Δ10 constructs show wild type DNA-binding characteristics, probably because of the similarity between the remaining residues and the deleted residues (RKL and KKLGN respectively).

II.G. Role of a putative PEST sequence and a phosphorylation site (alternative to milestone III.B, IV.A)
The presence of a putative PEST sequence within the hinge region, next to the Δ11 motif indicates a possible communication between these different functions. Also the AR is a phosphoprotein with a function of Serine 650 phosphorylation in nuclear export (Wong et al. 2004; Zou et al. 1995, Gioeli et al. 2006). However, deletion of the PEST sequence or mutations of the Ser 650 did not affect the transactivation of the AR in our assays (see Figure 6 of the 2005 report). It is interesting to note that a mutation of Serine 650 was recently found in a male fertility patient. This mutant is under further investigation (other project).

II.H. Role of the hinge region in AR stability (milestone II.B, IV.A)
In an earlier study, we analysed the effect of MG132, an inhibitor of the proteasome, on the activity of the AR versus the Δ1 construct (Tanner et al. 2004).
We have followed the steady state levels of the AR versus Δ1 and Δ11 by Western blotting for AR in cellular extracts made 1, 6 and 24 hours after addition of hormone (Figure 8A of the 2005 report). Although after one hour hormone stimulation, the effect is not outspoken, after six hours, the Δ1 and Δ11 constructs are expressed to a higher level as compared to the wtAR. After 24 hours, higher bands become visible on the Western blot. They can not be explained by sumoylation (Callewaert et al. 2004). Even in the absence of ligand, the Δ1 and Δ11 constructs are more expressed. This is not due to a enhancer-like element located in the hinge region coding cDNA part, since we did not observe androgen-regulation of a reporter gene with this fragment cloned upstream of the promotor (data not shown).
We have analysed the response in time to androgens in cells transfected with a C3(1)-based-luc reporter gene co-transfected with either wtAR, Δ1 or Δ11. The difference in responses to ligand on the C3(1) reporter parallel the increase in receptor levels (Figure 8B of the 2005 report), except that, although Δ1 and Δ11 seem equally expressed, Δ11 is more active.

II.I. Effect of hinge region mutations on nuclear translocation (Milestones III and IV)
The CTE overlaps with part of the nuclear translocation signal of the AR, and the two prostate cancer mutations coincide with this motif. Therefore, mutants and WT AR were fused to EGFP. EGFP-R629Q as well as EGFP-K630T have increased transcriptional activity (figure 4). In the absence of hormone, all EGFP fusion proteins show a mainly cytoplasmic distribution. After 1 h of hormone stimulation, the EGFP-WT is exclusively nuclear, while the mutants show an incomplete nuclear translocation. Again, this is counterintuitive since the AR has a function in the nucleus.

II.J. The basic character of the hinge region
It has been demonstrated that the AR can be modified by acetylation (Pestell 2003). The acetylation sites are located in the hinge region at residues 630, 632 and 633, which are all three located within the inhibitory region and one of the three is mutated in the prostate cancer mutant K630T. To reveal the potential role of the acetylation sites in the control of the super activity as revealed by the deletion of the inhibitory region, all three lysine residues were mutated into arginine or alanine, resulting in the following constructs, K630R, K632R, K633R, K630/632/633R and K630/632/633A (figure 5). A mutation of a lysine into arginine prevents possible acetylation, but retains the basic character of the residue while mutation into an alanine eliminates both properties. In functional assays with a C3(1) ARE based reporter, all constructs show a transcriptional activity comparable to the wild type AR, except the K630/632/633A mutant, which is four times more active (figure 5). In spite of this increase, the super activity level of ∆1 (34-fold induction) is not reached.

II.K. Relation of the hinge region with the transactivation functions

II.K.1.Activation function 1 and 2 of the AR mutant

For most nuclear receptors, two AFs have been characterized, AF1 in the NTD and AF2 in the LBD. The following experiments were designed to check whether the hinge affects AF1, AF2 or both. The AR-LBD was replaced by the DBD of the GAL4 yeast transcription factor, resulting in the chimerical WT/∆AF2 GAL4 fusion, and the corresponding mutant ∆1/∆AF2, deleted for the 628-646 fragment was created (figure 6A) The GAL4 DBD-fusions were developed to avoid any effects on DNA-binding and nuclear translocation via the AR-DBD. The activity of both chimerical constructs was measured by co-transfection with a GAL4-responsive luciferase reporter construct. When comparing the transcriptional activity of the WT/∆AF2 protein with that of the mutant ∆1/∆AF2 protein, no inhibitory effect of the hinge region on AF1 could be observed.
In a next step, we tried to determine whether the hinge region has an effect on the AR AF2. The AR DBD-hinge-LBD fragment was fused to the heterologous GAL4 DBD, resulting in the ∆AF1/WT construct and its activity was compared with that of the corresponding mutants ∆AF1/∆1, ∆AF1/∆11, ∆AF1/R629Q and ∆AF1/K630T (figure 6B). As expected, the wild type ∆AF1/WT construct showed no transcriptional potency at all. With the mutant ∆AF1/∆1 and ∆AF1/∆11 constructs, a moderate transcription activity was observed, reaching a maximal increase of 2-fold compared to the GAL4 DBD alone. No induction could be observed for the prostate cancer mutations.
Since AF2 is normally dependent on the recruitment of co-activators, we co-transfected the p160 coactivator TIF2 (figure 6C). In the absence of the hinge region, AF2 was co-activated by TIF2, while almost no co-activation was seen for the wild type as well as for the prostate cancer mutants under the conditions of this assay.

II.K.2. The N/C interactions in the hinge mutants

The role of the hinge region on the N/C interaction was tested in a mammalian double hybrid assay (figure 7A). ΔAF1/WT and the corresponding hinge mutants were co-expressed with the wild type NTD fused in frame with the VP16 protein in the presence of a GAL4-responsive luciferase reporter. The resulting luciferase values for the ΔAF1/Δ1 and ΔAF1/Δ11 double hybrids are 6- to 7-fold higher than for ΔAF1/WT, indicating a much stronger N/C interaction in the absence of the inhibitory hinge region. Similarly, the prostate cancer mutants also increased the N/C interaction, however to a much lower extend (about 2-fold increased luciferase activity). The VP16-NTD fusion deleted for the 23-FQNLF-27 motif has a deficient N/C interaction. Indeed, co-expression of the VP16-ΔFQNLF does not result in a luciferase increase which indicates the specificity of the test.

Subsequently, we investigated the N/C interaction in the full length AR context by means of transient transfection experiments. The transcriptional activities of the WT, Δ1, Δ11, R629Q and K630T constructs were compared with or without the 23-FQNLF-27 motif (figure 7B). After deletion of the 23-FQNLF-27 motif, and hence in the absence of a N/C interaction, the super activation due to the hinge mutations diminishes considerably, but is not completely abolished. Although relative expression levels are not identical, the observed differences are not sufficient to explain the big differences in transactivation.

II.K.3. Effect of hinge region on AF1

Because the AF1 in the AR is strong and constitutive (Jenster et al. 1995), meaning it can activate transcription in the absence of the LBD, we focussed on this receptor fragment. The AF1 comprises two activation functions Tau 1 (between residues 100 and 360) and Tau 5 (between residues 370 and 485) (Jenster et al. 1995). We assayed the effect of different CTE lenghts on the activity of the NTD in AR-NTD-DBD constructs (Figure 8). When we compare the constructs, depicted in figure 8A and B, truncated at 4, 12, 15 or 45 residues of the hinge region, the construct with the complete NTD is only 1.5-fold stronger compared to that containing the isolated tau5. Truncation of the first 171 or 100 residues results in a 4-fold stronger activation. The lower activity of the full NTD is not observed with the CTE15 and CTE45 constructs. The four-fold increase between the tau5 and the other constructs is observed for the CTE15 constructs but not for the CTE 45 constructs.

In comparison with the 2005 report, we have now been able to demonstrate expression for all constructs (figure 8C). The signals in the Western blots do not corroborate the thesis that activity of the different deletions mutants is strictly correlated with their expression levels. The fact that some CTE4 constructs are equally active as compared to CTE15 or CTE45 constructs is clearly discordant from their expression level.

II.K.4. Is the CTE a degron? (cfr milestones III.B, IV.B)

Degradation is an intricate part of the estrogen induced transcription (Reid et al. 2003). It has been reported for e.g. Myc that a motif which is responsible and sufficient to confer fast degradation, acts as a transactivating domain (Salghetti *et al.* 2000 and fig 9A). Since deletions in the hinge region results in A. an increased transactivation and B. a higher expression level, we wanted to test the hypothesis that maybe the CTE is an autonomous transactivation domain and can confer fast degradation to a heterologous protein. We have fused one, two or three copies of the CTE to the GAL4 DNA binding domain and tested the transactivation of a GAL4-reponsive luciferase reporter gene. Although the control constructs with the degradation signal of the VP16 activation domain indeed resulted in a strong transactivation, this was not seen for the CTE constructs. As for the degradation, we observed in Western blot that the constructs

obtained Dr. Tansey indeed show no signal when using their GAL4-fusion plasmid. When cloning the VP16 AD degradation signal in our GAL4-DBD fusion plasmid, this degradation was not as apparent, although strong transactivation is observed in a functional assay (figure 9B). For these constructs, as well as for our CTE-GAL4-DBD fusions constructs, expression was confirmed in Western blot (figure 9B and C).
We conclude that the CTE is not an activation motif acting as a degradation motif.

III. A. Mutation analysis of Arginine 629 and Lysines 630, 632 and 633 (Milestone III.B, III.C, IV.B)

The AR can be acetylated *in vivo* in the hinge region at Lysines 630, 632 and 633 (Fu *et al.* 2000, 2002; Pestell 2003). Lysine 630 was proposed to be involved in transcriptional regulation through an enhanced recruitment of p300 and reduced affinities for N-CoR and Smad3 after its acetylation. The mutation of this lysine to glutamine or threonine (mimics acetylation), when expressed in DU145 cells promoted cell survival and growth of cancer cells in soft agar and nude mice (Fu *et al.* 2003). More recently, the group of Robson proposed a link between Mdm2 mediated degradation and deacetylation (Gaughan et al. 2005). In the earlier reports, we showed data on AR mutated in several residues of the hinge.
We have mutated R629, K630, K632 and K633 to Valine separately and in different combinations, and tested the effect on transactivation of a TAT-GRE-luciferase reporter gene. Compared to wild type any single mutant was 2 to 3-fold more active. Surprisingly the R629/K630V mutant is less active, while the K632/K633V mutant is 7-fold more active. The quadruple mutant shows less than 50% activity of wild type AR (figure 10A). Western blots show expression of all mutants, with some higher expression of R629V, R629/K630V, K632/633V and the quadruple mutant (figure 10B). The expression levels are therefore not correlated with the functional androgen responses in figure 1A.
We subsequently analysed subcellular localisation of the mutants by fusing them to GFP (Figure 11). The R629V and K630V mutants show impaired nuclear translocation, which is even more pronounced for the double R629/K630V mutant. Surprisingly, the K632V and K633V mutants have wild type cellular distribution, while the double mutant K632/K633V or the quadruple mutant shows defective nuclear translocation. It should be noted that the R629/K630V and the quadruple mutant seem to be excluded from the nucleus in the presence of hormone. This correlated with their impaired function as shown in figure 1A. These tests, however, do not explain the higher activity of the other mutants.

III. B. Valine scanning of the hinge region (cfr milestone III.B.)

All residues of the hinge region from position 628 to 648 where mutated into Valine (Figure 12). Some of these mutants were reported in earlier reports. The mutants discussed in section II.A. give similar results. In addition, mutation of Lysine 638 also results in a more active AR. None of the single mutantions inactivated the receptor. The mutation of Leucine 634 did not affect AR activity, although a change into Lysine affects the activity on selective AREs. It has been a very demanding job to produce this figure. As stated in earlier reports, the hinge region has been proven to be very difficult to mutate. It has taken many efforts to obtain all mutations in the full size AR.

Figure 1: Effect of prostate cancer mutations on DNA binding

A. DNA-binding analysis by band shift assays. The band shift experiments were performed by incubating either the labelled non-selective C3(1)ARE or the androgen-selective PB-ARE2 probe with the whole cell extracts of the cells transfected with the indicated construct. Extracts of non-transfected cells were added to the first lanes as a negative control. The supershifts were obtained by adding a specific anti-body against AR (αAR). The positions of the free unbound probe (FP), the retarded complex (RC) and the supershifted complex (SC) are indicated with arrows. Non-specific complexes are indicated with an asterisk.

B. Immunoblotting of whole cell extracts. The expression plasmids for the flag-tagged WT and the mutant R629Q and K630T constructs were transiently transfected into COS cells (3 µg), plated into 6-cm Petri dishes and stimulated for 1 h with 10 nM of the synthetic androgen R1881.Whole cell extracts were made by lysing hormone-stimulated cells in 50 µl extraction buffer. The extracts were immunoblotted and the expressed proteins were detected using an anti-flag antibody.

Figure 2: Functional analysis of hinge region mutant ARs.

A. Function of full length AR and Δ1 (described in legend of figure 3) were compared. Different types of androgen-responsive luciferase reporter constructs were transfected into HeLa cells (100 ng) plated into 96-wells. The C3(1) ARE is an non-selective androgen-responsive oligonucleotide reporter constructs, the SC ARE and PB-ARE2 are androgen-selective. The MMTV reporter is a non-selective promoter reporter constructs. An expression plasmid for either WT or Δ1 was co-transfected (10 ng). Transfected cells were stimulated for 24 h with 0.1, 1 or 10 nM of the synthetic androgen R1881. The results are shown as relative induction factors representing the luciferase activity of hormone-stimulated cells relative to the activity of non-stimulated cells. The induction factor of WT stimulated with 0.1 nM is set at 1. The error bars represent the SEM.

B. Comparison of AR and the mutant constructs R629Q and K630T. Transfected HeLa cells were stimulated for 24 h with 10 nM of the synthetic androgen R1881. The results are shown as induction factor representing the luciferase activity of hormone-stimulated cells relative to the activity of non-stimulated cells. The error bars represent the SEM.

C.Functional analysis of full length WT and deletion mutants using the MMTV reporter The hinge region which stretches out from aa 628 to 669 is represented (A). The protein sequence of the N-terminal part of the hinge from aa 628 to 646 is shown. The borders of the C-terminal extensions (CTE) of the DBD of 4 and 12 residues, required for proper DNA-binding to respectively non-selective and androgen-selective AREs, are indicated with arrows. The nuclear localization signal is boxed; the potential acetylation sites are underlined. The borders of the deleted parts are indicated, the deleted aa are represented by a thin line. The expression plasmids for the WT and the mutant constructs were transfected into HeLa cells (10 ng) plated into 96-wells. The androgen-responsive MMTV luciferase reporter was co-transfected (100 ng). Transfected cells were stimulated for 24 h with 10 nM of the synthetic androgen R1881. The results are shown as induction factor representing the luciferase activity of hormone-stimulated cells relative to the activity of non-stimulated cells. The error bars represent the SEM. The expression of the AR proteins was analyzed by immunoblotting.

Figure 3: Function and DNA-binding of deletion mutants using canonical and androgen-selective AREs

A.Functional analysis of the WT and the mutant constructs Δ1 to Δ11 cfr legend figure 2. The results are shown as induction factor representing the luciferase activity of hormone-stimulated cells relative to the activity of non-stimulated cells. The error bars represent the SEM.

B.Immunoblotting of the whole cell extracts. WT and the mutant constructs Δ1 to Δ11 were expressed in COS cells stimulated for 1 h with 10 nM of the synthetic androgen R1881. Equal amount of the extracts were immunoblotted and the expressed proteins were detected using an anti-flag antibody.

C. DNA-binding analysis by band shift assays. The band shift experiments were performed by incubating either the labeled non-selective C3(1)ARE or the androgen-selective PB-ARE2 probe with the whole cell extracts of the cells transfected with the indicated construct. No protein was added to the first lanes as a negative control. The supershifts were obtained by adding a specific anti-body against AR (αAR). The positions of the free unbound probe (FP), the retarded complex (RC) and the supershifted complex (SC) are indicated with arrows. Non-specific complexes are indicated with an asterisk.

Figure 4: Localisation of AR and AR mutants

Either EGFP-WT, EGFP-R629Q or EGFP-K630T expression plasmid were transfected into HeLa cells (500 ng), which were plated into a chambered cover glass 4-well. The subcellular distribution of the EGFP fusion proteins without hormone stimulation and after 1 h stimulation with 10 nM R1881, was analyzed by fluorescence microscopy.

Figure 5: Functional analysis of the AR mutants R629Q and K630T in HeLa cells

Experiments were performed as described in the legend of figure 2. The results are shown as induction factor representing the luciferase activity of hormone-stimulated cells relative to the activity of non-stimulated cells. The error bars represent the SEM.
Immunoblotting of equal amounts of the whole cell extracts are done as described in figure 1. The results are depicted under the histogram of the corresponding induction factors.

Figure 6: Effect of the hinge region on activation function 1 and 2

A. WT/ΔAF2 and Δ1/ΔAF2, are schematically represented on the left. The results of function tests are depicted on the right (cfr legend of figure 2). The pAB-GAL4 plasmid was used as a reference control. The GAL4-resonsive $(GAL)_5$TATA-luc luciferase reporter construct was co-transfected (100 ng). The results are shown as luciferase activity of WT/ΔAF2 and Δ1/ΔAF2 relative to the pAB-GAL4. The error bars represent the SEM. Expression was checked by immunoblotting.

B.ΔAF1/WT and the ΔAF1 mutants are schematically represented and functionally tested in transient transfections. HeLa cells were plated into 96-wells and transfected with different expression plasmids for either ΔAF1/WT or ΔAF1/Δ1 the (50 ng). The pAB-GAL4-flag plasmid was used as a reference control. The GAL4-resonsive $pUAS_4$TATA-luc luciferase reporter construct was co-transfected (100 ng). Transfected cells were treated as described in the legend of figure 2. The error bars represent the SEM. Expression was checked by immunoblotting.

C. Effect of TIF-2: HeLa cells were plated into 96-wells and transiently transfected with expression plasmids for either ΔAF1/WT or the hinge mutants (50 ng). The pAB-GAL4-flag plasmid was used as a reference control. The co-activator TIF2 was co-transfected (50 ng). The GAL4-resonsive pUAS$_4$TATA-luc luciferase reporter construct was co-transfected (100 ng). Transfected cells were treated and induction factors represented as described in the legend of figure 2.

Figure 7: Effect of the hinge region on N/C interaction

A. Double hybrid. HeLa cells were plated into 96-wells and transiently transfected with 50 ng expression plasmids for either ΔAF1/WT or ΔAF1/Δ1 and 50 ng of the VP16-NTD or VP16-ΔFQNLF. The (GAL)$_5$TATA-luc luciferase construct was used as reporter (100 ng). The constructs used are represented at the top. The results are depicted as in figure 2.
B. Full size context. The functionality of the expression plasmids for the WT and the mutant constructs, schematically represented at the right, were tested in transient transfection experiments. The constructs were transfected into HeLa cells (10 ng) plated into 96-wells. The androgen-responsive MMTV luciferase or the C3(1) ARE reporters were co-transfected (100 ng). The cells were stimulated for 24 h with 10 nM of the synthetic androgen R1881. The results are shown as induction factor representing the luciferase activity of hormone-stimulated cells relative to the activity of non-stimulated cells. The error bars represent the SEM. For the immunoblotting, the expression plasmids for the different constructs were transiently transfected into HeLa cells (1 µg) plated into 24-wells and stimulated for 1 h with 10 nM of the synthetic androgen R1881. Whole cell extracts were made by lysing stimulated cells with 20 µl SDS-loading buffer. The extracts were immunoblotted and the expressed proteins were detected using an anti-flag antibody.

Figure 8: The effect of the hinge region on AF1.

A. Schematic representation of the NTD of the AR, indicating the FQNLF motif, Qr region, Tau-1 and Tau-5. NTD deletions for plasmid contructs are indicated with solid black bars: (i) NTD, constructs that contain the full-length NTD; (ii) 100NTD, contructs that contain the full Tau-1 and Tau-5 domains but are deleted of the FQNLF motif and Qr region; (iii) 360NTD, contructs containing only the Tau-5 domain; and (iv) DBD, contructs where the entire NTD had been deleted (not indicated in the figure).
B. Amino acid sequences of the various hinge region truncations used to create plasmid contructs that express AR proteins with variable NTD deletions, the DBD, and various carboxy-terminal extensions (CTE). Contructs described to possess CTE4, CTE12, CTE15 and CTE45 are truncated at amino acids 628, 636, 639 and 669 respectively. The inhibitory region of the hinge is indicated in italics.
C. Transient transfections were performed in HeLa cells as described in figure 1. 100 ng of the GRE-TAT-luciferase reporter construct was co-transfected with 10 ng of the various NTD-DBD-CTE expression plasmids and 10 ng of a pCMV-β-galactosidase construct. The constructs contain one of the four possible N-terminal deletions (DBD, 360NTD, 100NTD or NTD, described in A above); the full DBD; and one of the four possible C-terminal extensions (CTE4, CTE12, CTE15 or CTE45, described in B above). Cells were incubated in the absence of hormone (as all constructs are devoid of the ligand binding domain) for 24 hours before being harvested for assays. Data represent the means of at least two independent experiments performed in triplicate. Luciferase values are corrected for β-galactosidase expression levels and expressed as relative light units (rlu). The NTD-DBD-CTE constructs (tagged at the N-terminus with the Flag peptide) were expressed in HeLa cells seeded into 24-well plates at 10^5 cells/well. Extracts

were prepared after a 24 hour incubation in the absence of hormone and expression analysed by Western Blotting as described in figure 1.

Figure 9: A comparison of the transcriptional activation and protein expression levels of Gal4 DBD fusion proteins with either one or more degron or inhibitory region motifs.

Transient transfections were performed in HeLa cells as described in figure 1. Here 100 ng of the GRE-TAT-luciferase reporter construct was co-transfected with 10 ng of the appropriate Gal4DBD fusion expression plasmids and 10 ng of a pCMV-β-galactosidase construct. Cells were refreshed 24 hrs after transfection and harvested for assays after an additional 24 hr incubation period. Data represent the means of at least two independent experiments performed in triplicate. Luciferase values are corrected for β-galactosidase expression levels and expressed as relative light units (rlu). Protein expression levels were determined by Western Blotting (as described in figure 1) using the appropriate primary anti-bodies as Gal4DBD domains were either C-terminally tagged with the HA peptide or N-terminally tagged with the Flag peptide.
A. Constructs used were obtained from W. Tansey (previously described in Salghetti, S.E., *et al*, 2000). The plasmids used were composed of pCG-Gal4DBD-HA with zero, one, two or three copies of the VN8 module which encodes an amino acid sequence of DFDLDMLG (also referred to as the VP16 transactivation domain or degron).
B. Constructs used were created as controls and for comparative purposes. The plasmids were composed of pSG5-flag-Gal4DBD with zero, one, two or three copies of the degron motif (encoding the same amino acid sequence as the VN8 module described in A, namely DFDLDMLG).
C. Constructs used were created to investigate the transcriptional activity and degradation potential of the inhibitory region of the hinge region. The plasmids were composed of pSG5-flag-Gal4DBD with zero, one, two, three or four copies of the inhibitory region (encoding an amino acid sequence of RKLKKLGN).

Figure 10: The role of R629, K630, K632 and K633 on the transcriptional activation, expression and cellular localisation of the androgen receptor.

A. Transient transfections were performed using HeLa cells plated in 96-well plates at a density of 10^4 cells/well. 100 ng of the GRE-TAT-driven luciferase reporter construct was co-transfected with 10 ng of receptor expression plasmid and 10 ng of a pCMV-β-galactosidase construct. Receptor expression plasmids used encoded either the full-length wild-type AR (wtAR) or full-length AR with single, double or quadruple valine mutants of the positively charged residues in the inhibitory region of the hinge region. Cells were incubated in the absence or presence of 10 nM R1881 (a synthetic androgen) for 24 hours. Cells were then harvested and luciferase and β-galactosidase values were measured. Relative inductions are the ratio's of the luciferase values (means of at least two independent experiments performed in triplicate, and corrected for β-galactosidase expression levels) of extracts from stimulated and unstimulated cells.
B. The receptor expression plasmids (described in A) were expressed in HeLa cells seeded into 24-well plates at 10^5 cells/well. Twenty-four hours after transfection of 250 ng of the respective plasmids, cells were incubated in the absence or presence of 10 nM R1881. Extracts were prepared 24 hours after addition of hormone. Equal amounts of extracts were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and blotted onto Hybond-P nitrocellulose membrane. All the full-length constructs are tagged at the N-terminus with the Flag peptide and

so the membrane was probed with anti-Flag antibody, receptor proteins were then detected by ECL and autoradiography performed.

Figure 11: The cellular localisation of the wild-type and mutant AR proteins (described in A) was determined by immunocytochemistry. HeLa cells were seeded into 4-well coverslip chamber plates at 6 x 10^5 cells/well. Cells were transfected with 250 ng of the appropriate receptor expression plasmid. Twenty-four hours after transfection, cells were stimulated with 1 nM R1881 for 1 hour. Cells were then fixed and permeabilized. Proteins were probed with the anti-Flag antibody, followed by goat-anti-mouse IgG TRITC-conjugated. Cellular localisation was visualised using a flouresence microscope.

Figure 12: Valine screen of the hinge region.
A. Transient transfections were performed using HeLa cells plated in 96-well plates at a density of 10^4 cells/well. 100 ng of the GRE-TAT-driven luciferase reporter construct was co-transfected with 10 ng of receptor expression plasmid and 10 ng of a pCMV-β-galactosidase construct. Receptor expression plasmids used encoded either the full-length wild-type AR (wtAR) or full-length AR with single valine mutants of the first 21 amino acids of the hinge region. Cells were incubated in the absence or presence of 10 nM R1881 (a synthetic androgen) for 24 hours. Cells were then harvested and luciferase and β-galactosidase values were measured. Relative inductions are the ratio's of the luciferase values (means of at least two independent experiments performed in triplicate, and corrected for β-galactosidase expression levels) of extracts from stimulated and unstimulated cells.

IV. Milestones reached?
The numbering refers to that of the 'Statement of work' of the grant application

First year
i.a. Crystals: have been made (report 2004)
i.b. Translation of data: has been done (report 2004 and 2005)
i.c. Deletion of the hinge region: has been done (report 2004 and 2005)
i.d. Make a yeast expression vector: has been done (report 2004).

Second year
ii.a. Make a library of mutations in the hinge: has been done by PCR mediated mutagenesis.
ii.b. Bacterial expression vectors: have been done in part (report 2004 and 2005)
ii.c. Prokaryotic expression for crystallization: has been done.

Third year
iii.a. Finalise screening in yeast: was proven unfeasable (report 2004). As an alternative we performed a hypothesis driven mutation analyses (2004, 2005 and 2006 reports)
iii.b. Pro- and eukaryote expression constructs for specific mutants: has been done and is ongoing.
iii.c. Screening for dominant negative peptides. A first attempt has been reported in report 2004. This approach will be continued based on new information of the ongoing hinge region studies.

Fourth year
We are very greatfull that we could continue developing the experiments until April 2006. This enabled the confirmation of the hypothesis that the hinge is a major player in the control of the expression level, as well as the activation and nuclear translocation of the AR.

As anticipated in the Statement of work (first sentence), we deviated from the timeline, as well as from some of the milestones. However, we feel that the accomplished work is a major contribution to the knowledge on the molecular biology of action of the AR in normal physiology and prostate cancer. Part of the later work is under preparation for submission as manuscripts.

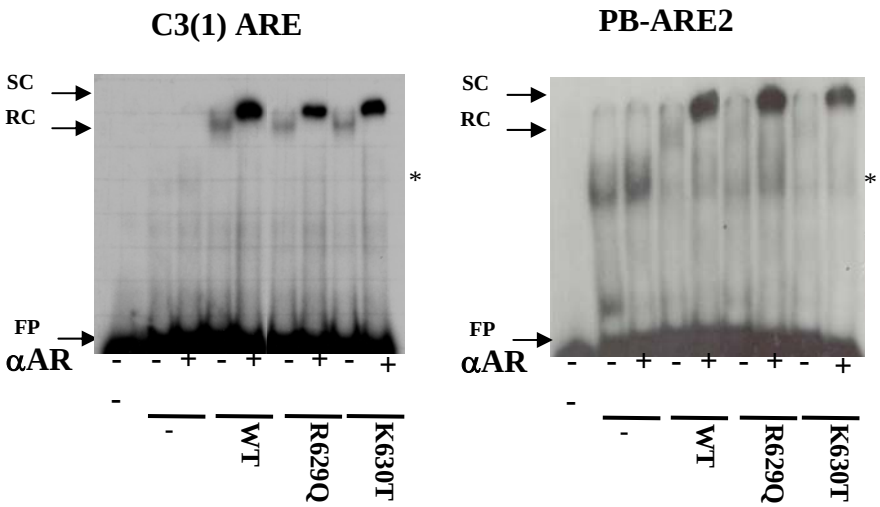
This work also led to collaborations with:
- 1. Dr. Adriaan Houtsmuller (Erasmus Rotterdam, The Netherlands) to analyse the nuclear mobility of the different (superactive) AR mutants (Farla et al. 2005)
- 2. Dr. Jiemin Wong (Houston U.S.A.) for chromatine template based assays. He observed that the N/C interactions are necessary for the activation of chromatine based templates (Li et al. 2006). In view of the effects of the hinge region mutations, this is a very promising collaboration.
- 3. Prof. K. Knudsen (Ohio, U.S.A.) for interaction with chromatine modifying complexes (Link et al. 2005).

Personel employed in relation to this grant

PhD student Tamzin Tanner (100 % salary for the whole period)
Postdoctoral Fellow Arnold dAlesio (100% salary for 6 months)
Postdoctoral Fellow Annemie Haelens (salary provided by the national fund for Scientific research Flanders 2002-2006)

Figure 1

A



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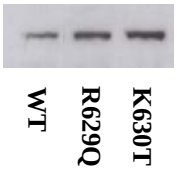
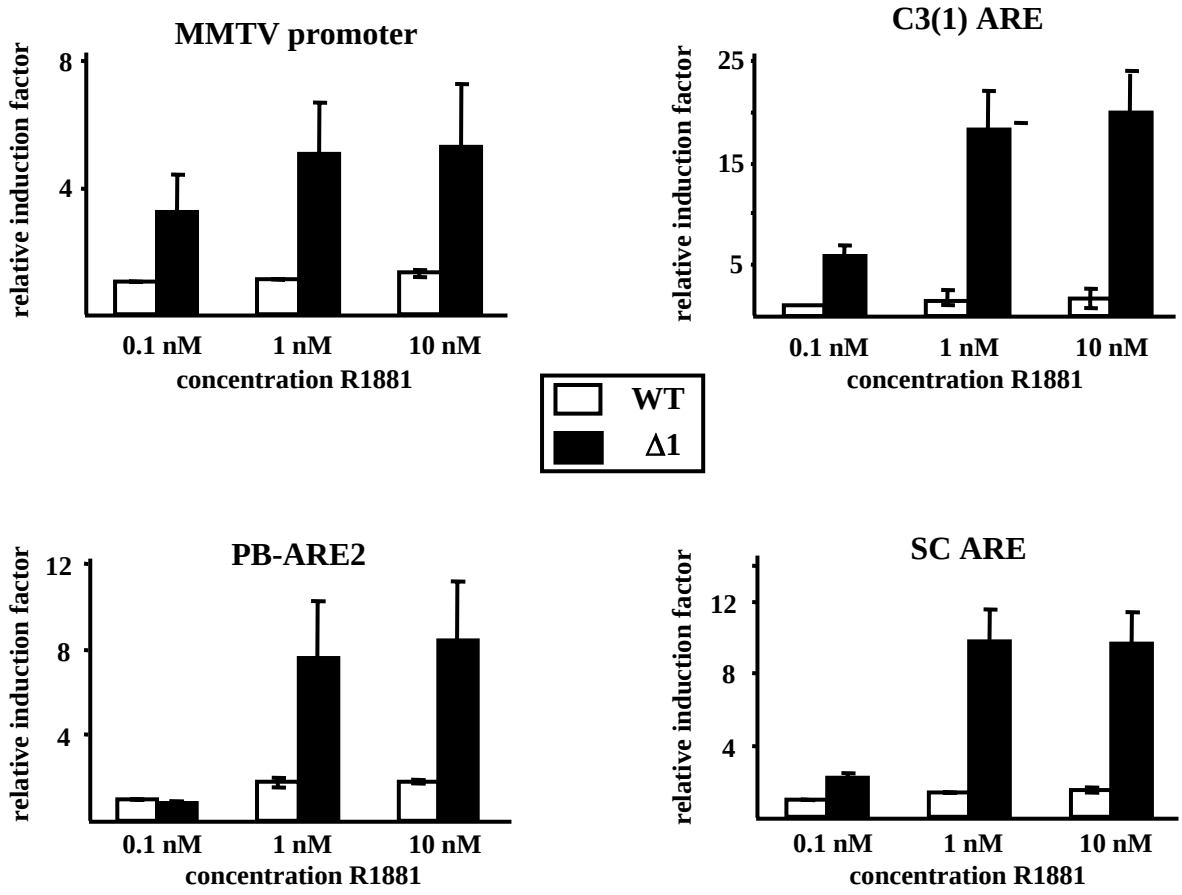


Figure 2A


MMTV promoter
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8
4
0.1 nM
1 nM
10 nM
concentration R1881
C3(1) ARE
relative induction factor
25
15
5
0.1 nM
1 nM
10 nM
concentration R1881
WT
Δ1
PB-ARE2
relative induction factor
12
8
4
0.1 nM
1 nM
10 nM
concentration R1881
SC ARE
relative induction factor
12
8
4
0.1 nM
1 nM
10 nM
concentration R1881

Figure 2B

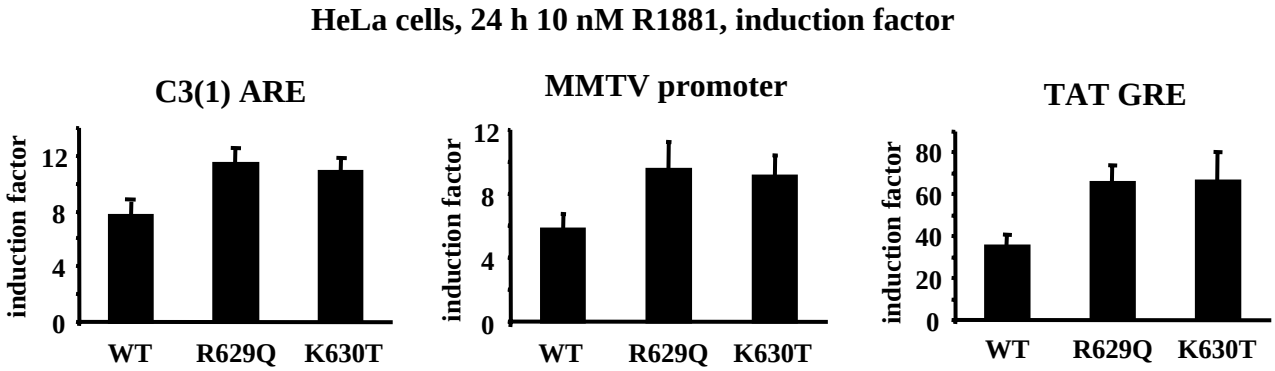


Figure 2C

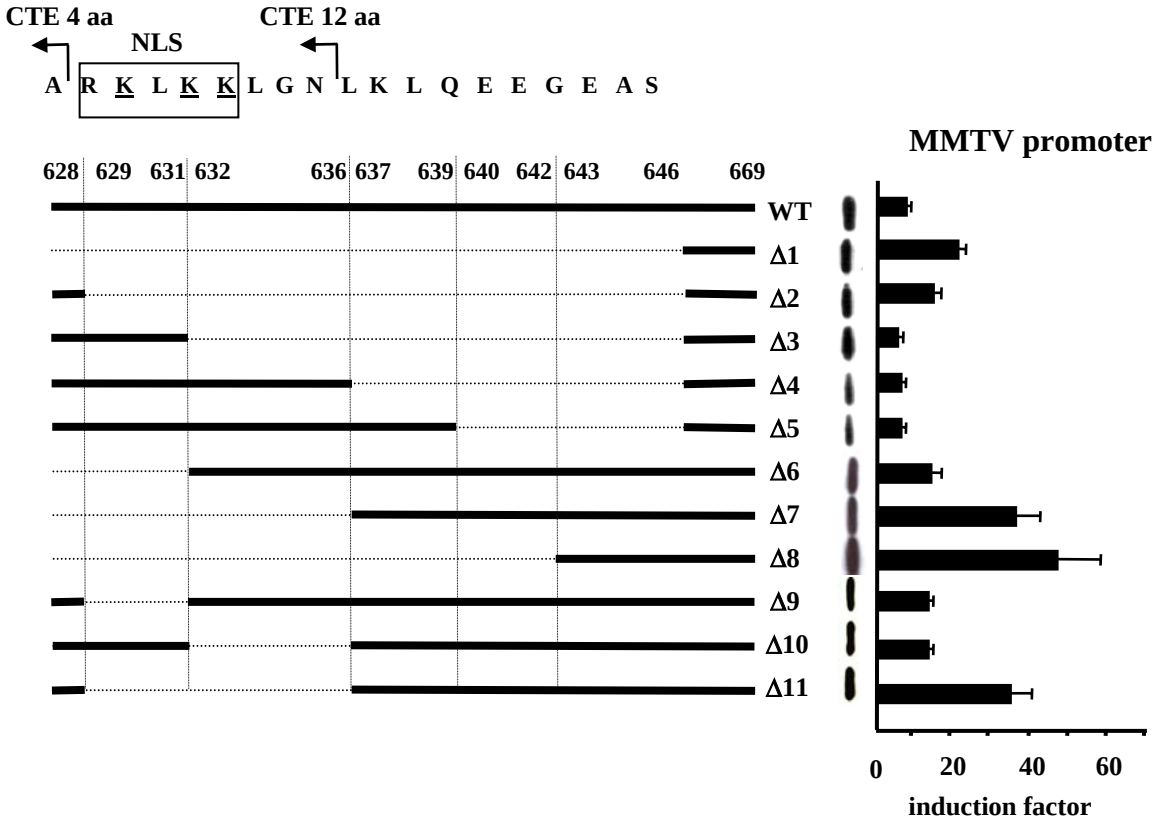
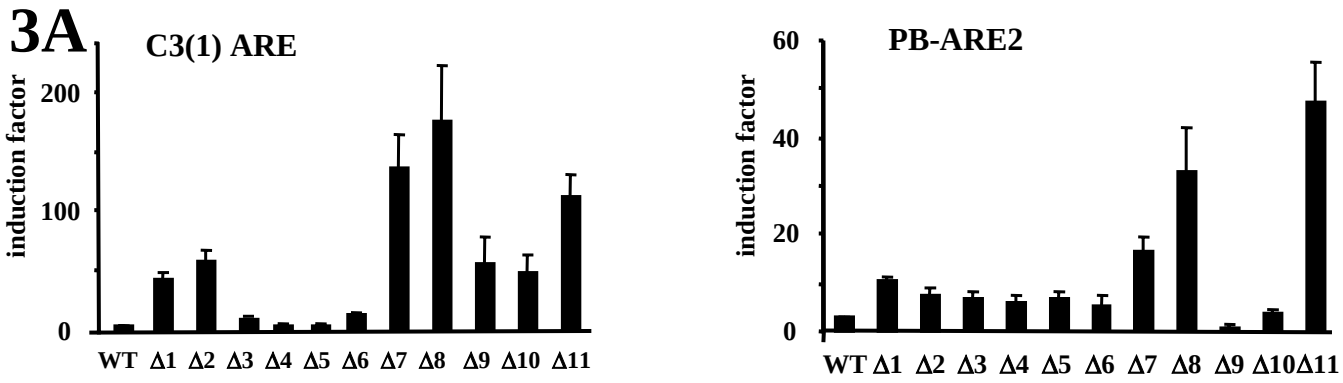
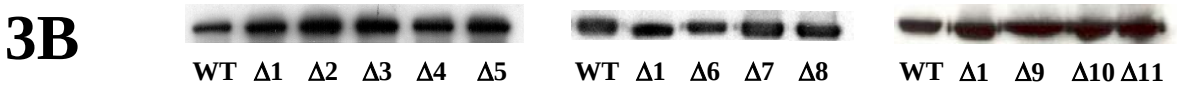


Figure 3


3A
C3(1) ARE
induction factor
200
100
0
WT Δ1 Δ2 Δ3 Δ4 Δ5 Δ6 Δ7 Δ8 Δ9 Δ10 Δ11
PB-ARE2
induction factor
60
40
20
0
WT Δ1 Δ2 Δ3 Δ4 Δ5 Δ6 Δ7 Δ8 Δ9 Δ10 Δ11


3B
WT Δ1 Δ2 Δ3 Δ4 Δ5
WT Δ1 Δ6 Δ7 Δ8
WT Δ1 Δ9 Δ10 Δ11

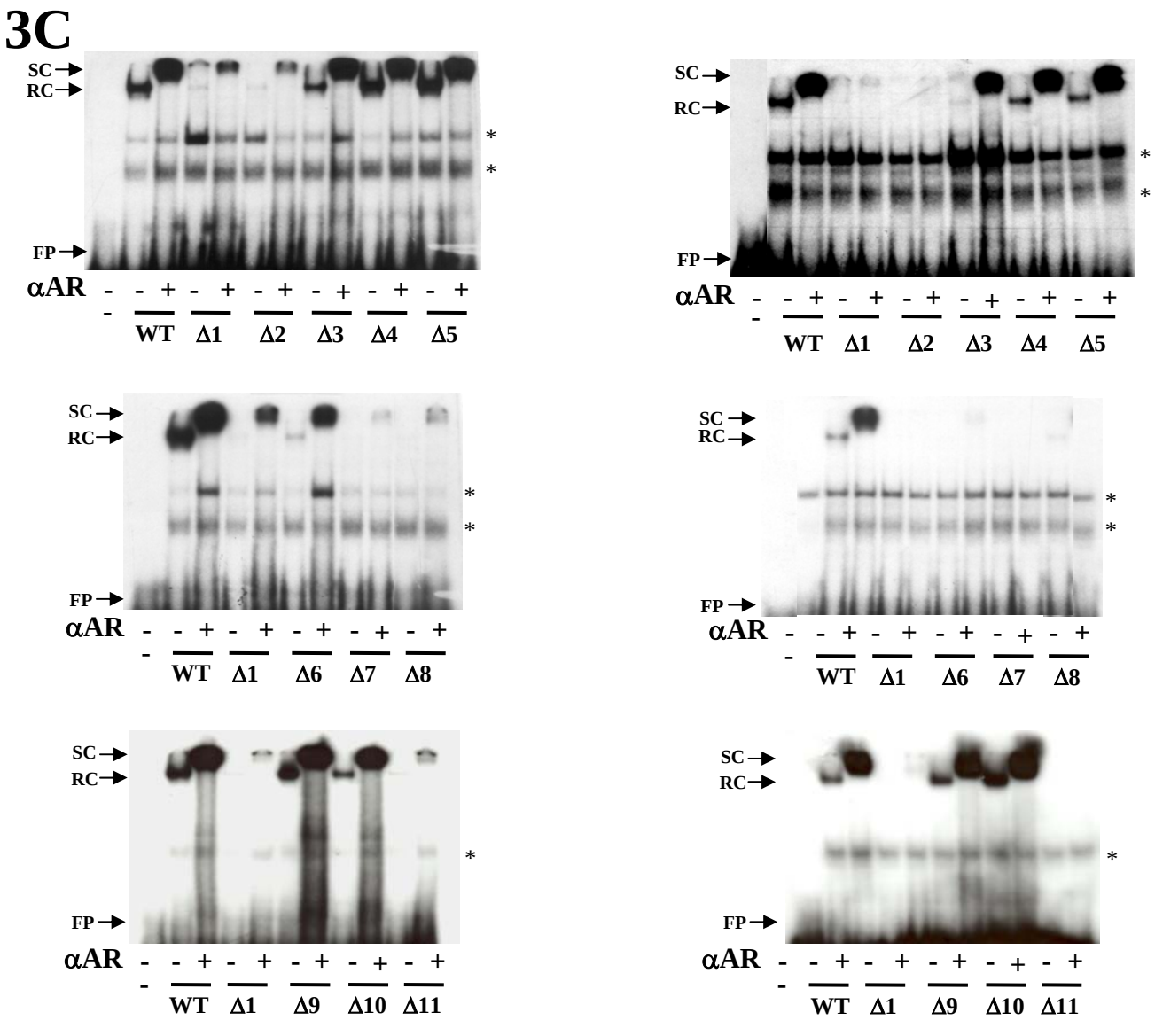

3C
SC
RC
FP
αAR
WT Δ1 Δ2 Δ3 Δ4 Δ5
WT Δ1 Δ6 Δ7 Δ8
WT Δ1 Δ9 Δ10 Δ11

Figure 4

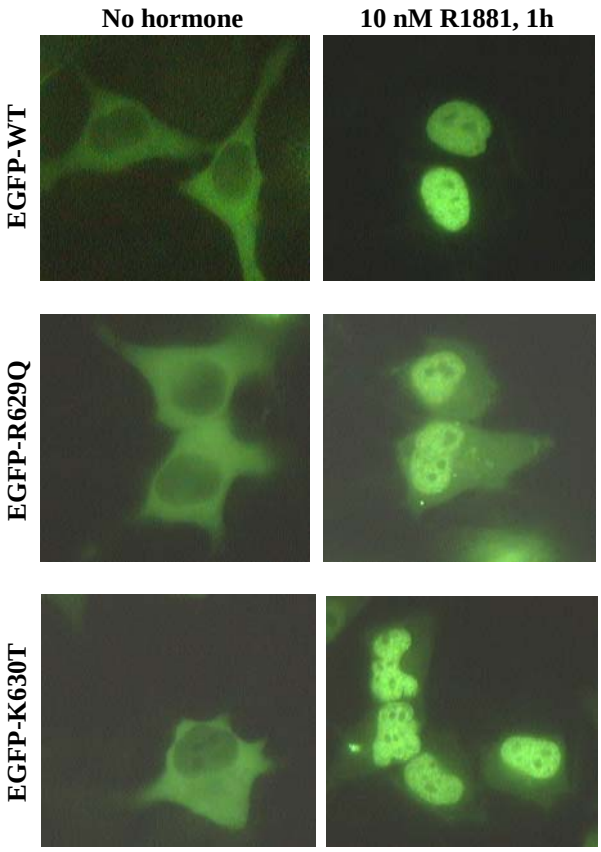
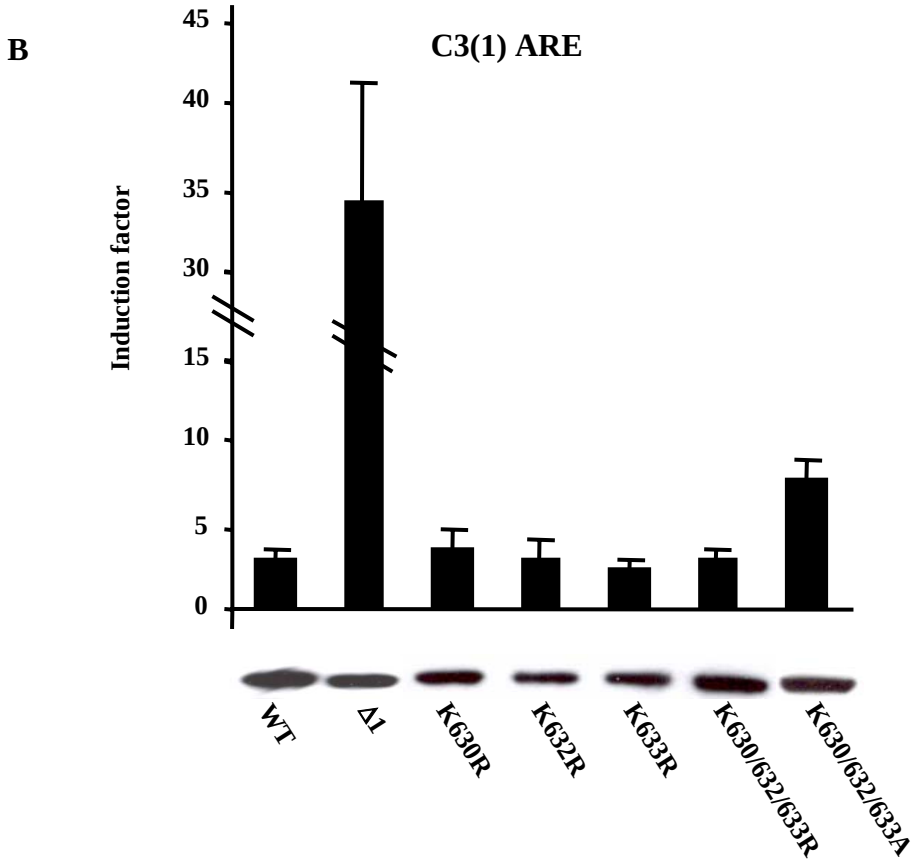


Figure 5


B
C3(1) ARE
Induction factor
45
40
35
30
15
10
5
0
WT
Δ1
K630R
K632R
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K630/632/633R
K630/632/633A

Figure 6

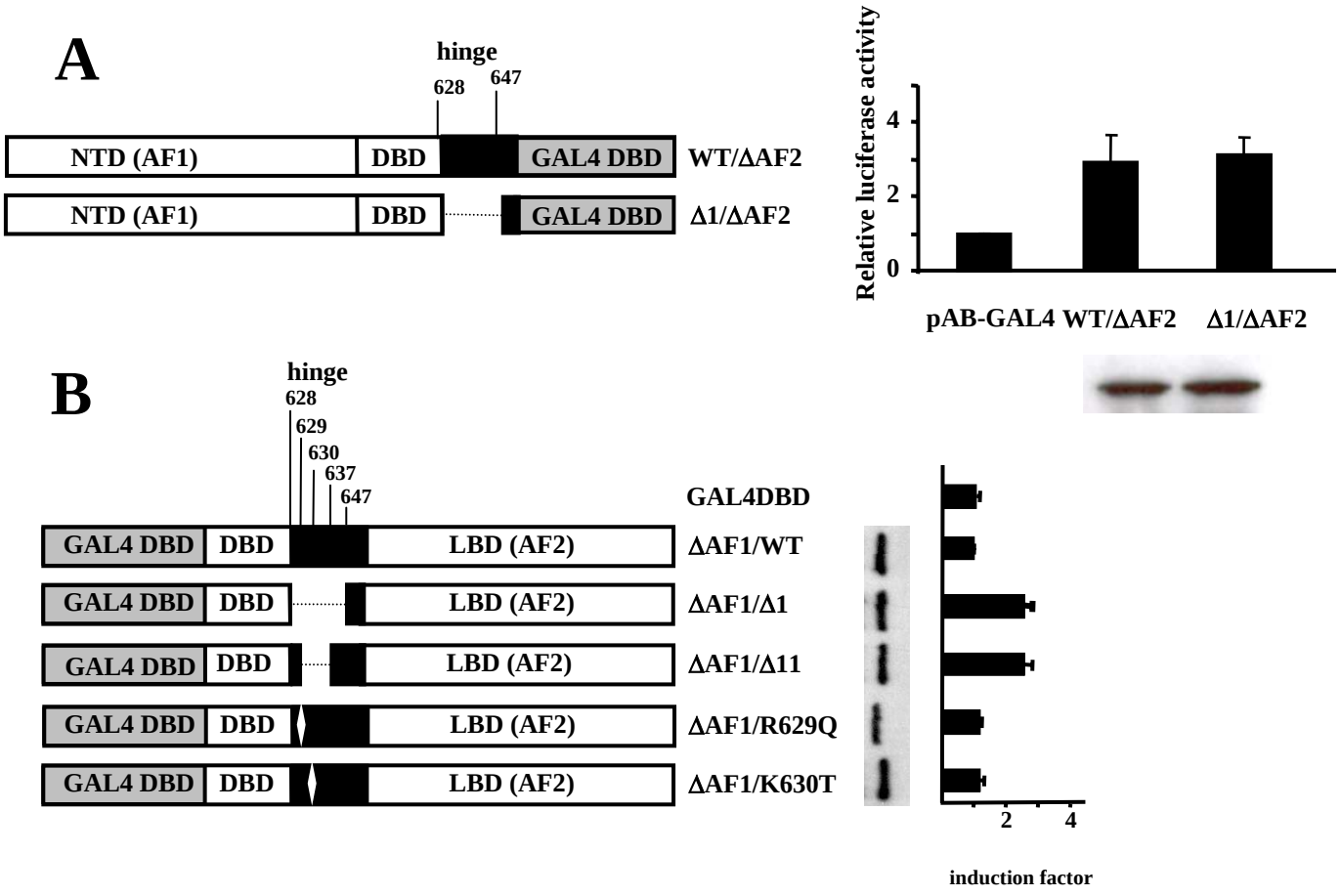

A
hinge
628
647
NTD (AF1)
DBD
GAL4 DBD
WT/ΔAF2
NTD (AF1)
DBD
GAL4 DBD
Δ1/ΔAF2
Relative luciferase activity
4
2
0
pAB-GAL4
WT/ΔAF2
Δ1/ΔAF2
B
hinge
628
629
630
637
647
GAL4DBD
GAL4 DBD
DBD
LBD (AF2)
ΔAF1/WT
ΔAF1/Δ1
ΔAF1/Δ11
ΔAF1/R629Q
ΔAF1/K630T
2
4
induction factor
C
induction factor
15
10
5
0
GAL4DBD
ΔAF1/WT
ΔAF1/Δ1
ΔAF1/Δ11
ΔAF1/R629Q
ΔAF1/K630T
-
TIF2

Figure 7

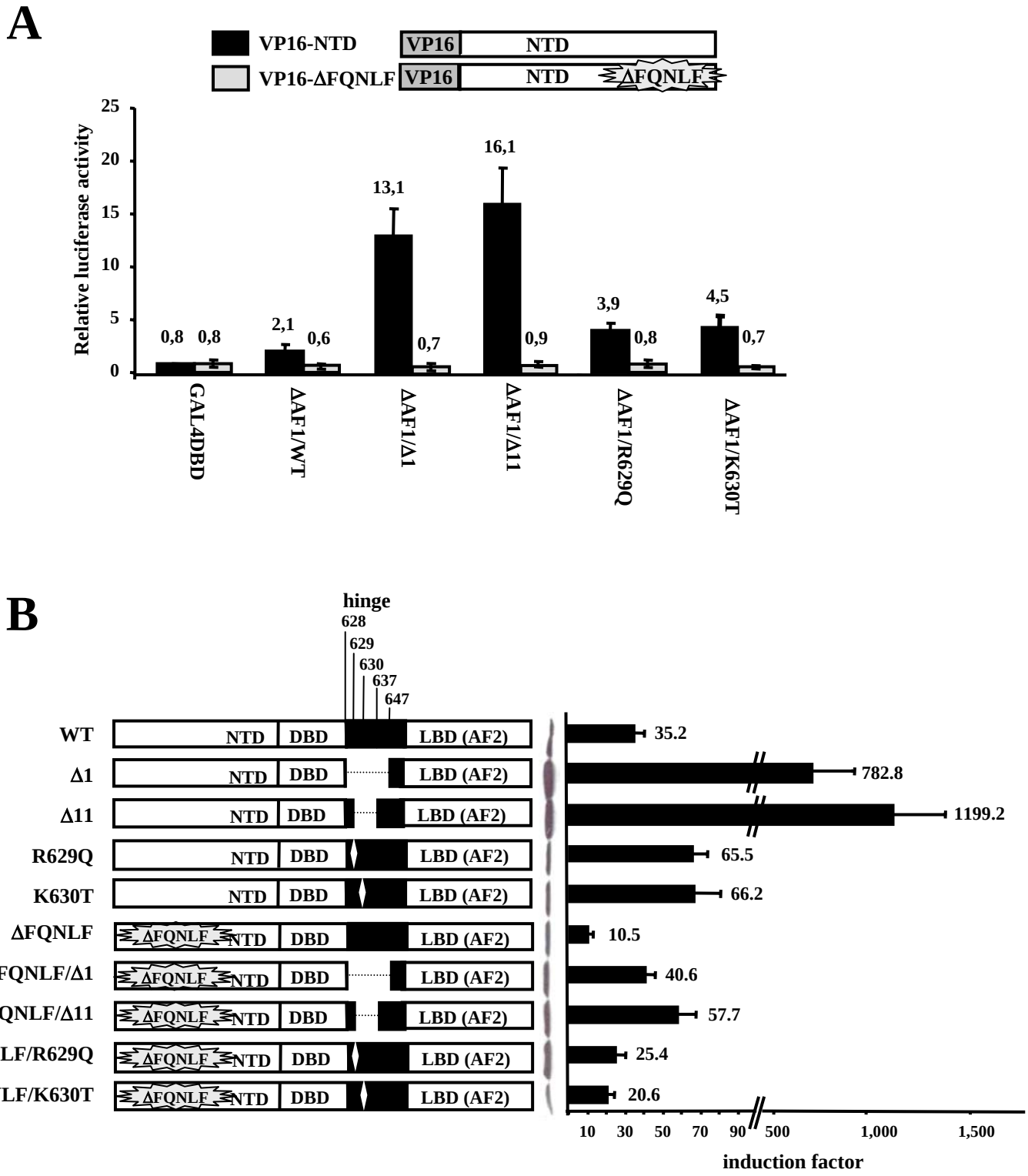
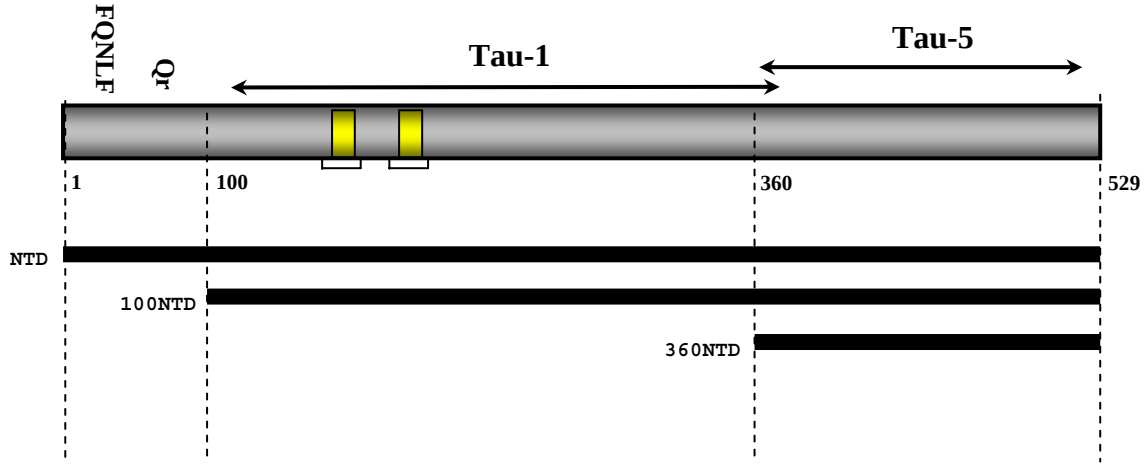

A
VP16-NTD
VP16-ΔFQNLF
VP16
NTD
ΔFQNLF
Relative luciferase activity
25
20
15
10
5
0
0,8
0,8
2,1
0,6
13,1
0,7
16,1
0,9
3,9
0,8
4,5
0,7
GAL4DBD
ΔAF1/WT
ΔAF1/Δ1
ΔAF1/Δ11
ΔAF1/R629Q
ΔAF1/K630T
B
hinge
628
629
630
637
647
WT
Δ1
Δ11
R629Q
K630T
ΔFQNLF
ΔFQNLF/Δ1
ΔFQNLF/Δ11
ΔFQNLF/R629Q
ΔFQNLF/K630T
NTD
DBD
LBD (AF2)
35.2
782.8
1199.2
65.5
66.2
10.5
40.6
57.7
25.4
20.6
10
30
50
70
90
500
1,000
1,500
induction factor

Figure 8

A NTD deletions



B CTE extensions

DBD-CTE4	DBD- TLGA
DBD-CTE12	DBD- TLGA R K L K K L G N
DBD-CTE15	DBD- TLGA R K L K K L G N LKL
DBD-CTE45	DBD- TLGA R K L K K L G N LKLQEEGEASSTTSPTEETTQKLTVSHIEGYEC

C

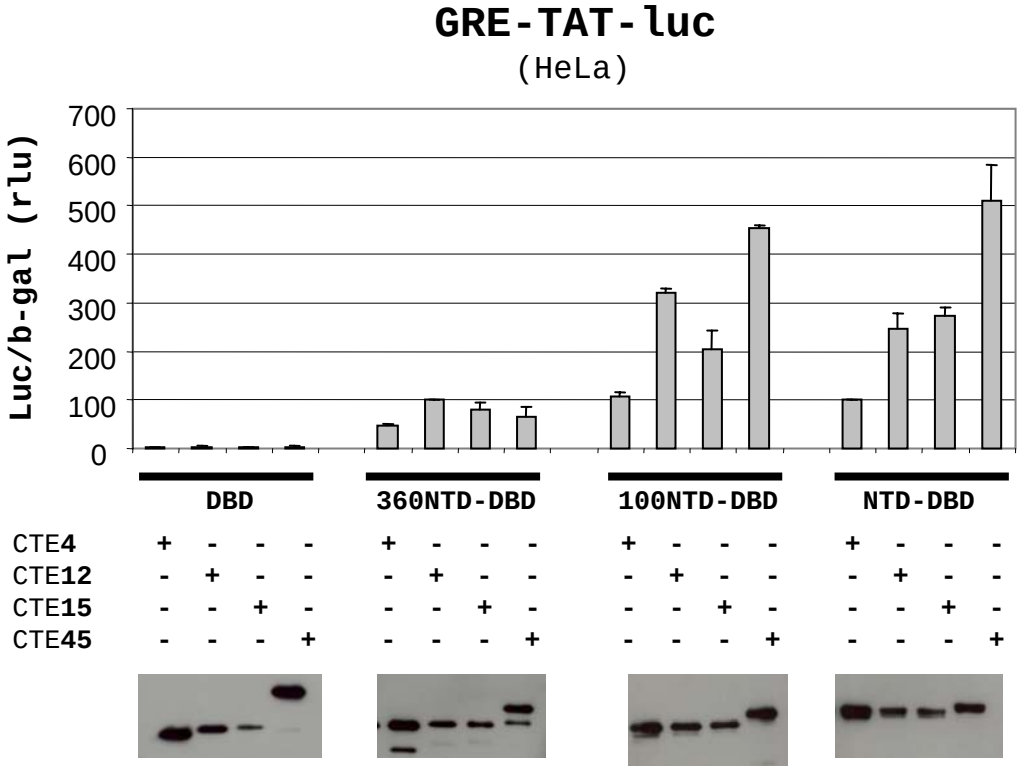


Figure 9

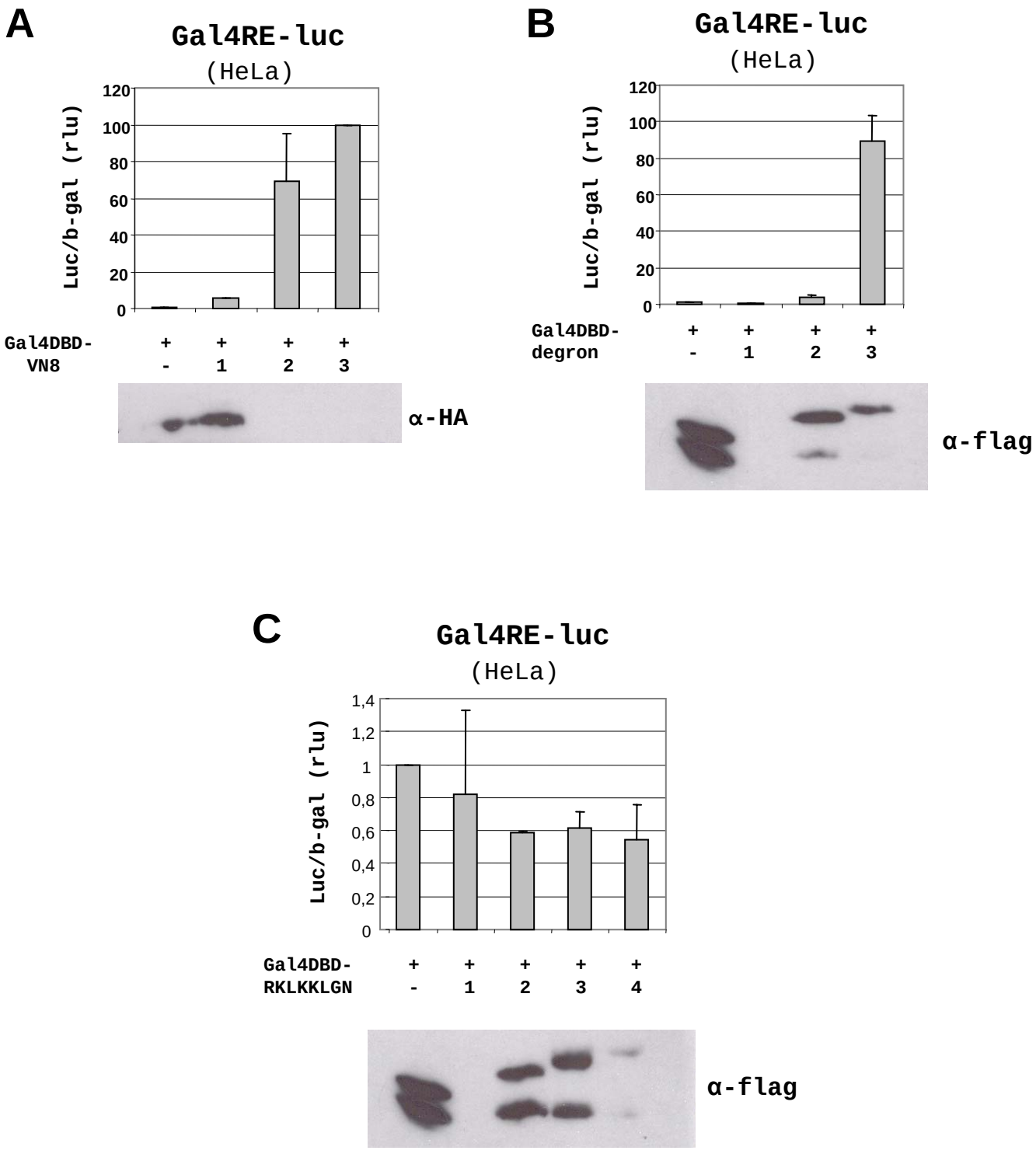
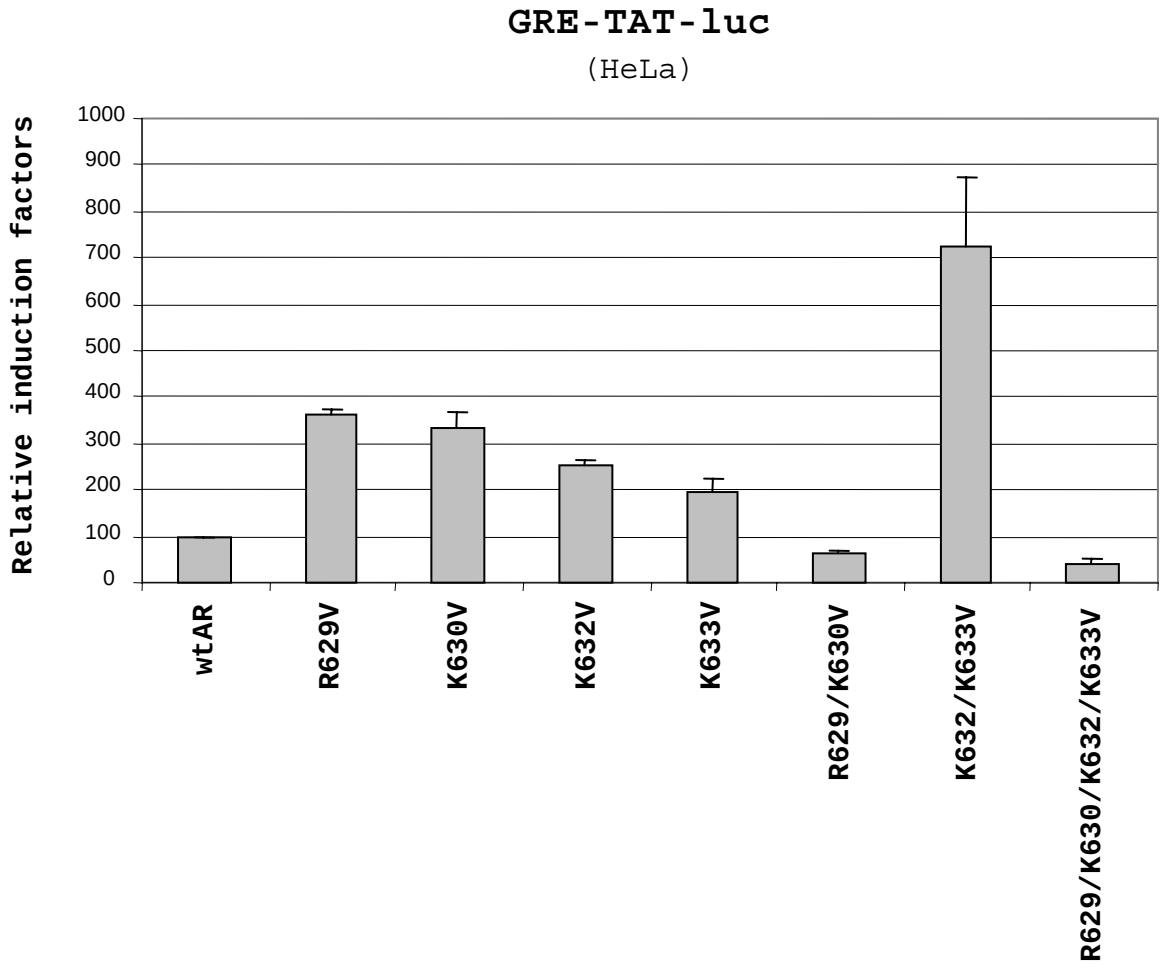

A
Gal4RE-luc
(HeLa)
Luc/b-gal (rlu)
120
100
80
60
40
20
0
Gal4DBD-
VN8
+ + + +
- 1 2 3
α-HA
B
Gal4RE-luc
(HeLa)
Luc/b-gal (rlu)
120
100
80
60
40
20
0
Gal4DBD-
degron
+ + + +
- 1 2 3
α-flag
C
Gal4RE-luc
(HeLa)
Luc/b-gal (rlu)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Gal4DBD-
RKLKKLGN
+ + + + +
- 1 2 3 4
α-flag

Figure 10

A


GRE-TAT-luc
(HeLa)
Relative induction factors
1000
900
800
700
600
500
400
300
200
100
0
wtAR
R629V
K630V
K632V
K633V
R629/K630V
K632/K633V
R629/K630/K632/K633V

B

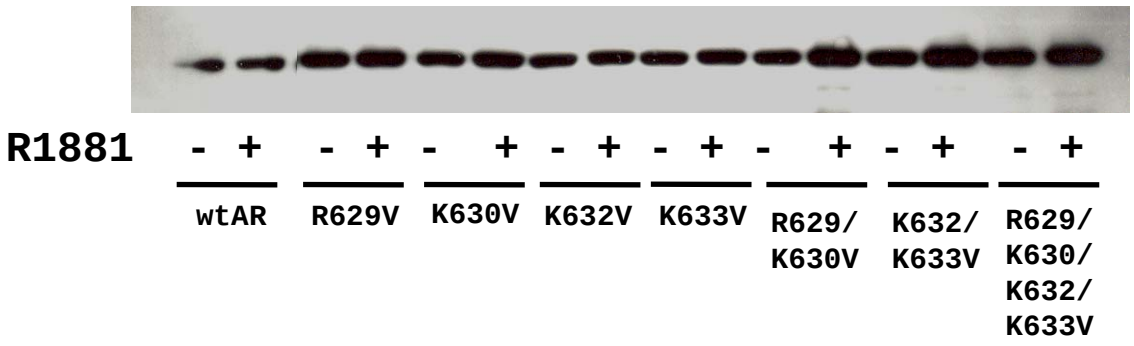

R1881
- + - + - + - + - + - + - + - +
wtAR
R629V
K630V
K632V
K633V
R629/ K630V
K632/ K633V
R629/ K630/ K632/ K633V

Figure 11

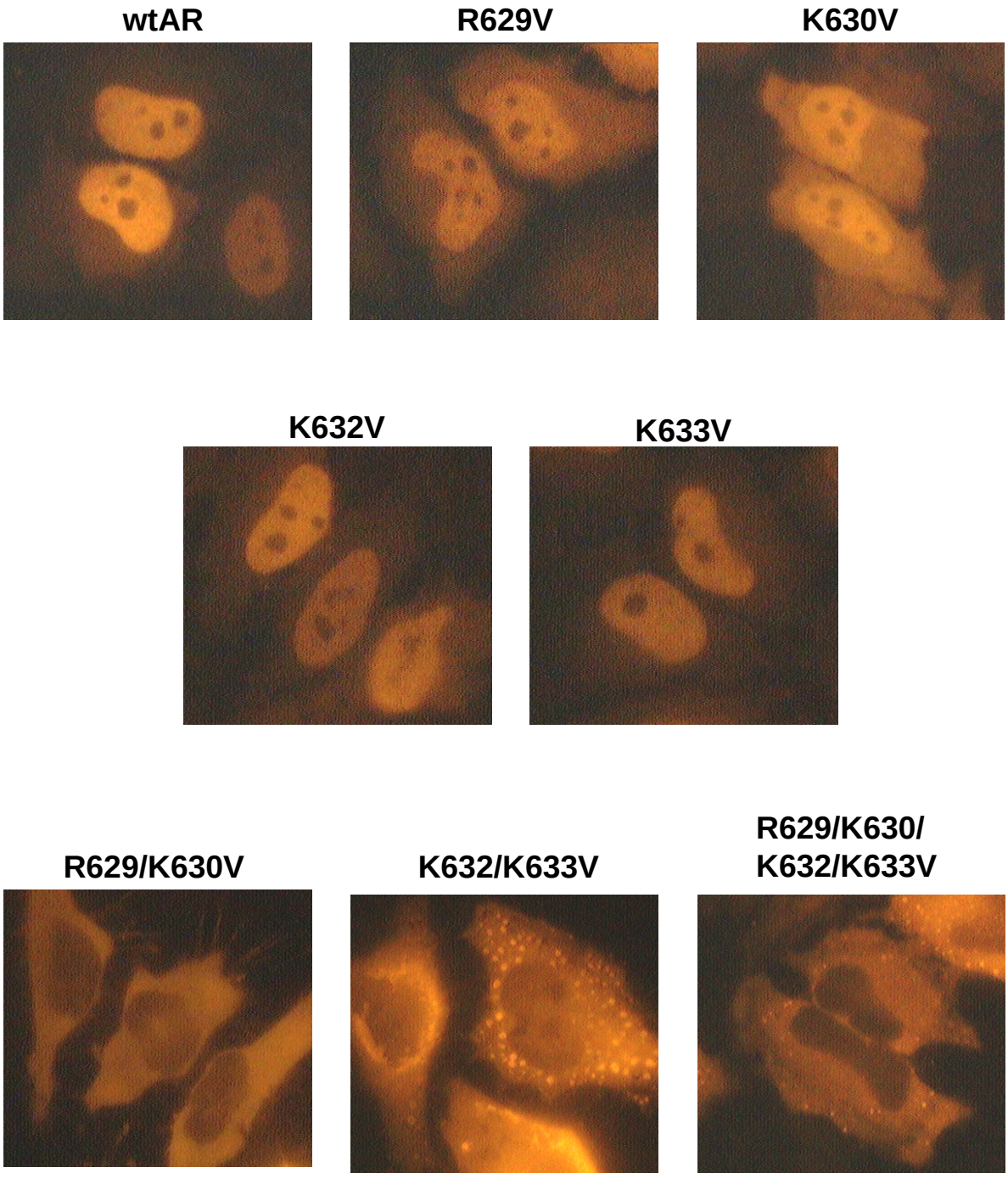
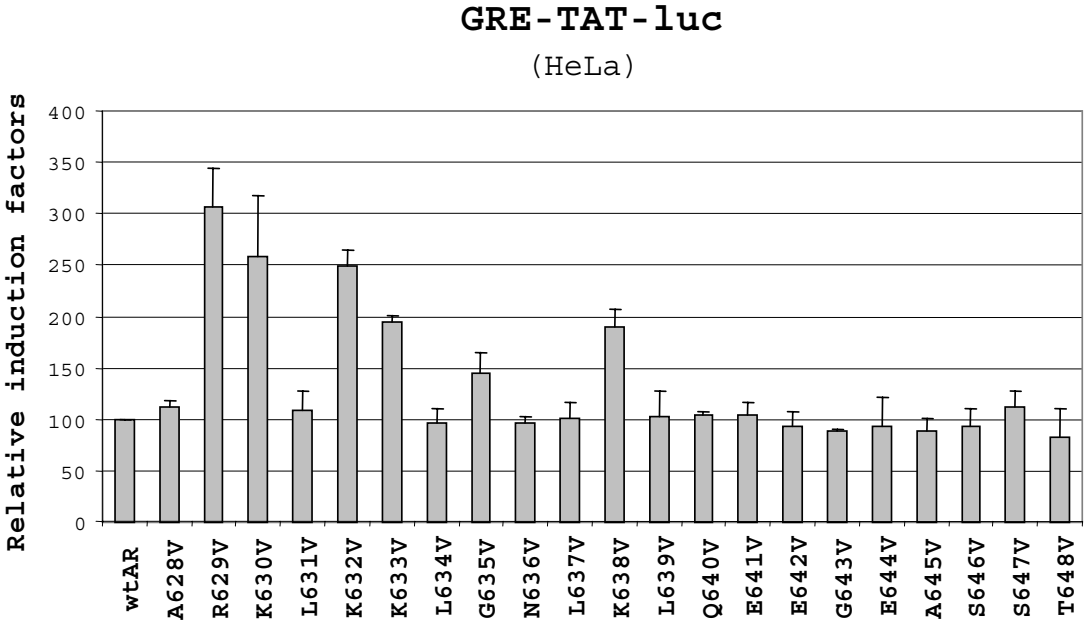

wtAR
R629V
K630V
K632V
K633V
R629/K630V
K632/K633V
R629/K630/
K632/K633V

Figure 12


GRE-TAT-luc
(HeLa)
Relative induction factors
400
350
300
250
200
150
100
50
0
wtAR
A628V
R629V
K630V
L631V
K632V
K633V
L634V
G635V
N636V
L637V
K638V
L639V
Q640V
E641V
E642V
G643V
E644V
A645V
S646V
S647V
T648V

KEY RESEARCH ACCOMPLISHMENTS

- The AR-DBD has a stronger dimerization interface. No data on the structure of the hinge could be extracted from the crystals.

- We learned that it is still possible that the AR can bind to selective AREs in a head-to-tail conformation. Surprisingly, the PR can also bind to selective AREs, but in male animals, the progesterone concentration is normally so low that this is unlikely to happen in many cases.

- Mutations in the hinge region can have very diverse effects on transactivation, protein steady state levels or nuclear localisation. Prostate cancer mutations enhance the activity of the receptor.

- Possibly, the increased androgen response can be explained by the higher expression level of some of the mutant ARs.

- Other mutations in the hinge region affect nuclear translocation, expression level and transactivation properties differentially. It is our current hypothesis that the deletion of the CTE results in a more potent AR despite impaired nuclear translocation because of a stronger N/C interaction, and because of a second effect on the AF-2.

ABBREVIATIONS

AF activation function
AR androgen receptor
ARE androgen response element
CMV cytomegalovirus
CTE carboxyterminal extension
DBD DNA-binding domain
DBD-LBD AR fragment lacking the amino-terminal domain
EGFP enhanced green fluorescent protein
ER estrogen receptor
FRET fluorescence Resonance Energy Transfer
GR glucocorticoid receptor
GRE glucocorticoid response element
LBD ligand)-binding domain
MMTV mouse mammary tumour virus
MR mineralocorticoid receptor
NTD-DBD AR fragment lacking the ligand-binding domain
PR progesterone receptor
SARG specific androgen-regulated gene
SC secretory component
SDS sodium dodecylsulfaat
Slp Sex limited protein
SRC 1 steroid receptor co-activator 1
TRITC Tetramethyl Rhodamine Iso-Thiocyanate

REPORTABLE OUTCOMES:

I. Papers in International refereed journals

Callewaert, L., Verrijdt, G., Haelens, A., Claessens, F. (2003) Differential effect of small ubiquitin-like modifyer (SUMO-ylation of the androgen receptor in the control of cooperativity on selective versus canonical response elements (2004) Mol. Endocrinol. 18, 1438-1449.

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Callewaert L., Van Tilborgh, N., Claessens, F. (2006) Interplay between two hormone-independent activation domains in the androgen receptor Cancer Res. 66, 543-553.

II. Lectures on invitation

Verrijdt, G., Peeters, A., Schauwaers, K. and Claessens, F. (2004) Mutational analysis of the dimerisation interfaces of the androgen and glucocorticoid receptor DNA-binding domains. Bioscience 2004 Meeting 'From molecules to organisms' Glasgow, UK, july 18 – 22th 2004.

Claessens F. Mécanismes moléculaires de l'action des androgènes. Journée d'Endocrinologie Sexuelle Alfred Jost, 7 februari 2004, Hôpital Cochin, Parijs

Claessens F. Molecular basis of androgen selectivity. Organon Oss Netherlands 22 September 2004.

Gewirth D. Structural basis of androgen receptor binding to selective androgen response elements. International Androgen 2004 Symposium at Berlin, 8 October 2004.

Claessens F. A crystal clear message on selective androgen response elements. International Androgen 2004 Symposium at Berlin, 8 October 2004.

Claessens F. The molecular biology of the androgen receptor: NTD, DBD, LBD and most of all 'the hinge' Lecture at the CelGen Division of Medical Faculty of the KULeuven.
- Lecture, Université Blaise Pascal, Clermont-Ferrand, Frankrijk, december 2005 : 'Le récepteur des androgènes : biologie moleculaire de Tau1 et Tau-5.'

- International Workshop 'Molecular Andrology', Giessen, Germany, October 7-9, 2005.
' Androgen receptor molecular biology'

- Short presentation at Keystone Symposium Nuclear Receptors : Steroid Sisters, Calgary, March 18-23, 2006 'The two hormone-independent activation domains of the human androgen receptor'

- Lecture at BioScience2006, Glasgow 23-27 July 2006 'Mutations in the human androgen receptor gene as a learning tool for molecular endocrinology'

III. Poster presentations at international meetings

-Callewaert, L., Christiaens, V., Schauwaers, K., Tanner, T., Verrijdt, G., Haelens, A., Bevan, C. and Claessens, F. (2003) An amino-terminal amphipathic helix in the AR has a dual function in transactivation. Poster presentation at the EMBO conference 'Biology of Nuclear Receptors' Villefranche sur Mer (Nice), France, June 4-7, 2003.

-Schauwaers, K., Verrijdt, G., Haelens, A., and Claessens, F. (2003) Importance of the second zinc finger of the androgen receptor in androgen-specific gene regulation through selective and non-selective response elements. Poster presentation at the EMBO conference 'Biology of Nuclear Receptors' Villefranche sur Mer (Nice), France, June 4-7, 2003.

-Claessens, F., Gewirth, D. Structure-function relations in the androgen receptor outside the ligand-binding domain. (2004) Nuclear receptors: Orphan brothers Steroid sisters February 28-March 4, 2004.

-Haelens, A., Tanner, T., Callewaert, L., Christiaens, V., Verrijdt, G. and Claessens, F. (2003) Functional analysis of the hinge region of the human androgen receptor. Special FEBS 2003 Meeting. Signal transduction: from membrane to gene expression, from structure to disease. Brussels Belgium, july 3 – 8th 2003.

-Christiaens, V., Callewaert, L., Haelens, A., Verrijdt, G., Bevan, C. and Claessens, F. (2003) The two coactivator interacting surfaces of the androgen receptor and their relative role in transcriptional control. Special FEBS 2003 Meeting. Signal transduction: from membrane to gene expression, from structure to disease. Brussels Belgium, july 3 – 8th 2003.

-Callewaert, L., Verrijdt, G., Haelens, A., Claessens, F. Different action mechanisms of the androgen receptor on selective versus canonical androgen response elements. Nuclear receptors. Stockholm, Zweden, 10-13 oktober 2004.

-Verrijdt, G., Peeters, A., Schauwaers, K. and Claessens, F. (2004) Mutational analysis of the dimerisation interfaces of the androgen and glucocorticoid receptor DNA-binding domains. Bioscience 2004 Meeting 'From molecules to organisms' Glasgow, UK, july 18 – 22th 2004.

-Callewaert, L., Verrijdt, G., Haelens, A., Claessens, F. Different action mechanisms of the androgen receptor on selective versus canonical androgen response elements. Androgens 2004. Symposium on androgen receptor function. Berlijn, Duitsland, 7-8 oktober 2004.

-Claessens, F., Callewaert, L., Van Tilborgh, N., Tanner, T., Verrijdt, G., Haelens, A. (2005) The androgen receptor activates transcription through two interdependent but hormone-independent activation domains EMBO conference Nuclear Receptors: from chromatine to disease sept. 29-oct1 2005 The hinge region of the human androgen receptor is a multifunctional domain

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-Claessens, F. (2006) The two hormone-independent activation domains of the human androgen receptor. Nuclear receptors: Orphan brothers and Steroid sisters March 18-23, Banff, Alberta, U.S.A.

IV. Internship reports

Master thesis in Biomedical Sciences Functionele analyse van de hinge-regio van de androgeenreceptor. Eindwerk voorgedragen tot het behalen van de graad van licenciaat in de Biomedische Wetenschappen door Kelly Gijsemans. (academiejaar 2003-2004)

Master thesis for Industrial Ingenieur Studie van de hinge-regio op de transcriptie-activatie van de humane androgeenreceptor. Ondernemingsproject voorgedragen tot het behalen van de graaad van industrieel ingenieur door Kelly Van der Sande. (academiejaar 2003-2004)

Master thesis in Pharmaceutical Sciences Annelies Peeters “De androgeen receptor: de invloed van D-box mutaties op DNA-herkenning”
Eindverhandeling ingediend tot het behalen van het Diploma van Apotheker.
Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen.academiejaar 2003-2004

Master thesis in Biomedical Sciences ‘ Moleculaire analyse van Tau5 van de androgeen receptor’ Eindwerk voorgedragen tot het behalen van de graad van licenciaat in de Biomedische Wetenschappen door Nora Van Tilborgh (academiejaar 2004-2005)

Master thesis in Pharmaceutical Sciences Lien Bockx Onderzoek van kandidaat androgen-responsieve elementen Eindverhandeling ingediend tot het behalen van het Diploma van Apotheker. Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen academiejaar 2004-2005.

V. PhD theses

PhD in Medical Sciences Leen Callewaert “Structure/function analysis of the amino-terminal domain of the androgen receptor”
Public defens and date of the degree 29 March 2004

PhD in Pharmaceutical Sciences Valerie Christiaens "Modulation of androgen receptor activity by p160 coactivators and a study of environmental contaminants"
Public defens and date of degree 23 March 2005

VI. Obtained grants

- A research grant (2006-2009) was obtained at the Flemish Fund for Scientific Research. The subject of this grant partly overlaps and continues on the here reported grant.

- A grant was obtained at the Association for International Cancer Research, a Scottish Charity. Although the subject of this grant differs from the here reported grant, similar techniques and constructs will be used.

- Leen Callewaert and Annemie Haelens obtained postdoctoral Fellowships of the Flemish Fund for Scientific Research. Leen Callewaert has done some experiments on the hinge region, but is now focussing on prostate cancer mutations and structure-function relations in the aminoterminal domain. Annemie Haelens has performed part of the experiments and supervised Tamzin Tanner and Arnold d'Alesio.

- Kris Schauwaers obtained a two-year grant of the Institute for Encouragement of Innovation through Science and Technology. Salary for the period 2003-2005 on 'Role of selective AREs in androgen-selective control of gene expression: in vivo mutagenesis of the AR gene' In which the second zinc finger and part of the CTE of the endogenous AR gene were swapped for those of the mGR. This results in mice in which the AR can only activate transcription through canonical AREs and not through selective AREs. Male transgenic mice are affected in reproductive organs and prostate.

CONCLUSIONS

1. The androgen receptor DNA binding domain co-crystallizes with a direct repeat of the 5'-TGTTCT-3' hexamer separated by three nucleotides in a head-to-head conformation. One monomer binds with high affinity to a 5'-TGTTCT-3' hexamer, while the other recognises the complementary strand in the second hexamer (5'-AGAACA-3'), in which the position of G and C are identical to those in the 5'-TGTTCT-3'-sequence. The lower affinity binding seems to be compensated for by a stronger dimerization interface in case of the AR, but not in case of the GR.

2. Mutation analyses have failed to confirm the hypothesis that the two residues that differ in the dimerization interface can explain the AR-specific recognition of direct repeat elements. We will now turn our attention back to the carboxy-terminal extension (CTE) of AR and GR.

2. There is a higher similarity between AR and PR in the CTE than between GR and AR or GR and PR. We therefore tested whether PR is also able to act through selective AREs. Although it clearly can, we still prefer to call the elements selective AREs, since the hormone concentration needed to activate the PR is higher than that normally observed in male serum. Whether this is correct will become apparent from the analyses of the transgenic ARE-reporter mice (based on a selective ARE).

3. A deletion of the hinge region results in an AR which is more potent, despite a strongly impaired DNA binding. This is due to the deletion of eight amino acids (ARKLKKLGN). This deletion mildly affects both activation functions AF1 and AF2, as well as the interaction between them.

4. Deletion of part of the hinge region has a dramatic effect on the N/C interactions which has been shown to be necessary for the activation of some reporter genes.

5. Since the eight amino acid motif overlaps with the nuclear localization signal, we have done localization studies with EGFP-fused ARs. As expected, this feature was impaired. Similar studies were done for two prostate cancer mutations in the hinge region, as well as several synthetic mutants. Conclusion: there is no strict (inverse) correlation between nuclear translocation efficiency and transactivation potential. Inactivating mutants seem to be excluded from the nucleus, activating mutants are partly nuclear/partly cytoplasmic.

6. Different residues of the hinge region are involved in transactivation, nuclear translocation and directly or indirectly in N/C interactions. The mutations described in biopsies from prostate cancer have an activating effect, which is in part explained by an increased N/C interaction.

7. In our current working model, the hinge region controls the cycling of AR at the level of chromatin or at the nuclear shuttling level. We therefore started collaborations to test these hypotheses (see further)

So What?

We hope that a better description of the functions of the AR, and the hinge region in particulate can lead to new targets for therapeutic strategies which could be used in hormone-dependent as well as hormone-refractory stages of prostate cancer.

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0888-8809/04/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 18(6):1438–1449

doi: 10.1210/me.2003-0313

Differential Effect of Small Ubiquitin-Like Modifier (SUMO)-ylation of the Androgen Receptor in the Control of Cooperativity on Selective *Versus* Canonical Response Elements

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The androgen receptor (AR) can be small ubiquitin-like modifier (SUMO)-ylated in its amino-terminal domain at lysines 385 and 511. This SUMO-ylation is responsive to several agonists, but is not induced by the pure antagonist hydroxyflutamide. We show that the main site of interaction of Ubc9, the SUMO-1 conjugating enzyme, resides in transcription activation unit 5.

Overexpression of SUMO-1 represses the AR-mediated transcription, and this effect is abolished after mutating both SUMO-1 acceptor sites. On the other hand, the mutation of lysine 385 clearly affects the cooperativity of the receptor on multiple hormone response elements. Lysine 511 is not implicated in this function. Surprisingly, these effects on cooperativity clearly depend on the nature of the response elements. When selective androgen response elements, which are organized as direct repeats of 5′-TGTTCT-3′-like sequences, were tested, the lysine 385 mutation did not increase the androgen response. Point mutations changing the direct-repeat elements into inverted-repeat elements restored the effects of the lysine 385 mutation on cooperativity. In conclusion, SUMO-ylation of the AR might have a differential function in the control of cooperativity, depending on the conformation of the AR dimer bound to DNA. (*Molecular Endocrinology* 18: 1438–1449, 2004)

THE ANDROGEN RECEPTOR (AR) is a ligand-dependent transcription factor and belongs to the family of the nuclear receptors (NRs). Like all other NRs, the AR consists of three major functional domains: an amino-terminal domain (NTD), a DNA-binding domain (DBD), and a ligand-binding domain (LBD) (1). The DBDs of the class I steroid receptors [AR, glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor] recognize similar inverted repeats of 5′-TGTTCT-3′-like core sequences, spaced by three nucleotides. These elements will be referred to as canonical androgen response elements (AREs). However, several elements have been described to be recognized by the AR, but by no other NR. This was proposed to contribute to the AR specificity of transcriptional responses (2). Such elements will be referred to as selective AREs. The three-dimensional structures of the LBDs of NRs are quite similar (3). In contrast to the other NR-LBDs, however, only a weak activation function 2 is observed in the AR-LBD (1, 4).

The AR-mediated response involves the recruitment of coactivators of which the group of the p160 or NR-interacting proteins are the best studied (5). Steroid receptor coactivator-1 (SRC-1), human and rat transcription-intermediary factor 2 and its mouse ortholog glucocorticoid receptor-interacting protein 1 (GRIP1), and receptor-associated coactivator 3 belong to this group (6–9). They interact with the NR-LBDs via highly conserved α-helical LxxLL motifs, arranged in a centrally located NR-interacting region (5, 10). For the AR, however, a glutamine-rich region of SRC-1 (Qr) is the main interaction site for the AR-NTD (11–13).

The NTD of the AR is about 530 amino acids (aa) long and contains a strong hormone-dependent transactivation unit 1, called Tau-1, residing between aa 100–370. When the LBD is deleted, this activation domain shifts more C terminally and is called the autonomous transactivation unit (Tau)-5 (aa 360–529) (14, 15). A strong amino/carboxy (N/C)-interaction is necessary for AR-mediated activation on canonical but not selective androgen response elements (16–18).

Abbreviations: aa, Amino acids; AR, Androgen receptor; ARE, androgen response element; CMV, cytomegalovirus; CPA, cyproterone acetate; DBD, DNA-binding domain; GR, glucocorticoid receptor; GRIP, glucocorticoid receptor-interacting protein; GST, glutathione-*S*-transferase; hAR, human AR; HRE, hormone response element; LBD, ligand-binding domain; MPA, medroxyprogesterone acetate; NR, nuclear receptor; NTD, amino-terminal domain; PR, progesterone receptor; N/C, amino/carboxy; OH-F, hydroxyflutamide; PIAS, protein inhibitor of activated signal transducer and activator of transcription; SDS, sodium dodecyl sulfate; SRC-1, steroid receptor coactivator-1; Tau, transcription activation unit; SUMO-1, small ubiquitin-like modifier-1; TK, thymidine kinase; rTAT, rat tyrosine aminotransferase; wtAR, wild-type AR.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

The transcriptional activity of the NRs can be controlled, or at least modulated, by posttranslational modifications such as phosphorylation and acetylation (19–23). Another posttranslational modification is ubiquitination (24, 25). Covalent attachment of at least four ubiquitin molecules targets the substrates to the proteasome where they undergo degradation. Recently, a new posttranslational modification system was discovered, which resembles, but is distinct from, the ubiquitination system. It was called SUMO-1 (small ubiquitin-like modifier-1) modification or SUMO-ylation. The lysine residue where SUMO-ylation can occur resides in a consensus motif φKxE where φ is a large hydrophobic residue, K the lysine of SUMO-1 attachment, x any amino acid, and E a glutamic acid (26–30). The conjugation pathway is mediated by three types of enzymes: an activating enzyme consisting of the Aos1/Uba2 dimer, a conjugation enzyme Ubc9, and ligation enzymes (26–30). Only one conjugating enzyme for SUMO-1 is known, but several SUMO-1 ligating enzymes have been discovered recently, *e.g.* the protein inhibitor of activated signal transducer and activator of transcription (PIAS) (31–32). Moreover, both Ubc9 and PIASxα/AR-interacting protein 3 have been demonstrated to interact with the AR (32).

A wide range of proteins are subject to SUMO-ylation, *e.g.* promyelocytic leukemia protein, inhibitor of nuclear factor κB, p53-related p73α protein, PIAS proteins, and RanGap1 (26–32). Recently, several steroid receptors have also been reported to be conjugated with SUMO-1. The glucocorticoid receptor has three major SUMO-1 attachment sites, two of which are situated in the NTD and one in the LBD (33, 34). The PR can be SUMO-ylated in the NTD, and this modification is thought to regulate its autoinhibition and transrepression (35). The AR-NTD has two SUMO-1 consensus modification sites at positions 385 and 511 (36). In this paper, we analyze the SUMO-1 conjugation of the AR and its impact on AR-mediated transcriptional activity.

RESULTS

The Two SUMO-ylation Acceptor Sites in the Human (h)AR Differ in Their Abilities to be Conjugated by SUMO-1

Poukka *et al.* (36) described AR SUMO-ylation at lysine 386 and 520. We can confirm these as the SUMO-1 targets in the AR. The numbering of the residues in this study is based on the AR cDNA sequence of the clone obtained from Brinkmann and co-workers (37).

We mutated one or both lysines of the SUMO-1 attachment sites in the hAR into arginines (K385R and K511R) and compared the SUMO-ylation efficiency to that of the wild-type (wt)AR in the presence of AR agonist methyltrienolone (R1881), pure AR antagonist hydroxyflutamide (OH-F), or partial AR antagonists medroxyprogesterone acetate (MPA) and cyproterone acetate (CPA). Clearly, both lysines are independent SUMO-1 acceptor sites, and the modification status of the AR depends on the nature of the ligand (Fig. 1).

In Fig. 1A, Flag-tagged hAR and mutant ARs were transiently expressed in COS-7 cells and coexpressed with c-*myc*-tagged SUMO-1 in the presence or absence of R1881 (10^{-8} M). When wild-type AR (wtAR) is coexpressed with SUMO-1, two major bands appear on Western blot in the absence of hormone (Fig. 1A, *upper panel*, lane 1). The fastest migrating band corresponds with the unmodified AR, whereas the slower band is dependent on coexpression with SUMO-1. As described by Poukka *et al.* (36), stimulation of the cells with R1881 enhances the SUMO-1 modification of the AR, whereby three bands appear (Fig. 1A, *upper panel*, lane 2). From immunoprecipitation with anti-Flag antibody and subsequent immunoblotting with anti-c-*myc* antibody, we can conclude that the slower migrating bands are the SUMO-ylated AR forms (Fig. 1A, *lower panel*). We assume that the middle band is explained by mono-SUMO-ylation, whereas the slowest band corresponds with di-SUMO-ylated AR. It is clear that these lysines are the only SUMO-1 conjugation targets in the wtAR because no modification is observed when both sites are mutated (K385R/K511R, lanes 7 and 8). When lysine 385 is mutated, mono-SUMO-ylation takes place only after stimulation with R1881 (lane 4 compared with lane 3). However, SUMO-ylation of the K511R construct already takes place in the absence of R1881 (lane 5) but is much more pronounced in the presence of the agonist (lane 6).

The SUMO-ylation pattern of the AR and AR mutants in the presence of 10^{-8} M OH-F is shown in Fig. 1B. The Western blots have been overexposed to detect low SUMO-ylation efficiencies. Clearly, SUMO-1 attachment to the wtAR or the K385R and K511R constructs is only very weakly enhanced by OH-F.

When the cells are treated with the partial antagonists MPA (Fig. 1C) or CPA (data not shown), SUMO-ylation of the AR constructs resembles the pattern obtained with agonist R1881.

Ubc9-Binding Sites in the hAR

Many of the proteins that can be SUMO-ylated interact with Ubc9, the SUMO-1 conjugating enzyme. The hinge region of the AR has been implicated in Ubc9 interaction (38). Therefore, we predicted that the deletion of the hinge region of the AR (ARΔH) should affect the SUMO-ylation efficiency. However, the Western blot in Fig. 2A shows that the SUMO-1 pattern for both wtAR and ARΔH are superimposable. To identify the Ubc9-interacting part of the AR, two-hybrid assays were performed in COS-7 cells (Fig. 2, B and C). Surprisingly, no interaction is observed between Ubc9 and the DBD/H/LBD under conditions in which a good interaction is observed between the NTD and the DBD/H/LBD. We therefore looked for additional Ubc9 interaction sites in the AR. Coexpression of AR-NTD fused to the DBDGal4 domain with Ubc9 fused to the VP16 activation domain clearly shows that Ubc9 binds well to the AR-NTD (Fig. 2C). There is

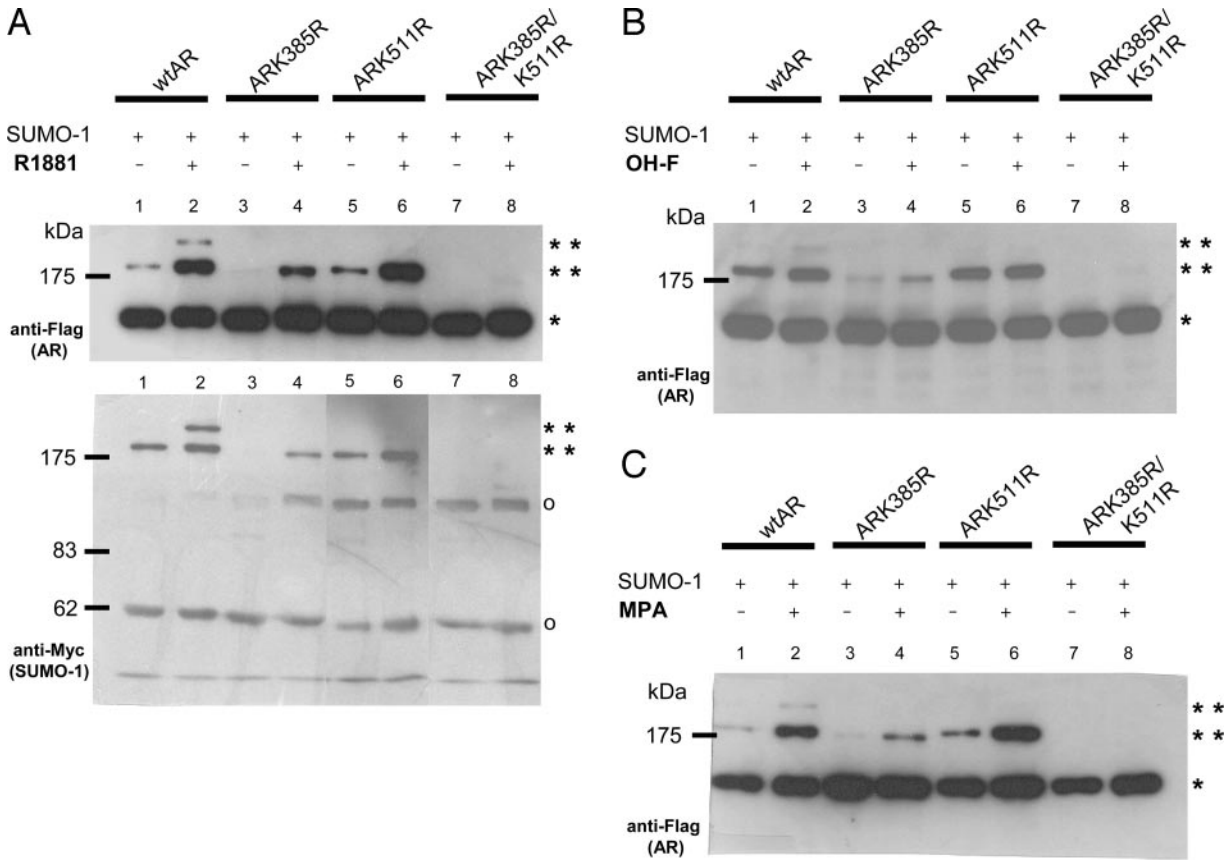

Fig. 1. Analysis of the Ligand Dependency of the Two SUMO-1 Acceptor Sites

A, The effect of the mutation of the SUMO-1 acceptor sites was analyzed in immunoprecipitation assays. COS-7 cells were transfected with Flag-tagged wtAR, ARK385R, ARK511R, or ARK385R/K511R and cotransfected with c-*myc*-tagged SUMO-1. After 24 h, cells were incubated with or without agonist R1881 (10^{-8} M) as indicated on *top*. The protein extracts (10%) were subjected to Western blotting, and AR was detected using the monoclonal M2 anti-Flag antibody (*upper panel*). AR was immunoprecipitated from 90% of the extracts with the anti-Flag antibody agarose, subjected to Western blotting, and probed with anti-c-Myc antibody to detect the SUMO-ylated AR forms (*lower panel*). The nonmodified ARs and the SUMO-ylated ARs are indicated on the *right* by an *asterisk* or a *double asterisk*, respectively. Nonspecific bands are indicated by an *open circle*. B and C, Experiments are performed as in panel A. Cells were stimulated with OH-F (10^{-8} M) (panel B) or MPA (10^{-8} M) (panel C).

already a high luciferase activity measured in the presence of the AR-NTD alone because of a strong constitutive active activation domain. To verify this interaction and to analyze the interaction of AR with Ubc9 *in vitro*, we performed glutathione-*S*-transferase (GST) pull-down experiments. Bacterially expressed GST or GST-Ubc9, immobilized on glutathione-Sepharose beads, was incubated with *in vitro* translated and [^{35}S]methionine-labeled hAR-NTD$_{1–529}$ or deletions hAR-NTD$_{\Delta 1–360}$ and hAR-NTD$_{\Delta 360–529}$ (Fig. 2D). The first deletion construct still contains both SUMO-1 consensus motifs in contrast to the latter deletion construct, which lacks both sites. In this assay the hAR-NTD bound specifically to GST-Ubc9 but not to GST alone. Other than the wtAR-NTD, only the fragment of the NTD encompassing the Tau-5 domain and the SUMO-1 sites (hAR-NTD$_{\Delta 1–360}$) showed an interaction with Ubc9, whereas the mutant AR NTD$_{\Delta 360–529}$ is not able to interact with the conjugating enzyme.

SUMO-ylation of the hAR-NTD

Following the study of SUMO-1 conjugation to the K385R and K511R constructs in context of the full size AR (Fig. 1), we have analyzed the SUMO-ylation of the separated NTD of the AR (Fig. 3). Flag-tagged hAR-NTD or its mutants (NTDK385R, NTDK511R and NTDK385R/K511R) are transiently expressed in COS-7 cells and coexpressed with either c-*myc*-tagged SUMO-1 or SUMO-1mut. SUMO-1mut lacks the two carboxy-terminal glycines so that SUMO-1 modification does not occur. The extracts were immunoprecipitated with anti-Flag antibody and subsequently immunoblotted with anti-Flag (Fig. 3, *upper panel*) or anti-c-*myc* antibody (Fig. 3, *lower panel*). After cotransfection of the AR-NTD with SUMO-1, a slower migrating band appears, indicating that the AR-NTD, when tested in isolation, is also SUMO-ylated. We can only detect mono-SUMO-ylation at

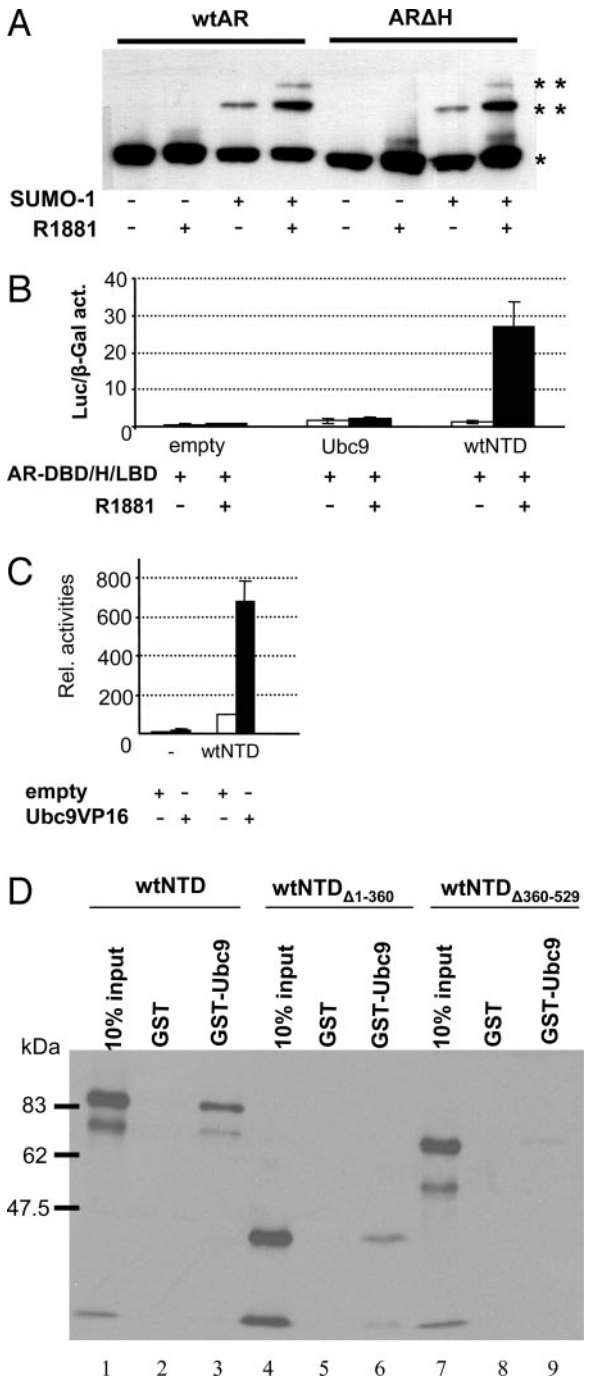

Fig. 2. Ubc9 Interaction with hAR and Effect on AR Activity

A, SUMO-ylation of wtAR and ARΔH. COS-7 cells were transfected with Flag-tagged AR or ARΔH and cotransfected with either empty vector pSG5 or with pSG5SUMO-1 and stimulated with or without hormone after 24 h. The extracts were resolved on a 6% SDS polyacrylamide gel and immunoblotted with monoclonal M2 anti-Flag antibody. The positions of nonmodified AR and SUMO-ylated AR are indicated by an *asterisk* or a *double asterisk*, respectively. B, Two-hybrid assay. PSG5AR-DBD/H/LBD (538–919aa) (50 ng/well) was coexpressed in COS-7 cells with either the empty pSNATCH-II expression vector or the same expression vector containing Ubc9 or AR-NTD (50 ng/well). Assays were performed using the 2×TAT-GRE(E1b)-Luc reporter (100 ng) and the cytomegalovirus (CMV)-β-Gal reporter (5 ng/well). *Bars* represent the luciferase/β-galactosidase values. C, Two-hybrid assay. Empty pABGal4 or pABGal4AR-NTD (50 ng/well) was coexpressed in COS-7 cells with 50 ng of empty pSNATCH-II or pSNATCHIIUbc9. Assays were performed using the $(Gal4)_5$-TATA-luciferase reporter (100 ng). Activities are depicted relative to the activity of the wtAR-NTD construct in the presence of empty vector, which was set to 100. D, GST pull-down assay. wtAR-NTD (lanes 1–3) and the deletion mutants NTD$_{\Delta 1-360}$ and NTD$_{\Delta 360-529}$ (lanes 4–6 and 7–9, respectively) were transcribed and translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with GST or GSTUbc9 beads. Elution was performed with SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. The amount of protein loaded in the input lane is equivalent to 10% of the amount of protein assayed in each binding experiment.

lysine 385 (Fig. 3, lane 6). However, when both Ubc9 and SUMO-1 are overexpressed with wtAR, we could detect di-SUMO-ylation (data not shown).

SUMO-ylation of the AR Does Not Depend on N/C Interactions

We and others demonstrated that an amphipatic helix N-terminal of the hAR-NTD, consisting of the FQNLF-motif, is necessary for N/C interaction and AR function (16–18). We investigated whether the SUMO-1 conjugation of the AR depends on the N/C interaction within the AR. Therefore, we compared the SUMO-ylation status of wtAR with that of ARΔFQNLF and ARG21E (Fig. 4), two mutants for which we know the N/C interaction is abolished (18). Immunoblotting transfected COS-7 cells with a monoclonal M2 anti-Flag antibody shows that the SUMO-1 pattern for ARΔFQNLF and ARG21E is the same as for wtAR and hence, SUMO-ylation of the AR is independent of the N/C-interaction.

SUMO-ylation Does Not Affect DNA Binding

We investigated whether SUMO-1 could affect the DNA binding of the AR. Gel retardations were performed using either wtAR or ARK385R/K511R (Fig. 5A). COS-7 cells were transfected with expression vectors for wtAR or ARK385R/K511R in the presence of pSG5SUMO-1 or pSG5SUMO-1mut. As DNA probe, we used oligonucleotides covering the rat tyrosine aminotransferase (rTAT)-GRE sequence. The band shift assays showed no decreased binding of wtAR when cotransfected with SUMO-1. Also no difference in DNA binding is observed for the mutant ARs. The SUMO-ylation status of the AR in the extracts was verified by Western blot analysis (data not shown).

We subsequently investigated whether the SUMO-ylated AR form is still able to bind the DNA (Fig. 5B). The extracts were obtained from COS-7 cells, transfected with expression vectors for non-Flag-tagged wtAR in the presence of Flag-tagged SUMO-1 or

SUMO-1mut. Western blot confirmed the presence of SUMO-ylated wtAR (data not shown). Anti-Flag antibody induced a partial supershift of the retarded rTAT-GRE probe, indicating that SUMO-ylated AR indeed binds DNA (Fig. 5B, lane 5 compared with lane 3).

SUMO-1-Effect on the Transcriptional Activity of the AR

It has already been suggested that SUMO-1 modification negatively regulates the AR transactivation capacity (36). We analyzed this by cotransfecting wtAR or its mutants (K385R, K511R, and K385R/K511R) with an expression vector for either SUMO-1 or SUMO-1mut and the reporter construct 2×rTAT-GRE(E1b)-Luc (Fig. 6A). Indeed, the transcriptional activity of wtAR or the single mutants decreases with approximately 50% when SUMO-1 is coexpressed. The repressive effect of SUMO-1 for the double mutant (ARK385R/K511R), however, is much smaller. We further investigated this using several other hormone response elements (HREs) (Fig. 6B). As selective AREs, we used *slp*-HRE2 and *sc*-ARE1.2 (Table 1). As canonical AREs, we introduced mutations in *slp*-HRE2 (*slp*-HRE2 mut-4T-A; +2A-T) and *sc*-ARE1.2 (*sc*-ARE1.2 mut-4T-A; −2T-A), leading to a loss of selectivity of these elements (39). Here too, SUMO-1 overexpression leads to a decrease in AR activity.

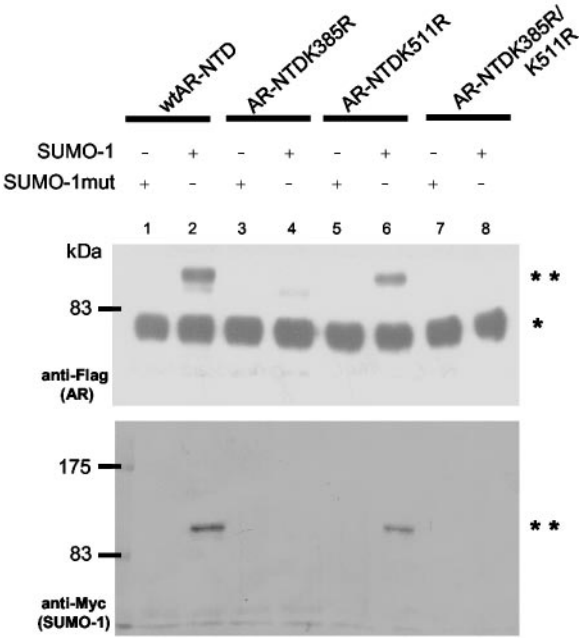

Fig. 3. SUMO-1 Conjugation of ARNTD and Its Mutants

COS-7 cells were transfected with Flag-tagged wtAR-NTD, NTDK385R, NTDK511R, or NTDK385R/K511R and cotransfected with c-*myc*-tagged SUMO-1 or SUMO-1mut. Protein extracts were immunoprecipitated with the anti-Flag antibody agarose, subjected to Western blotting, and probed with the monoclonal M2 anti-Flag antibody (*upper panel*) or anti-c-Myc antibody (*lower panel*). The nonmodified ARs and the SUMO-ylated ARs are indicated on the *right* by an *asterisk* or a *double asterisk*, respectively.

Role of the Synergy Control Motif in AR Transactivation through Canonical *vs.* Selective HREs

Initially, the SUMO-1 consensus sites in GR and AR have been described as synergy control motifs. The disruption of the SUMO-1 consensus sites was shown to lead to enhancement of the NR-dependent transcription on promotors with an increasing number of HREs (36, 40). We tested reporter constructs containing several different AREs. First, COS-7 cells were transfected with constructs expressing wtAR or mutated ARs together with a luciferase reporter construct driven by the minimal thymidine kinase (TK) promotor and containing one, two, or four copies of the rTAT-GRE. No effect of the lysine mutations on AR activity is seen when one copy of the rTAT-GRE was used (results not shown). In agreement with previous studies, a small increase in AR activity is observed on two copies of the rTAT-GRE when lysine 385 (K385R) or both lysines (K385R/K511R) were mutated (36, 40). Those effects are even more pronounced when a reporter containing four HRE copies was studied, as shown in Fig. 7. Clearly, mutating lysine 511 does not affect the androgen responses.

We tested whether the same is true for reporters containing multiple copies of the androgen-selective AREs, *slp*-HRE2 and *sc*-ARE1.2 (Fig. 8A). For four copies of *slp*-HRE2, there is a more than 10-fold in-

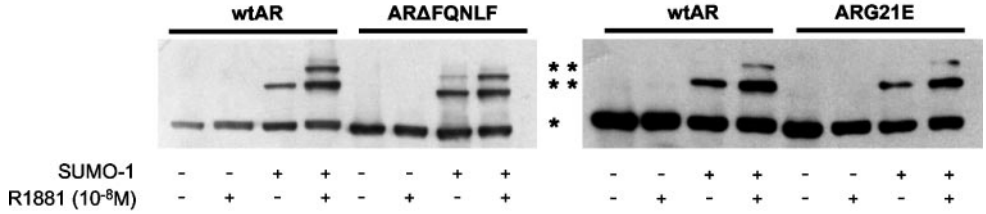

Fig. 4. SUMO-ylation of wtAR, ARΔFQNLF, and ARG21E

COS-7 cells were transfected with Flag-tagged AR, ARΔFQNLF, or ARG21E and cotransfected with either empty vector pSG5 or with pSG5SUMO-1. Cells were treated, and extracts were made and analyzed as dictated for Fig. 1 and detected with the monoclonal M2 anti-Flag antibody. The positions of nonmodified AR and SUMO-ylated AR are indicated by an *asterisk* or a *double asterisk*, respectively.

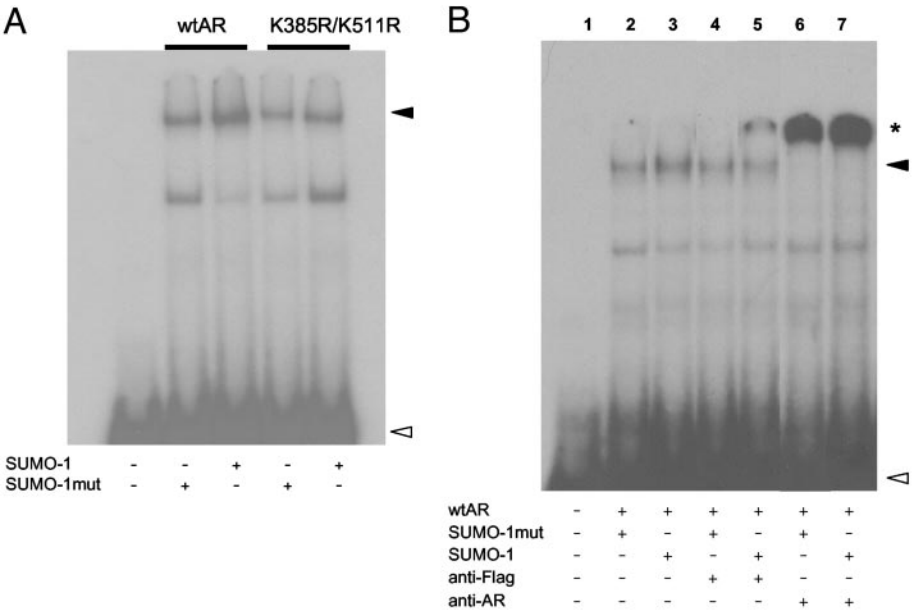

Fig. 5. DNA-Binding Analysis of SUMO-ylated AR

A, DNA-binding assay of the rTAT-GRE with wtAR or ARK385R/K511R. Labeled probe was incubated with similar amounts of COS-7 extracts containing wtAR or ARK385R/K511R indicated at the *top* in the presence of SUMO-1 or SUMO-1mut as indicated at the *bottom*. Cells were stimulated with hormone (R1881, 10^{-8} M) for 24 h. Free probe and bound probes are indicated on the *right* by an *open arrow* or a *solid arrow*, respectively. B, DNA-binding analysis and supershift assays of the SUMO-ylated AR forms. The same labeled probe was used as in Fig. 5A and was incubated with equal amount of COS-7 extracts containing wtAR (non Flag tagged) cotransfected with Flag-tagged SUMO-1 or SUMO-1mut, as indicated at the *bottom*. Cells were stimulated with hormone (R1881, 10^{-8} M) for 24 h. For the supershifts, the M2 anti-Flag antibody to detect SUMO-SUMO-1 (lanes 4 and 5) and a rabbit antiserum against hAR (lanes 6 and 7) were used. Free probe and shifted complexes are indicated on the *right* by an *open arrow* or a *solid arrow*, respectively. Supershifts are marked by an *asterisk*.

crease in wtAR activity compared with two copies (*upper panel*). Also a 5-fold higher androgen induction is observed for 4×*sc*-ARE1.2 compared with 2×*sc*-ARE1.2 (*lower panel*). However, in contrast to multiple copies of canonical AREs, mutating the SUMO-1 acceptor sites separately or together did not affect this synergistic effect.

Interestingly, as shown in Fig. 8B, the loss of specificity after mutation of the AR-specific HREs (*slp*-HRE2 mut-4T-A; +2A-T and *sc*-ARE1.2 mut-4T-A; −2T-A) indeed correlates with an increased transactivation by ARK385R and K385R/K511R in comparison with wtAR activity.

DISCUSSION

The AR has two SUMO-1 consensus sites in its amino-terminal domain, at lysine 385 and lysine 511 (36). Mutation of one of these sites prevents di-SUMO-ylation, whereas mutating both sites abolishes SUMO-1 conjugation (Fig. 1). SUMO-ylation of lysine 511 is agonist dependent. SUMO-ylation of lysine 385, although partly hormone independent, certainly is also a ligand-responsive event. Lysine 385 is the main site, but both lysines 385 and 511 can be SUMO-ylated independently from each other. In the presence of the pure antagonist OH-F, there is no SUMO-1 conjugation at lysine 511, nor enhanced SUMO-1 conjugation at lysine 385. Interestingly, we observed that the SUMO-ylation of the AR mutant T877A, seen in LNCaP cells, is comparable to that of wtAR (data not shown).

Most of the SUMO-1 protein targets interact with Ubc9, and it is likely that substrate recognition is achieved by Ubc9 (41, 42). For the AR, it has been suggested that the hinge region is implicated in Ubc9 interaction because it was isolated in double-hybrid screening with this region as a bait (38). Our assays, however, did not reveal clear interaction between Ubc9 and AR-DBD/H/LBD. Moreover, an AR in which the hinge region has been deleted is as efficiently SUMO-ylated as the wild-type receptor (Fig. 2A). In addition, SUMO-ylation assay of the AR-NTD in Fig. 3 confirms that SUMO-1 attachment at lysine 385 can happen in the absence of the hinge region. We concluded that Ubc9 must interact with the AR-NTD. It has been reported that SUMO-1 consensus motifs are not only necessary for the covalent binding of SUMO-1, but they can also serve as the site of interaction with Ubc9 (43). Indeed, from mammalian double-hybrid assays as well as GST-pull downs, we deduce that the major interaction site for Ubc9 in the AR is the Tau-5 constitutive active activation domain (Fig. 2). Remarkably, the two consensus motifs for SUMO-ylation that lie in Tau-5 are predicted to form

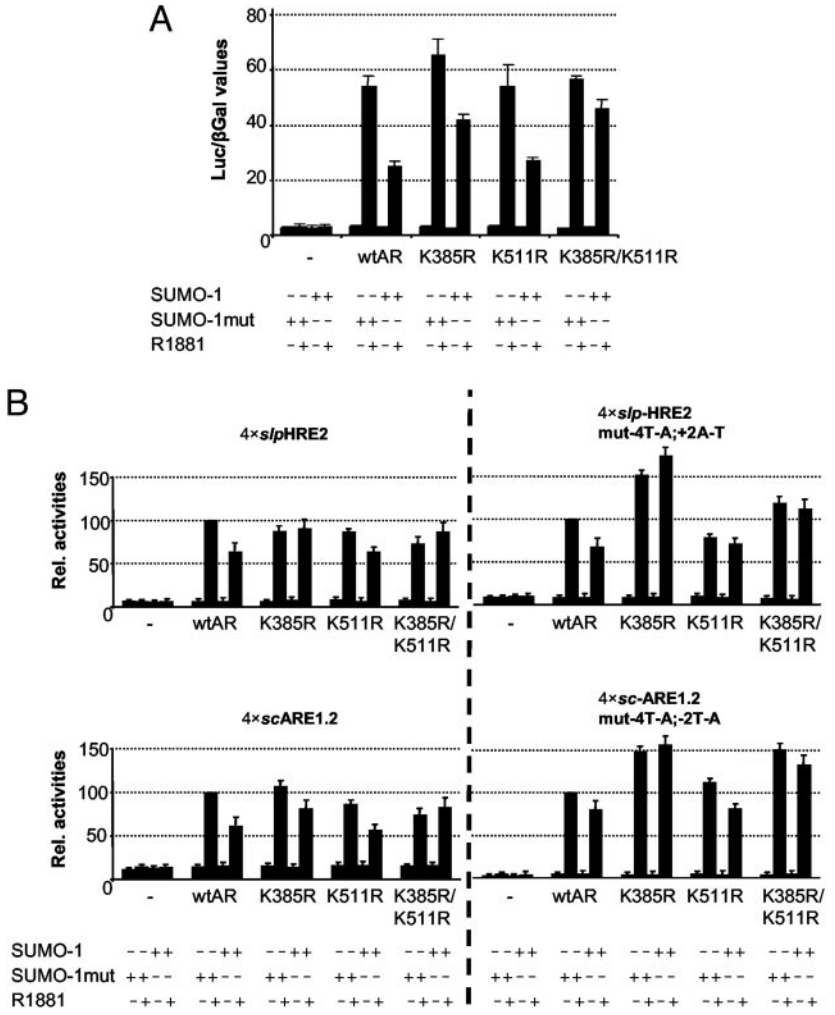

Fig. 6. SUMO-1 Affects AR Activity

A, Luciferase reporter construct (100 ng) driven by the E1b promotor containing two copies of the rTAT-GRE and 5 ng CMV-βGal reporter construct were transiently transfected into COS-7 cells. Cotransfection was performed with 20 ng of empty vector pSG5, pSG5wtAR, pSG5ARK385R, pSG5ARK511R, or pSG5ARK385R/K511R as indicated and with 20 ng pSG5SUMO-1 or pSG5SUMO-1mut. Cells were incubated for 24 h without or with hormone (R1881, 10^{-8} M). *Bars* represent the luciferase/β-galactosidase values. B, The transfection assays were performed as in Fig. 6A, using luciferase reporter constructs (100 ng) as indicated on *top*. The sequences of the AREs are given in Table 1. *Bars* represent the luciferase/β-galactosidase values measured in extracts, relative to the activity in the extracts of cells transfected with wtAR, which was set at 100.

a loop structure, which might fit in the catalytic cleft of Ubc9, as demonstrated in the RanGAP1-Ubc9 complex (44).

Ubc9 has been reported to be a potent coactivator of the AR (38), whereas in other studies Ubc9 was shown to enhance AR activity modestly on some reporter constructs but not on others (45). In our hands, cotransfection of low amounts of Ubc9 increased the AR activity only moderately on all constructs tested, but increasing amounts of Ubc9 lead to a repressive effect. Whether this correlates directly with the intrinsic transcription repressing functions of Ubc9 when fused to Gal4DBD is not clear (data not shown).

PIASxα has been shown to function as a E3-type SUMO-1 protein ligase and enhances SUMO-ylation of the AR *in vitro* (31, 32). In COS-7 cells, we could not show enhanced AR-SUMO-ylation after cotransfecting the AR with PIASxα, although a clear interaction of PIASxα with ARDBD/H/LBD is seen in a two-hybrid assay (data not shown). This may be explained by the fact that SUMO-ylation of the AR is already optimal in COS-7 cells even in the absence of overexpressed PIASxα. In functional assays, PIASxα represses or activates AR activity on the canonical TAT-GRE and *slp*-HRE2 mut-4T-A;-2A-T respectively, whereas on the selective *slp*-HRE2, no effect is observed (data not shown). We therefore agree with literature that overexpressing the SUMO-ylation ligase PIASxα can affect AR activity to different extents depending on the response elements tested (45, 46). We postulate that the PIASxα-mediated effects are indirect because we could not see a correlation with the SUMO-ylation status of the AR.

Table 1. Canonical and AR-Selective Steroid Receptor Binding Motifs

Name	Sequence	Specificity
TAT-GRE	5′-TGTACAggaTGTTCT-3′	Canonical
*slp*HRE2	5′-TGGTCAgccAGTTCT-3′	AR-selective
*slp*HRE2 mut-4T-A; +2T-A	5′-TGGACAgccTGTTCT-3′	Canonical
*sc*ARE1.2	5′-GGCTCTttcAGTTCT-3′	AR-selective
*sc*ARE1.2mut-4T-A; −2T-A	5′-GGCACAttcAGTTCT-3′	Canonical

Trivial names and sequences of the different motifs, used in this study, are indicated. Numbering of the nucleotides is relative to the central nucleotide of the three-nucleotide spacer. Mutated nucleotides are *underlined*.

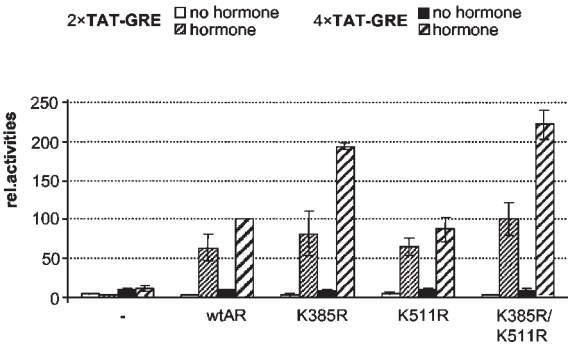

Fig. 7. Effect of Mutation of the SUMO-1 Acceptor Sites on AR Activity on 2×rTAT-GRE and 4×rTAT-GRE

TK minimal promotor-driven luciferase reporter constructs (100 ng) containing two or four copies of the response element, indicated on the *top*, were transiently transfected into COS-7 cells and cotransfected with 20 ng of empty vector, pSG5wtAR, pSG5ARK385R, pSG5ARK511R, or pSG5ARK385R/K511R as indicated. Cells were incubated for 24 h without hormone or with hormone (R1881, 10^{-8} M). *Bars* represent the luciferase/β-galactosidase values measured in extracts, relative to the activity in the extracts of cells transfected with wtAR and the luciferase reporter construct containing four copies of the response element, which was set at 100.

We then examined the effect of SUMO-ylation on the transcriptional activity of the AR by coexpression of SUMO-1. The observed effect was dependent on the presence of one or both SUMO-1 acceptor sites (Fig. 6). Similar to Poukka *et al.* (36), SUMO-1, but not SUMO-1mut, has a negative effect on AR activity.

For the PR, the repression of the transcriptional activity by SUMO-ylation of its NTD requires the liganded LBD, suggesting that the N/C interaction is involved (35). For the AR, the ligand-dependent interaction of the LBD with the NTD is strongly agonist dependent, whereas OH-F, MPA, and CPA fail to induce N/C interaction (47). The SUMO-ylation pattern of the AR after stimulation with MPA and CPA resembles that after stimulation with agonist (R1881) and not after stimulation of antagonist OH-F (Fig. 1). It is therefore not surprising that in contrast to the PR, SUMO-ylation efficiency of the AR is not influenced by N/C interaction nor by the enhanced recruitment of the p160s, induced by the G21E mutation (Fig. 4) (18). Whether the ligand responsiveness of the SUMO-ylation is indirectly a result of a conformational change of the AR-NTD, induced by a ligand-occupied LBD, or whether other modulating proteins are recruited by the latter is still an open question.

One possible explanation for the observed reduction in AR transactivation by SUMO-ylation would be that SUMO-1 modification alters its DNA-binding ability. This has been demonstrated for heat shock transcription factor 2, a transcription factor that regulates heat shock protein gene expression. SUMO-1 attachment to heat shock transcription factor 2 converts this factor to the active DNA-binding form (48). However, the DNA-binding assays in Fig. 5 show that the reduced AR activity seen when SUMO-1 is coexpressed does not reduce the DNA binding. Indeed, the amount of retarded probe is even slightly higher when the AR is SUMO-ylated.

More recently, the p160 coactivators GRIP1 and SRC-1 have been shown to be SUMO-ylated at a site in the nuclear receptor interaction domain (49, 50). The group of Kotaja *et al*. (49) has shown that mutation of the SUMO-1 attachment sites in this domain of GRIP1 is correlated with a decreased colocalization of GRIP1 with the AR, a diminished coactivator capacity, and a diminished AR-LBD/GRIP1 interaction. It seems unlikely that such SUMO-ylation of GRIP1 or SRC-1 could be responsible for the decreased AR activity seen in our experiments, because the disruption of the SUMO-1 attachment sites (K385R/K511R) in the AR leads to reversal of the negative effects. It could, however, provide an explanation for the residual repression of the AR double mutant by overexpressed SUMO-1 on all AREs tested (Fig. 6, A and B).

The possibility that AR stability, and thus the outcome of these transfection experiments, is affected by SUMO-ylation was contradicted by the immunoblotting results, which revealed no increased proteolysis of the SUMO-1-modified AR and no change in steady-state levels (Fig. 1).

The SUMO-1 consensus modification sites of the GR overlap with the synergy control motifs (33, 40). Disrupting these motifs increases the transcriptional activity of the GR on promotors containing more that one hormone response element. Also the substitutions in the SUMO-1 acceptor sites affect AR activity on reporter constructs with multiple HREs (36). In our experiments, mutation of lysine 385 and the double mutation indeed lead to an increased activity on the reporter construct containing two copies of the rTAT-

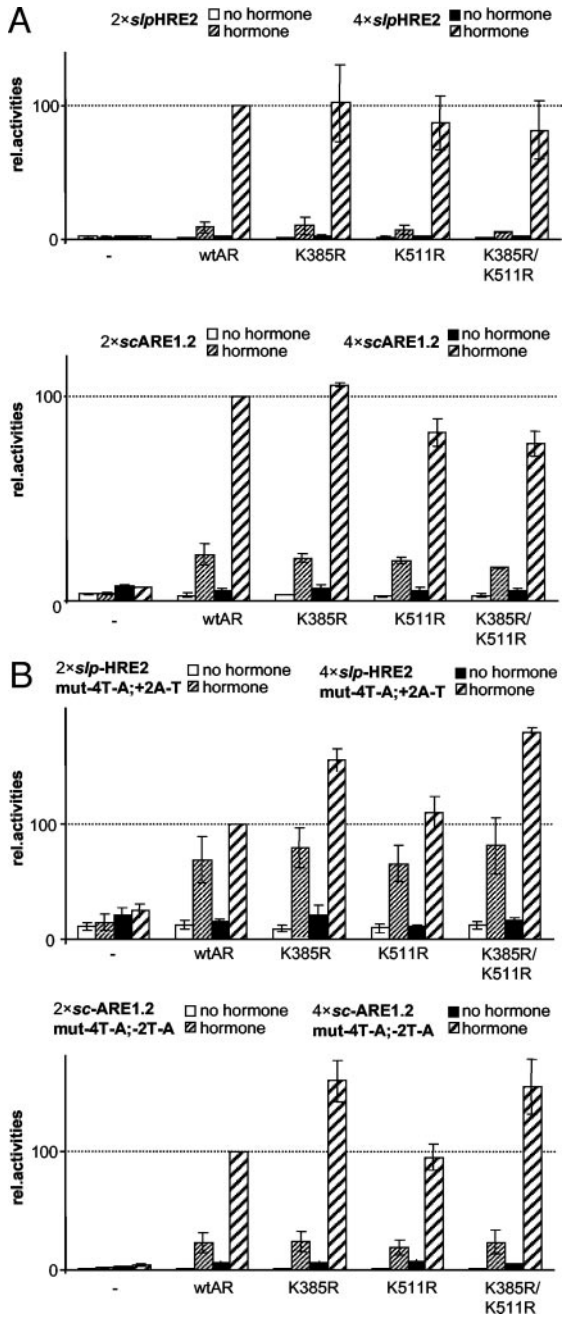

Fig. 8. Synergy Control Motif in AR Transactivation through Canonical *vs.* Selective HREs

A, Effect of mutation of the SUMO-1 acceptor sites on AR activity on selective AREs. TK minimal promotor-driven luciferase reporter constructs (100 ng) containing either two or four copies of the AR-selective response elements *slp*HRE2 or *sc*ARE1.2 (*upper* and *lower panel*, respectively), were transiently transfected into COS-7 cells and cotransfected with 20 ng empty vector, pSG5wtAR, pSG5ARK385R, pSG5ARK511R, or pSG5ARK385R/K511R as indicated. Cells were incubated for 24 h without hormone or with hormone (R1881, 10^{-8} M). The sequences of the AREs are given in Table 1. The experimental values are presented as in Fig. 7. B, Effect of mutation of the SUMO-1 acceptor sites on AR activity on mutant AREs. Luciferase reporter constructs containing four copies of the mutated *slp*-HRE2 and *sc*-ARE1.2 motifs were transiently transfected into COS-7 cells and cotransfected with 20 ng empty vector, pSG5wtAR, pSG5ARK385R, pSG5ARK511R, or pSG5ARK385R/K511R as indicated. The sequences of the AREs are given in Table 1. The experimental values are presented as in Fig. 7.

GRE, and this is even more pronounced when four copies are present (Fig. 7). It seems that lysine 385 plays an important role in this synergy control, whereas lysine 511 is not implicated in synergy.

We further characterized this synergy control in AR transactivation. From earlier experiments, we concluded that the AR transactivation mechanisms on canonical AREs differ from these on selective AREs, because the disruption of the N/C interaction or deletion of the glutamine repeat has a negative or positive effect on AR activity on canonical AREs whereas no change is seen on selective elements (18, 51). Here, we observed cooperativity of the AR on reporter constructs containing multiple selective motifs (Fig. 8A), but when the SUMO-ylation sites in the AR were mutated, no increase in transactivation was seen. It is difficult to compare the experimental data obtained after overexpression of SUMO-1 (Fig. 6), which will affect a multitude of factors, with those obtained when single SUMO-ylation sites are mutated (Fig. 8).

Clearly, lysine 385 is not acting as a synergy control element on selective AREs (Fig. 8A). However, when the selective AREs are mutated into canonical AREs, mutation of the SUMO-ylation sites again resulted in an increased synergy in the androgen response (Fig. 8B). This indicates that the underlying mechanism for cooperation and/or transcription activation and the role of SUMO-ylation in it on selective AREs might be different from that on canonical response elements.

In conclusion, we provide evidence that SUMO-ylation of lysines 385 and 511 is noncooperative, and independent from N/C interactions and the hinge region. We give evidence that Tau-5 of the AR-NTD is the main interaction site for Ubc9 rather than the AR hinge region. This is important because the hinge region is also involved in the recognition of selective AREs (2), and we report differences in the role of SUMO-ylation of lysine 385 in cooperativity on AR-selective *vs.* canonical elements. These observations must be taken into account in future experiments, *e.g*. on coactivators and corepressors. It has recently been suggested that both AR SUMO-ylation sites are involved in the binding of silencing mediator of retinoid and thyroid hormone receptor (52, 53) and SRC-1 to the AR-NTD (12, 13). The cell-specific levels of SUMO-1, Ubc9 and PIASxα, corepressors, and coactivators, as well as the nature of the response elements, will determine the extent of the androgen responses. Future experiments will also have to direct the issues of the chronological order of events and the regulatory role of SUMO-ylation at the level of AREs integrated into chromatin.

MATERIALS AND METHODS

Plasmid Constructs

The expression vectors pSG5AR (expressing full-length hAR either Flag tagged or not), pSG5ARG21E, pSG5ARΔFQNLF, pSG5AR-DBD/H/$LBD_{538–919}$ and the fusion constructs NTD with VP16 or DBDGal4 are described elsewhere (18, 54). The point mutations K385R, K511R, and K385R/K511R were made by site-directed mutagenesis using the PCR-based method. The generated fragments were cloned into the $pSG5(Flag)_3$ (expression of the full-size AR or AR-NTDs) or pABGal4 (generating AR-DBDGal4NTD fusions) vector. The expression vector for SUMO-1 and Ubc9 was a kind gift of Dr. A. Dejean (Unité de Recombinaison et Expression Génétique, Institut Pasteur, Paris, France). A c-*myc*-tagged or Flag-tagged SUMO-1 and SUMO-1mut (lacking the two C-terminal glycines) and the expression vector for flag-tagged ARΔH (hAR lacking the first 56 nucleotides of exon 4) were made by a PCR-cloning method. Similarly, Ubc9 was cloned into the GST expression vector, pGEX-5X-1 (Amersham Pharmacia Biotech, Arlington Heights, IL) and the VP16 expression vector pSNATCHII (15).

Restriction and modifying enzymes were obtained from MBI Fermentas GmbH (St. Leon-Rot, Germany). The luciferase reporter constructs containing the isolated elements TAT-GRE, *slp*-HRE2, *sc*-ARE1.2, *slp*-HRE2 mut-4T-A; +2A-T and *sc*-ARE1.2 mut-4T-A;−2A-T (Table 1) are driven by the TK minimal promotor or the E1b promotor and have been described elsewhere (Ref. 41 and references herein). The pCMV-βGal vector was obtained from Stratagene (La Jolla, CA).

Transfections

All transfections were performed in COS-7 African green monkey kidney cells, obtained from the American Type Tissue Culture Collection (ATCC, Manassas, VA). The cells were seeded in 96-well culture plates and transfected as described elsewhere (18). The amount of luciferase reporter construct was fixed at 100 ng per well, and the amount of pCMV-β-Gal was fixed at 5 ng per well. After transfection, the cells were incubated for 24 h with medium containing 5% dextran-coated charcoal and supplemented or not with 10^{-8} M of the synthetic androgen R1881 (methyltrienolone) (PerkinElmer, Boston, MA), the antagonist OH-F (a kind gift of Dr. Neri, Schering Plough, Kenilworth, NJ), or the partial antagonist MPA (Sigma-Aldrich Corp., St. Louis, MO). After 24 h, the cells were lysed in 25 μl of passive lysis buffer (Promega Corp., Madison, WI). The luciferase and β-galactosidase activities were measured in 2.5 μl of the extracts using the assay systems from Promega and Tropix (Westburg, The Netherlands), respectively. The luciferase activity in cell extracts was corrected for transfection efficiency by normalizing it according to the corresponding β-galactosidase activity. The values shown are the averages of at least three independent experiments performed in triplicate. *Error bars* indicate the SEM values.

Preparation of COS-7 Whole-Cell Extracts

COS-7 cells were plated in six-well culture plates (6-cm Petri dishes for immunoprecipitation experiments) and were transiently transfected with 0.5 μg of Flag-tagged AR or AR mutants (full-size AR or AR-NTDs) and 1.0 μg of c-*myc*-tagged SUMO-1 or SUMO-1mut. At 24 h after transfection, cells were stimulated for 24 h with or without hormone. The cells were treated and lysed as described earlier (51).

Immunoprecipitation and Western Blots

For immunoprecipitation, each protein extract was incubated with anti-Flag M2 agarose beads (10 μl) for 2 h at 4 C. After centrifugation (1 min, 5000 rpm), the supernatant was removed, and the cells were washed three times with Tris-buffered saline (10 mM Tris-HCl, pH 8.0; 150 mM NaCl). The bound proteins were released from the beads in 2 × sodium dodecyl sulfate (SDS) sample buffer. For Western blotting, equal amounts of protein extracts were separated on a 6% or a 8% SDS-PAGE gel (for full-size AR or AR-NTDs, respectively) and blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). The membranes were probed with a monoclonal M2 anti-Flag antibody (Stratagene) or with c-Myc antibody 9E10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive proteins were visualized with the chemiluminescence reagent plus (PerkinElmer) or with the chromogenic reagent for horseradish peroxidase detection (4CN reagent, PerkinElmer).

DNA-Binding Assays and Supershift Assays

Synthetic complementary oligonucleotides were hybridized, radioactively labeled, and used in band-shift assays as described previously (52). In brief, 15 μg of total cell extract were preincubated with 1 μl poly(dI:dC) (1 μg/μl), 10 μl D100 (20 mM HEPES, 5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 100 mM NaCl), 1 μl dithiothreitol (20 mM), 1 μl Triton X-100 (1%), and 1 μl of water. Subsequently, the probe is added and incubated for 20 min on ice. Bound probe was separated from the free by nondenaturing electrophoresis for 2 h at 120 V in a 5% polyacrylamide gel. To obtain supershifts, a rabbit antiserum against hAR (55) or the monoclonal M2 anti-Flag antibody was added before the probe.

Protein Expression and *in Vitro* Binding Assay

In vitro transcription and translation of full-size AR or AR fragments were performed in rabbit reticulocyte lysate in the presence of [^{35}S]methionine in a total volume of 25 μl as described by the manufacturer (Promega Corp.). The *in vitro* translated proteins were diluted to 500 μl with binding buffer (20 mM Tris, pH 7.5; 150 mM NaCl; and 0.1% Tween 20). GST or GSTUbc9 was expressed in the BL21 bacterial strain and bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech). Nonspecific protein-binding sites were blocked by incubation with 2% BSA for 1 h at 4 C, and 50 μl of each *in vitro* translated protein were incubated with the beads in 250 μl of binding buffer for 30 min at room temperature. Beads were washed three times with binding buffer. Bound proteins were eluted with 2× SDS sample buffer. After SDS-PAGE electrophoresis, the gel was fixed in 10% acetic acid-25% isopropanol for 30 min, incubated in Amplify NAMP 100 (Amersham Pharmacia Biotech) for another 30 min, and dried; finally, labeled proteins were visualized by exposure to autoradiographic film (Hyperfilm ECL, Amersham Pharmacia Biotech).

Acknowledgments

We thank R. Bollen, H. Debruyn, and K. Bosmans for their excellent technical assistance. We are indebted to Dr. A. Dejean for providing plasmids.

Received August 19, 2003. Accepted March 10, 2004.

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This work was supported in part by the "Geconcerteerde Onderzoeksactie van de Vlaamse Gemeenschap" and by grants from the "Fonds voor Wetenschappelijk Onderzoek, Vlaanderen" and by a grant of the Association for International Cancer Research. G.V. and A.H. are Holders of a Postdoctoral Fellowship of the "Fonds voor Wetenschappelijk Onderzoek-Vlaanderen."

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Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

5

DNA recognition by nuclear receptors

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Abstract

The nuclear receptors constitute a large family of ligand-inducible transcription factors. The control of many genetic pathways requires the assembly of these nuclear receptors in defined transcription-activating complexes within control regions of ligand-responsive genes. An essential step is the interaction of the receptors with specific DNA sequences, called hormone-response elements (HREs). These response elements position the receptors, and the complexes recruited by them, close to the genes of which transcription is affected. HREs are bipartite elements that are composed of two hexameric core half-site motifs. The identity of the response elements resides in three features: the nucleotide sequence of the two core motif half-sites, the number of base pairs separating them and the relative orientation of the motifs. The DNA-binding domains of nuclear receptors consist of two zinc-nucleated modules and a C-terminal extension. Residues in the first module determine the specificity of the DNA recognition, while residues in the second module are involved in dimerization. Indeed, nuclear receptors bind to their HREs as either homodimers or heterodimers. Depending on the type of receptor, the C-terminal extension plays a role in sequence recognition, dimerization, or both. The DNA-binding domain is furthermore involved in several other

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functions including nuclear localization, and interaction with transcription factors and co-activators. It is also the target of post-translational modifications. The DNA-binding domain therefore plays a central role, not only in the correct binding of the receptors to the target genes, but also in the control of other steps of the action mechanism of nuclear receptors.

Introduction

Transcription factors occupy the functional nexus between the stored information of the DNA genome and the extraction and expression of that information in the life activity of the cell. The superfamily of nuclear hormone receptors, of which there are at least 150 known members, has evolved to combine the disparate functions of signal responsiveness, DNA binding and transcriptional activation into one protein composed of functionally separate domains. DNA-binding domains (DBDs), the subject of this review, must overcome the challenge of finding a small cognate response element amid an 8×10^9 bp sea of cellular DNA. This would ordinarily be a formidable-enough challenge, but it is made even more difficult by the fact that the set of hormone-response elements (HREs) is itself quite limited in both structure and sequence. Moreover, a distinguishing feature of nuclear receptor DBDs is that they are highly conserved, even among the most distant relatives within the family [1–4]. A long-standing question is, therefore, how site selection is achieved, given that nuclear receptors employ a highly conserved DBD and target one of only two types of hexameric half-site sequence. In this chapter, we will discuss in more detail the current status and specifics of the HREs and of the DNA recognition by nuclear receptors.

Figure 1. Consensus binding sequences for the different classes of nuclear receptors

(**A**) The nuclear receptor superfamily has been divided into four classes depending on the type of interaction with the HREs. The DNA-binding sites for nuclear receptors exist as one or two copies of hexamer sequences, which resemble either 5′-AGAACA-3′ for the class I receptors or 5′-AGGTCA-3′ for the other receptors. Steroid receptors only bind inverted repeats with a three-nucleotide spacer (IR3), except for the androgen receptor (AR) which also binds DR3 [19–22]. DRs of the 5′-AGGTCA-3′ core are recognized by heterodimers of 9-*cis* retinoic acid receptor (RXR) with other nuclear receptors, except for those receptors indicated with an asterisk which can homodimerize on the cognate response element. The number of spacing nucleotides restricts the species of receptor dimers that can activate through these HREs; this was called the 1 to 5 rule [4]. Monomer-binding sites for class IV receptors are extended along their 5′ side as indicated by the nucleotides given in lower case. It should be noted that this is not a complete listing. CAR, constitutively active receptor; ERR, ER-related receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; ER, oestrogen receptor; PPAR, peroxisome-proliferator-activated receptor; COUP, chicken ovalbumin upstream promotor receptor; RAR, all-*trans* retinoic acid receptor; VDR, vitamin D receptor; T3R, thyroid receptor; PXR, pregnane X receptor; LXR, liver X receptor; NGFI-B, nerve growth factor-inducible factor I-B. (**B**) Illustration of the hypothesis of the conformational changes taking place during DNA-dependent dimerization of the DBDs. As an example, two DBDs of a class I receptor is shown (light blue boxes), one of which first binds to the most conserved hexamer with high affinity. The subsequent conformational change in the DBD–DNA complex (step 1, dark blue) results in a co-operative recruitment of the dimeric partner (step 2) to a downstream hexamer. The conformational change, however, does not allow co-operative binding to an upstream-situated hexamer [20,21]. ☞

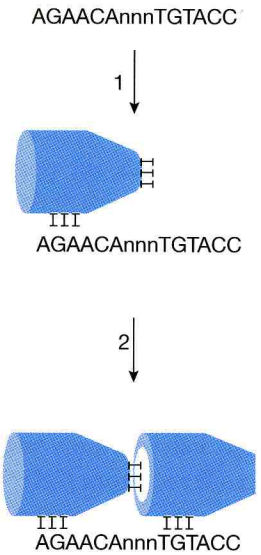
The HREs

A comparison of consensus HRE sequences for the steroid receptors (Figure 1A) reveals that they typically recognize palindromic inverted repeats of two hexameric core sequences, separated by three nucleotides (IR3). Early experiments showed that the androgen receptor (AR), glucocorticoid receptor (GR), mineralocorticoid receptor and progesterone receptor (PR), also called the class I receptors, all recognize a 5′-AGAACA-3′ core, while the oestrogen receptors (ERs), also called the class II receptors, recognize a 5′-AGGTCA-3′ core [2,5]. The class I receptor core sequence has proven to be the exception in

(A)

Inverted repeats		
AR, GR, MR, PR	**AGAACAnnnTGTACC**	Class I receptors
AR to DR3	**AGAACAnnnAGAACA**	
ER, ERR	**AGGTCAnnnTGACCT**	Class II receptors
Direct repeats		
RXR*, PPAR, COUP, RAR	**AGGTCAn1AGGTCA**	
PPAR, Rev-erb*	**AGGTCA n2 AGGTCA**	Class III receptors
VDR*, PXR	**AGGTCA n3 AGGTCA**	
T3R, LXR, CAR	**AGGTCA n4 AGGTCA**	
RAR, NGFI-B	**AGGTCA n5 AGGTCA**	
Monomeric sites		
NGFI-B	**aaaAGGTCA**	Class IV receptors
Rev-erb	**actAGGTCA**	

(B)



the nuclear receptor superfamily, as all other nuclear receptors bind preferably to the 5′-AGGTCA-3′ sequence (Figure 1A).

With only two consensus half-site sequences and over 150 different hormone receptors, additional diversity must be generated topologically, and this is achieved largely by varying the arrangement of the half-sites relative to one another. Inverted, everted and direct repeats (DRs) all restrict the dimeric receptor species that can bind. Among the best-characterized non-palindromic arrangements are the DRs of 5′-AGGTCA-3′, which are targets for the nuclear receptors that form heterodimers with RXR (9-*cis* retinoic acid receptor) [3,4]. The repeats vary in the length of the spacer that separates the two hexameric half-sites, and in this way, DRs 1, 2, 3, 4 and 5 are each recognized by specific receptor dimers. This was named the 1 to 5 rule (Figure 1A).

The spacing between the cores affects the position of the two bound nuclear receptor monomers since the addition of 1 bp results in a relative rotation of approx. 35° and a 3.4 Å (1Å = 0.1 nm) increase in their separation. This means that the dimerization surfaces of the nuclear receptors on different DR targets must be very different. This contrasts with the class I receptors, which all bind to IR3 repeats of the 5′-AGAACA-3′ core element. Since the spacing of these cores is always 3 bp, the dimerization interface for these receptors is likely to be unchanged from one steroid receptor to another (Figure 1A).

Although there are only two consensus core sequences for HREs, the two halves of the bipartite response element are not equal. Indeed, early analyses comparing all natural steroid-response elements revealed that one half-site is on average more like the consensus sequence while the other can diverge from this consensus considerably [2]. Together with the crystal structure of the GR DBD complexed with DNA [6], a mechanism of receptor binding was proposed wherein the consensus half-site is recognized with high affinity first. The binding of the first DBD results in a conformational change in the protein that supports the formation of the proper dimerization interface, thereby enabling a second monomer to bind co-operatively to the second, less conserved, half-site element (Figure 1B). Remarkably, the DBD dimerization interface was found to be of such strength that when challenged with an IR4 response element instead of the preferred IR3, the protein–protein interface was preserved, even at the expense of forcing the second DBD to bind to a non-cognate half-site. This suggested a model whereby the set of two half-sites that together allows the lowest energy DBD dimerization interface has the highest overall affinity, even though the half-site elements individually may not have the highest affinity. Thus the second half site, although it is far less conserved, can modulate the affinity of the receptor for the HRE. For example, while some mutations in the consensus half-site abolish binding completely, the same mutations in the second half-site only moderately increase or decrease the binding affinities of steroid receptors [7,8]. This model, that distortions of the DBD dimer interface are energetically more costly than sub-optimal DBD–half-site interactions, also explains why the consensus high-

affinity binding site (5′-GGTACANNNTGTTCT-3′) for GR, PR and AR is not a perfect inverted repeat [7,9]. Support for this model also comes from several studies that have shown that the nucleotide identity in the spacer of steroid-response elements, which can affect the precise stereochemical relationship of the two half-site elements, is not neutral to DNA binding by the DBDs and to transactivation. Indeed, specific preferences in PCR-mediated selections of receptor-binding elements as well as in natural elements correlate with increased steroid responsiveness in transfection experiments (e.g. [7,10,11]).

The DBDs

When the cDNAs encoding the ER and GR were first compared, the DBDs were identified on the basis of their sequence conservation and high proportion of basic residues. The DBD is centrally located within the steroid receptors and contains eight cysteine residues whose conserved spacing immediately suggested that this domain would bind zinc ions. Indeed, the minimum DBD consists of approx. 63 amino acids and contains two zinc-binding modules, with each zinc ion in a tetrahedral arrangement co-ordinated by four cysteine residues [1]. The DBD contains several conserved hydrophobic and aromatic residues that form a compact, hydrophobic core [9]. Together with the two Cys_4-Zn clusters, the hydrophobic core stabilizes the globular structure of the DBD. The combination of a hydrophobic core and the co-ordinated zinc ion distinguishes the nuclear hormone receptors from the classical zinc finger structures.

The two zinc-nucleated modules are encoded by separate exons and have different functions. Module-swapping experiments between different steroid receptors showed that the first zinc-binding module is involved in sequence specificity, i.e. the discrimination between 5′-AGAACA-3′ and 5′-AGGTCA-3′ (e.g. [12]). Within this first module, mutation analysis revealed a proximal box or P-box, which contains the residues necessary for sequence discrimination (Figure 2A). In contrast, the second module does not confer sequence specificity but instead contains several residues which form the distal box or D-box segment involved in a DNA-dependent dimerization of the class I and class II DBDs (Figure 2B) [9].

Discrimination of the core hexamers

Luisi et al. [6] solved the structure of the GR DBD in complex with DNA. When bound to DNA, the DBD makes extensive contacts with the sugar-phosphate backbone of both strands such that the recognition helix rests in the major groove roughly perpendicular to the helical axis. In this orientation, the P-box residues are in position to make either direct or water-mediated contacts with the major groove faces of the base pairs. For the GR, and by inference all class I receptors, this involves contacts between the residues KVXXXR and

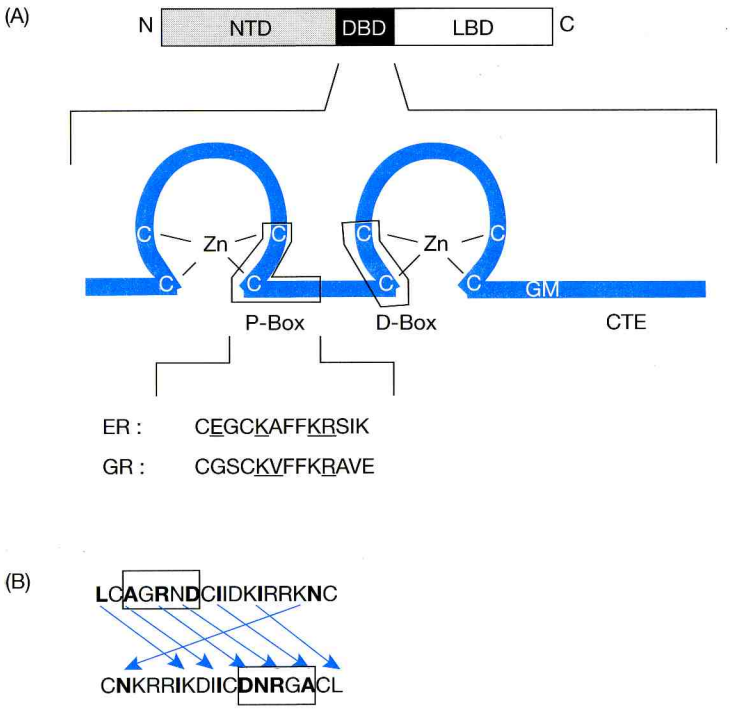


Figure 2. (A) Two types of P-box sequence and (B) GR dimerization interface
(**A**) The DBDs of nuclear receptors are located in the centre of the protein. Two zinc-coordinated modules are drawn as zinc fingers, and the locations of the P- and D-boxes are indicated. The DNA-binding specificity by nuclear receptors is dictated by a DNA-recognition helix which inserts in the major groove of the DNA helix. For the class I receptors (represented by the GR) on the one hand, and the class II, III and IV receptors (represented by the ER) on the other hand, the DNA-recognition helices differ in sequence as indicated. The residues making contact with the DNA or contributing to the sequence selectivity are underlined (based on [6,13]). NTD, N-terminal domain; LBD, ligand-binding domain. (**B**) The peptide fragments that encompass the D-box (in boxes) are given and the different contacts between the residues (in bold) of two dimerizing GR DBDs are indicated by arrows (based on [6]). The lower D-box sequence is reversed to represent the conformational alignment in the monomers. Dimerization of the ER DBDs involves very similar complementarity of shape of the corresponding region [13].

the three bases underlined in the upper (5′-AGAACA-3′) and lower strand (5′-TGTTCT-3′) of the half-site (Figure 3A). The ER has a different set of P-box amino acids on its recognition helix, and in this case the structure of the ER DBD–DNA complex showed that contacts are made between residues EXXKXXXKR and the bases underlined in the upper (5′-AGGTCA-3′) and lower strand (5′-TGACCT-3′; Figure 3B) [13].

Discrimination between the 5′-AGAACA-3′ class I element and the 5′-AGGTCA-3′ ER-/nuclear receptor-response element by the DBDs is mainly due to differences in the helical structure of the response elements, which leads to the inclusion of additional water molecules between the DNA and the protein when the DBD is bound to the incorrect core element. This destabilizes

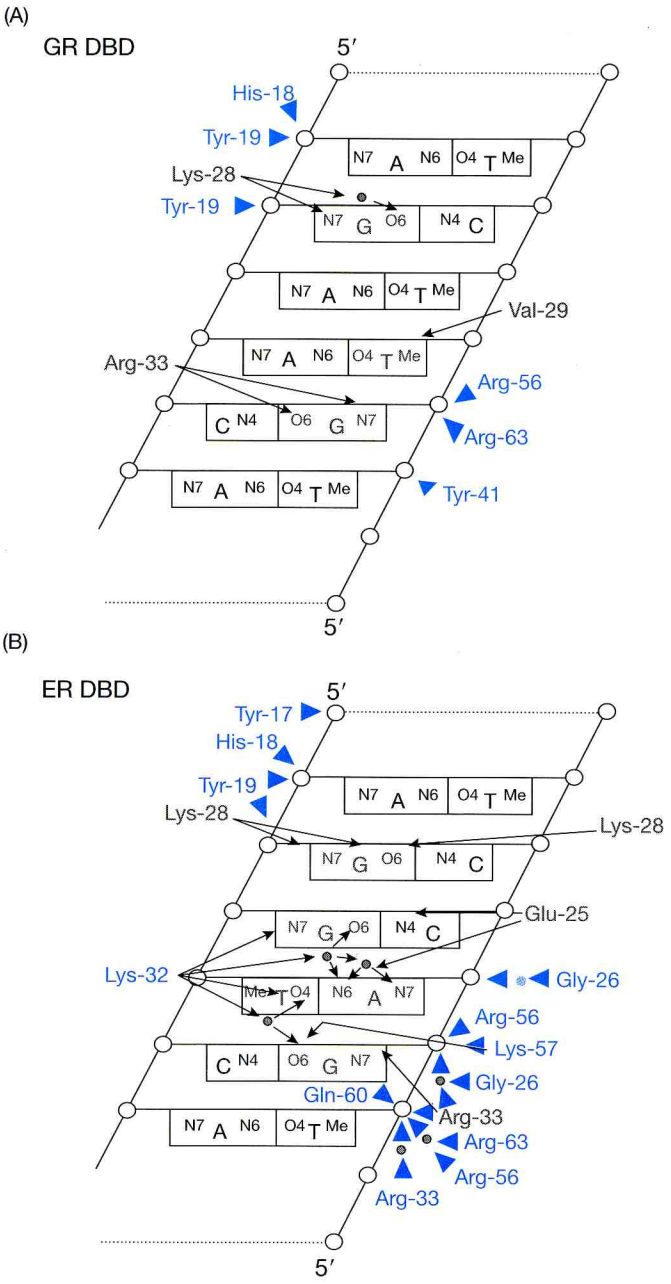


Figure 3. Schematic representation of the protein–DNA contacts between the DNA-recognition helices of the DBDs of GR (A) versus ER (B) to 5′-AGAACA-3′ and 5′-AGGTCA-3′ respectively

The major groove of the hexamer is projected and the interactions between receptor residues and the DNA are indicated by arrows. Phosphate-contacting residues are given in blue, base-contacting residues are given in grey. Small filled circles represent water molecules. Several amino acid side chains can make multiple contacts with DNA (e.g. Arg-33 in the ER).

the interaction due to the increased entropic burden [14]. An additional increase in the discriminative power of the DBDs has been suggested to involve negative interactions of P-box residues, which prevent binding to non-cognate response elements (reviewed in [9]).

Dimerization controls specificity

An important feature of many nuclear receptor DBDs is their ability to dimerize in the presence of their cognate DNA sites. In the absence of DNA, protein–protein interactions between DBDs do not occur because the potential dimerization surfaces are small. DNA-dependent dimerization thus adds to the specificity of sequence recognition on the one hand, and to the diversity of sequence elements which can be recognized on the other. For the steroid receptors, the separation of the hexamer cores by three nucleotides is a prerequisite for high-affinity binding (e.g. [7,10]). By forming a co-operative dimer, the receptors measure both the spacing and the helical repeat of the response element, thus greatly increasing the specificity of the interaction. For the class III receptors, the possibility of heterodimerization with RXR expands the diversity of the recognition sequences (Figure 1A) [15].

So far, we have mainly discussed the binding of nuclear receptor DBDs to IRs. However, many nuclear receptors recognize DRs either as homodimers or, more commonly, as heterodimers with RXR. The mechanism of spacer discrimination on DR response elements was revealed by the structures of several nuclear receptor DBDs bound to cognate DR targets [15–17]. This series of structures highlighted the role of the C-terminal extension (CTE) to the core DBD. The CTEs of nuclear receptor DBDs bound to DR-response elements are arranged such that the CTE of the downstream partner is positioned to make specifying contacts with the upstream protein. By contrast, the CTEs of the steroid receptors (GR, ER) bound to palindromic targets are on opposing faces of the DBD dimer and are not positioned to make cross-dimer interactions [6,13].

CTEs vary widely in sequence between receptors, and this is reflected in considerable structural variation. In the case of vitamin D receptor (VDR) and thyroid hormone receptor (TR) [15–17], the CTE forms a 24-residue α-helix that extends nearly to the start of the ligand-binding domain. The seven residues of the CTE between the DBD core and the α-helix make protein–protein contacts with the second zinc-coordinating module of the RXR partner, thus forming part of the dimerization interface. For the TR–RXR heterodimer, the TR CTE is also involved in contacts with the minor groove of the spacer sequence, thus explaining the sequence preferences of this heterodimer in this part of the HRE. The long α-helix serves as a steric 'ruler' that restricts binding to correctly spaced core elements by making van der Waals clashes with the upstream partner on incorrectly spaced targets. The CTEs of some other nuclear receptors do not employ a rigid secondary structural element for spacer

selectivity and dimerization. Instead, as in the case of orphan receptors of class IV (Figure 1A) that bind DNA as monomers, they use specific CTE–DNA contacts to enlarge their recognition sequence, enabling further discrimination on the basis of nucleotide identity immediately upstream of the core hexamer [15].

While the role of the CTE is clear for the class III and class IV receptors, its role in steroid receptor DNA binding is less well understood. The CTEs in the structures of the GR DBD and ER DBD were not ordered [6,13], even though deletion studies implicated the CTE in DNA binding, if not DNA recognition [18]. As discussed next, the AR might be an exception to the rules on DNA binding by the steroid receptors as inferred from GR and ER DBD structural data.

Exceptions to the rules

It has been known for some time that steroid receptors bind with high affinity to IR3s, and most steroid response elements fall into this category. Not surprisingly, DNA targets containing IR3s of the 5′-AGAACA-3′ core confer simultaneous androgen, glucocorticoid, mineralocorticoid and progesterone responsiveness to heterologous promoters [5,7–10]. However, some elements characterized in androgen target genes are exclusively activated by the AR, a puzzling result given that all formerly known androgen-response elements were IR3s, and that these IR3 elements were recognized equally well by all steroid receptors [7,19,20]. Swapping experiments were subsequently able to show that the second zinc-binding module and the CTE are involved in the binding to these AR-specific elements [21]. Because these parts of the DBD are not well positioned to make specifying contacts with the DNA bases, this suggested that an alternative dimerization mode, rather than alternative sequence specificity, was the basis for this AR-selective phenomenon. Indeed, upon careful examination of the selective androgen-response elements, it was noted that they might be DR3s of the 5′-AGAACA-3′ core element, rather than IRs [22]. The surprising implication of this analysis is that the AR DBD may have two dimerization surfaces, allowing it to bind to IR3s as a classical steroid receptor, or, alternatively, to DR3s in a mode that resembles a nuclear receptor, VDR (Figure 4) [17].

For the other steroid receptors, *in vitro* assays have demonstrated they do not bind to DR elements [8,11]. Steroid receptors have evolved from a predecessor in common with the other nuclear receptors. This predecessor probably bound DNA as a monomer and subsequently evolved into nuclear receptors, which gained the ability to bind dimerically to DRs. We hypothesize that while the other steroid receptors have lost their ability to bind DRs, the AR seems to have retained this characteristic. Therefore, although many other mechanisms are involved in the specificity of the *in vivo* responses of steroids,

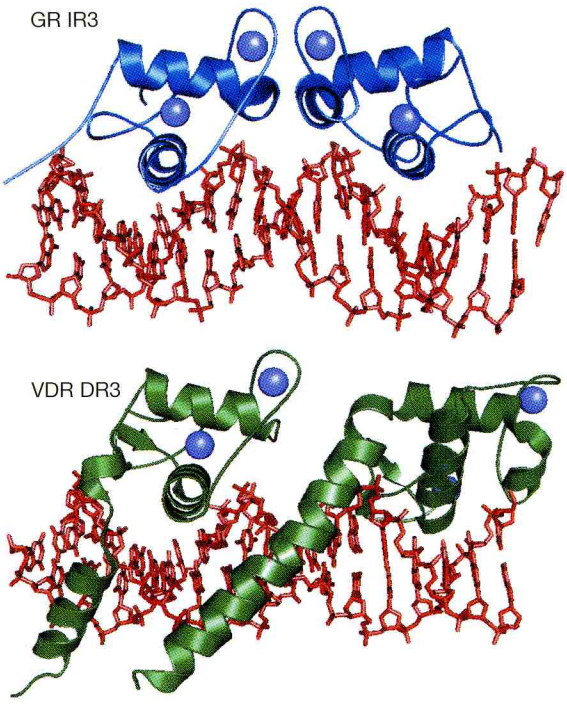


Figure 4. Structure of the GR DBD dimer (blue) binding to a three nucleotide spaced IR (top panel) clearly shows the head-to-head dimerization and the α-helix entering the major groove of the HRE [6]

The two zinc residues are indicated by purple spheres. The structure of the dimer of the VDR DBD (green) on a DR separated by three nucleotides is shown in the lower panel [16]. The long α-helical CTE of the right-hand monomer folding back and making protein–protein contacts with the left-hand monomer is clearly visible.

alternative DNA recognition by the AR explains part of the androgen-selective activation of target genes [20].

Allosteric effects of HREs

The sequence of the HRE not only serves as a docking site for receptor dimers. It can also dictate, probably via allosteric effects, whether the receptor will activate or repress transcription of a neighbouring promoter. Indeed, GR binding to sites in the prolactin and the proliferin genes result in a glucocorticoid-mediated repression, and single-base-pair mutations convert these negative response elements into positive ones [23]. AR binding to different androgen-response elements leads to alternative mechanisms not only in the dimerization of the DBDs, but also in subsequent steps in gene activation, e.g. co-activator recruitment by the activation functions 1 and 2 (AF1 and AF2) [24,25]. In general, allosteric effects of ligand and DNA on nuclear receptors seems to determine which co-activator complexes are recruited and more importantly what activities the recruited complexes will have [26].

Other functions of the DBD

Nuclear receptors do not occupy their response elements continuously, and the free DBD mediates other functions such as nuclear localization, nuclear export, interactions with chaperones and communication with other transcription factors (reviewed in [27]). Similarly, even DNA-bound DBDs can have additional functions. For example, the C-terminal half of the DBD and hinge region, by virtue of not making extensive contacts with the DNA, are able to serve as the recruitment site for several co-activators [28]. ARIP (AR-interacting protein), as a putative co-activator, as well as enzymes involved in sumoylation of the receptor, have been isolated in double-hybrid screening experiments with AR fragments. Specific lysine residues within the CTE of the ER and AR have been shown to be substrates for acetylation by pCAF (p300/CBP-interacting factor) and Tip60 (Tat interactive protein of 60 kDa). This acetylation is clearly involved in a regulation of the activity of these receptors, but the exact molecular mechanisms remain elusive [29].

General conclusions

HREs are embedded in complex enhancers to which several transcription factors can bind. Nuclear receptors will therefore become part of larger protein complexes, sometimes called enhanceosomes, that affect chromatin structure and hence transcription of the underlying genes [30]. It will be an exciting challenge to find out how the different signals coming through the nuclear receptors via the binding of ligands and DNA sequences, through the post-translational modifications, and through the recruited co-activator or co-repressor complexes, all become integrated with those coming from other signal pathways at the sites of these enhanceosomes.

Note added in proof

A crystal structure of the AR DBD bound to a three-spacer direct repeat steroid response element has been determined recently [31]. Surprisingly, this structure shows that the AR DBD binds in a symmetrical head-to-head arrangement, like the GR and ER DBDs, instead of the expected head-to-tail arrangement. In this conformation, the AR DBD dimer interface is more stable than the equivalent interface in the GR DBD. This implies that selective HREs that appear to have alternative arrangements of their hexameric half sites may be further examples of the ability of these receptors to exploit the strength of their DBD dimerization interfaces to accommodate sub-optimal protein–half-site interactions. This is likely to be not only a mechanism of response element discrimination, but also an effective way of modulating transcription from different hormone responsive genes. Finally, it will be interesting to study the exact contribution of the C-terminal extension of the DBD, of which the structure remains unsolved, to DNA binding and transactivation by the AR.

Summary

- *Nuclear receptors are ligand-inducible transcription factors which recruit a series of transcription-modulating complexes to specific DNA sequences located near their target gene.*
- *The DNA elements are typically organized as direct or inverted repeats of the hexamers 5'-AGAACA-3' or 5'-AGGTCA-3', and the relative orientation and distance between the repeats is the main determinant for receptor specificity.*
- *The DBDs are organized as two zinc-coordinating modules, the first of which makes specific contacts with the hexamer sequences. The second zinc-coordinating module serves as a dimerization interface.*
- *Depending on the type of receptor, a short CTE of the DBD plays a crucial role in sequence recognition through direct DNA interactions, or through a dimerization. The latter fixes the relative positions of the DNA-recognition residues and hence dictates the optimal distance between the repeats in the HREs.*
- *The AR is the only steroid receptor that recognizes both IRs and DRs of the 5'-AGAACA-3' hexamer, probably through two alternative dimerization interfaces.*
- *Besides DNA binding and dimerization, the DBDs have multiple functions in transcriptional control.*

We thank the Belgian Fund for Scientific Research, the Catholic University of Leuven (K.U. Leuven, Belgium) and the DOD Prostate Cancer Research Program (DAMD17-02-1-0082) for support of the work which led to some of the findings and concepts described in this chapter. We are grateful to the members of our laboratories for fruitful discussions and interactions.

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Structural basis of androgen receptor binding to selective androgen response elements

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Communicated by Jane S. Richardson, Duke University Medical Center, Durham, NC, February 17, 2004 (received for review October 29, 2003)

Steroid receptors bind as dimers to a degenerate set of response elements containing inverted repeats of a hexameric half-site separated by 3 bp of spacer (IR3). Naturally occurring selective androgen response elements have recently been identified that resemble direct repeats of the hexameric half-site (ADR3). The 3D crystal structure of the androgen receptor (AR) DNA-binding domain bound to a selective ADR3 reveals an unexpected head-to-head arrangement of the two protomers rather than the expected head-to-tail arrangement seen in nuclear receptors bound to response elements of similar geometry. Compared with the glucocorticoid receptor, the DNA-binding domain dimer interface of the AR has additional interactions that stabilize the AR dimer and increase the affinity for nonconsensus response elements. This increased interfacial stability compared with the other steroid receptors may account for the selective binding of AR to ADR3 response elements.

The androgen receptor (AR) is a ligand-activated transcription factor that plays a central role in male sexual development and in the etiology of prostate cancer (1, 2). It is a member of the steroid and nuclear hormone receptor superfamily, which also includes receptors for glucocorticoids (GR), mineralocorticoids (MR), progesterone (PR), estrogens (ER), and vitamin D (VDR) (3). Members of this family contain conserved, discrete, DNA-binding domains (DBDs) and ligand-binding domains. The amino-terminal domain and the hinge region connecting the central DBD to the C-terminal ligand-binding domain diverge among family members.

The hormone receptor DBD consists of a highly conserved 66-residue core made up of two zinc-nucleated modules, shown schematically in Fig. 1*A* (4, 5). With VDR as the only reported exception (6), the isolated DBD and associated C-terminal extension are necessary and sufficient to generate the same pattern of DNA response element selectivity, partner selection, and dimerization as the full-length receptor from which it is derived (6–11).

Although ligand binding elicits distinct hormone-specific responses, all classical steroid receptors (AR, PR, MR, and GR) recognize identical DNA response elements, which consist of two hexameric half-sites (5′-AGAACA-3′) arranged as inverted repeats with 3 bp of separating DNA, producing the 2-fold IR3 sequence pattern (Fig. 1*B*) (12). A question that continues to engage the steroid receptor field is how these transcription factors achieve DNA target specificity despite this degeneracy. As seen in the structures of the GR and ER DBDs bound to IR3 elements (4, 13), the receptors bind as "head-to-head" homodimers whose symmetric displacement across the DNA pseudodyad reflects the underlying half-site arrangement. Differences in steroid metabolism, receptor expression, local chromatin structure, and the availability of cofactors all contribute to steroid-specific responses (14–17). However, recent work has now also identified selective androgen response elements (AREs). The AREs consist of two hexameric half-sites arranged as an androgen direct repeat separated by 3 bp of spacer (ADR3) (18–21), with the half-site repeating on the same strand (Fig. 1*B*). The expanded binding repertoire of AR, including both the common IR3 and specific ADR3 elements, breaks the degeneracy of the steroid response elements, allowing specific AR activation from certain response elements but disfavoring interaction with PR, MR, or GR. This finding could further account for steroid-specific actions *in vivo*.

The crystal structures of nuclear receptors bound to direct-repeat elements, including the VDR DBD bound to a similar DR3 element, reveal a "head-to-tail" protein dimer bound to the DNA (6, 22–24). For AR to bind to ADR3-type elements in a head-to-tail orientation, the DBD would require a second dimerization interface that is distinct from the canonical D box region used to dimerize on IR3 elements (25). To visualize this unusual homodimeric assembly, we have solved the crystal structure of an AR DBD homodimer bound to an ADR3 response element. The structure we report here reveals that the proteins do not adopt the expected head-to-tail orientation on the DNA, but, instead, they retain the symmetric mode of dimerization observed previously for the GR DBD bound to an IR3 DNA element. We describe the protein–protein and protein–DNA interactions that allow for this unexpected arrangement, and we propose that AR-specific dimerization contacts account for the AR specificity of ADR3 elements.

Materials and Methods

Protein and DNA Purification. The rat AR DBD (residues 533–637, C552A) was expressed in *Escherichia coli* BL21/DE3 cells as a GST fusion and purified with a glutathione-Sepharose column (Sigma). The GST was cleaved with thrombin at 4°C overnight. Further purification was performed with SP Sepharose FastFlow (pH 7.4) and Source 15S (pH 6.9) columns. Protein concentration and purity was determined by UV absorbance and SDS/PAGE.

Synthetic oligonucleotides (W. M. Keck Facility, Yale University) were detritylated and purified by reversed-phase HPLC (Rainin Dynamax-300). Concentrated, purified strands were annealed by heating to 95°C and slowly cooling to room temperature.

Crystallization and Data Collection. Samples for cocrystallization contained DNA and protein concentrations of 0.15 and 0.30 mM, respectively, in 5 mM Tris (pH 7.6)/150 mM LiCl/10 mM DTT. Crystals were grown by hanging drop vapor diffusion at 18°C with the addition of 2 μl of the complex to an equal volume of reservoir solution (50 mM Mes, pH 5.6/0–20 mM $MgCl_2$/0–2% polyethylene glycol 400). Diffraction quality crystals ($0.15 \times 0.15 \times 0.4$ mm) grew in 2–6 weeks.

Crystals were equilibrated into reservoir solution supplemented with 35% glycerol before being flash-cooled in liquid

Abbreviations: AR, androgen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; ER, estrogen receptor; VDR, vitamin D receptor; DBD, DNA-binding domain; ARE, androgen response element.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 1R4I).

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www.pnas.org/cgi/doi/10.1073/pnas.0401123101

nitrogen. Diffraction data were collected at −180°C on beamline 22ID at the Advanced Photon Source with a CCD detector (Marresearch, Norderstedt, Germany). Data were indexed and reduced by using HKL2000 (26).

Structure Determination and Refinement. Four zinc sites were found by using SOLVE (27) and data from the peak anomalous wavelength. Experimental phases were generated with these sites; and, in the anomalous difference Fourier maps, the four zinc sites had peaks of >30 σ, whereas the next highest peak was 3 σ, indicating one AR dimer was in the asymmetric unit. Only one of the two possible enantiomeric space group choices yielded zinc sites that corresponded to possible AR dimers. Visual inspection of the zinc sites revealed that the proteins were arranged in a palindromic orientation. This finding led to construction of a molecular replacement model by using the ER DBD-IR3 structure (13) (PDB ID code 1HCQ). Because of its higher sequence homology to AR, the ER DBD was replaced with the core GR DBD (4) (PDB ID code 1GLU) by using least-squares fitting. A molecular replacement solution was obtained by using MOLREP (28).

Multiwavelength anomalous dispersion phases were calculated by using the remote and peak wavelength data to 3.4 Å and also used in refinement, which was done in CNS (29) by using the maximum likelihood Hendrickson–Lattman target. Model building was done by using O (30). Even at 3.1 Å, the number of unique reflections used was eight times the number of modeled atoms because of the very large (>80%) solvent content of the crystal, allowing for restrained individual B factor refinement in later rounds. Visualization of hydrogen bonds, van der Waals interactions, and clashes was aided by use of all atom contacts in KING and PROBE (31). Graphics used RIBBONS (32) and PYMOL (DeLano Scientific, San Carlos, CA).

Results

Crystallization and Structure Solution. Initial crystals of AR DBD–ADR3 complexes grew as thin needles from complexes containing AR DBD (residues 533–619) and diffracted to 4 Å with synchrotron radiation. These crystals were resistant to dissolution, suggesting crosslinking within the lattice. The AR DBD contains a nonconserved cysteine at position 552[11] (common receptor DBD numbering is given in brackets), which was predicted to be solvent-exposed based on modeling from the GR DBD structure. When Cys-552[11] in the AR DBD was changed to alanine, complexes containing this mutant yielded bar-shaped crystals that were isomorphous with the initial crystal form. These crystals were used to determine the structure of the AR DBD–DNA complex (PDB ID code 1R4I).

The structure of AR DBD(533–637)Cys552Ala in complex with ADR3 DNA (Fig. 1) was determined at 3.1 Å by a combined MAD and molecular replacement approach with diffraction data collected at the zinc anomalous edge. The arrangement of the proteins on the ADR3 DNA was determined from zinc anomalous data that revealed the location of the four zinc atoms in the complex. Data collection and refinement statistics are presented in Table 1, and representative electron density maps are shown Fig. 7, which is published as supporting information on the PNAS web site.

Anomalous difference Fourier maps confirmed that the asymmetric unit consists of just one AR DBD homodimer–DNA complex, yielding a Matthews number of 6.9 and a solvent content of 82%. The main crystal-packing interactions are made by the junction near protomer A, which contains neither a pseudocontinuous DNA interaction nor a biologically plausible alternative protein dimer interface. The downstream AR DBD (protomer B) makes only two crystal contacts by residues Phe-589[48] and Arg-590[49] and, except for the interaction with

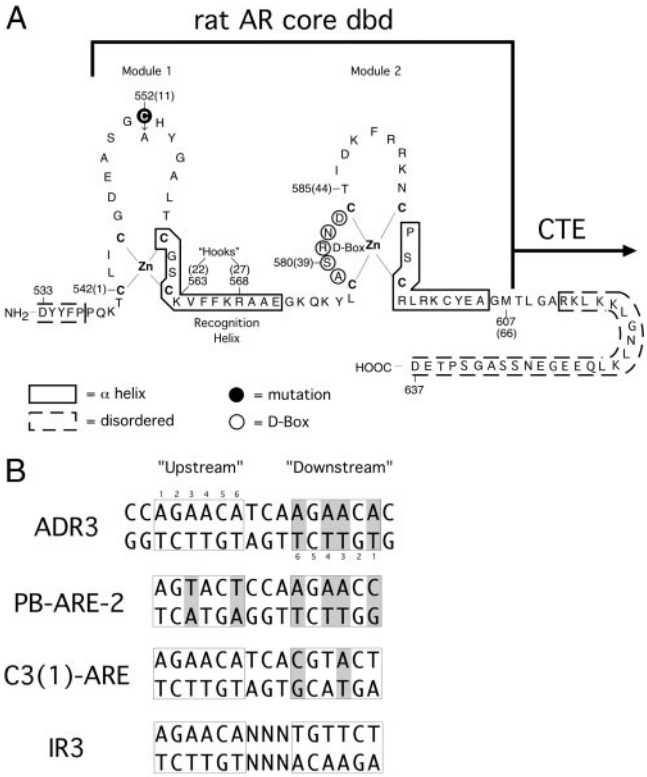

Fig. 1. Protein and DNA constructs. (*A*) The rat AR DBD. Sequence numbers in parentheses refer to the common receptor DBD-numbering scheme. Residues in dashed boxes are disordered in both protomers of the homodimeric complex. (*B*) The DNA used in cocrystallization, labeled ADR3, two naturally occurring AR response elements, PB-ARE-2 and C3 (1)-ARE, and a canonical IR3 steroid response element. Differences from the IR3 sequence are shaded gray.

protomer A and the DNA, it is otherwise completely exposed to the large solvent channels (Fig. 2).

Examination of the crystal-packing interactions can explain the refractory effect of C552[11] on crystallization. Residue

Table 1. Summary of data collection and refinement

Diffraction data		
Space group,*† Å	$P3_221$ 137.89, 85.71	
Data set	Native/remote	Zn peak
Wavelength, Å	1.0000	1.2831
Resolution, Å	50–3.1	50–3.4
Last shell, Å	3.21–3.1	3.52–3.4
Unique reflections	17,313	25,060
Completeness, % (last shell)	99.7 (99.1)	99.5 (98.9)
Average I/σ_σ (last shell)	20.4 (2.5)	19.6 (2.0)
R_{merge}, % (last shell)	9.6 (62)	7.8 (58)
FOM (after DM)‡	0.41 (0.96)	
Crystallographic refinement		
Resolution range, Å	50–3.1	
Reflections ($F > 2\sigma_F$)	14,839 (12,418)	
Atoms	1,813	
rms bond lengths, Å	0.0076	
rms bond angles, °	1.29	
R value ($F > 2\sigma_F$)†	24.6 (22.7)	
R_{free} ($F > 2\sigma_F$)	26.4 (24.9)	

*$R_{merge} = \Sigma_{hkl}\Sigma_i|I_{i(hkl)} - \langle I_{(hkl)}\rangle|/\Sigma_{hkl}\Sigma_i I_{(hkl)}$.
†$R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. 5% of the reflections were used for R_{free}.
‡Figure of merit = $\langle|\Sigma P(\alpha)e^{i\alpha}/\Sigma P(\alpha)|\rangle$, where α is the phase and $P(\alpha)$ is the phase-probability distribution.

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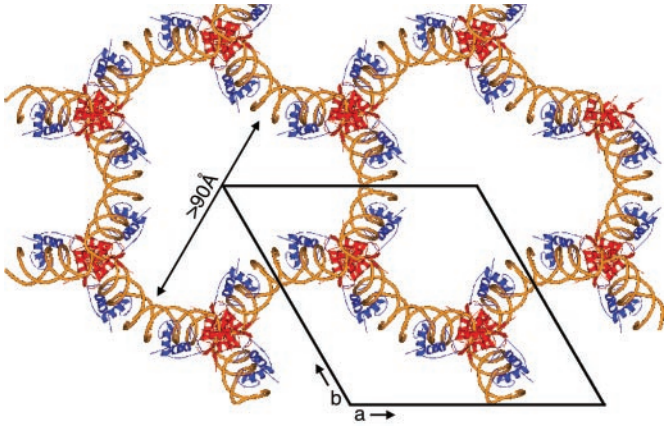

Fig. 2. Crystal packing of the AR DBD–ADR3 complex. Red and blue ribbons are the upstream and downstream subunits, respectively, with the DNA backbone shown in gold. The view is parallel to the *c* axis of the crystal, and the unit cell is shown.

552[11] from protomer A is in position to crosslink with Cys-578[37] of protomer A in the adjacent symmetry-related complex. Cys-578[37] coordinates a zinc atom in the first Zn module. Formation of a C552[11]-C578[37] disulfide link is likely to disrupt the native AR DBD conformation and adversely affect crystal order.

The AR DBDs Are Arranged as an Inverted Repeat on a Direct-Repeat DNA Target. In all the dimeric hormone receptor DBD–DNA complexes determined to date, the two DBDs adopt the same relative orientation as that of the underlying DNA target. Surprisingly, however, in the structure of AR DBD bound to ADR3 DNA, the two AR DBD protomers are not arranged as a head-to-tail dimer, as would be expected of receptors bound to a direct-repeat DNA element. Instead, the proteins form a symmetric, head-to-head dimer that is nearly identical with the dimer seen in the ER DBD–DNA and GR DBD–DNA structures (rms deviation for α-carbons of 1.09 and 0.89 Å, respectively) (4, 13). This finding was confirmed unambiguously by inspection of the positions of the four zinc sites determined from anomalous difference maps calculated from single wavelength anomalous dispersion phases (Fig. 3). The arrangement of the AR dimer is unlikely to be an artifact of crystal packing, because there are only two small crystal contacts between the downstream DBD (protomer B) and the neighboring molecules in the crystal lattice (Fig. 2).

The AR DBD Homodimer Interface. The subunit interface of the AR DBD homodimer is symmetric and closely resembles that seen in the GR DBD–DNA complex (4). As in the GR DBD– and ER DBD–DNA complexes, the majority of the cross-subunit contacts are made in the D box region of the second zinc module. In the GR homodimer, the subunit interface is stabilized both by a network of hydrogen bonds between D box residues and by an extensive complementary surface. As seen in Fig. 4*B*, however, the GR interface contains a void formed where the Gly-478[39] from the opposing subunits face each other. This "glycine hole" is also a feature of the MR and PR. In the AR DBD, however, glycine is replaced by Ser-580[39]. This serine packs into the glycine hole of the dimer interface, filling the void and making van der Waals contact with its counterpart in the other subunit. In addition, the arrangement of the two serines is optimal for the formation of a hydrogen bond across the molecular pseudodyad. The substitution of serine for glycine in the AR D box is likely

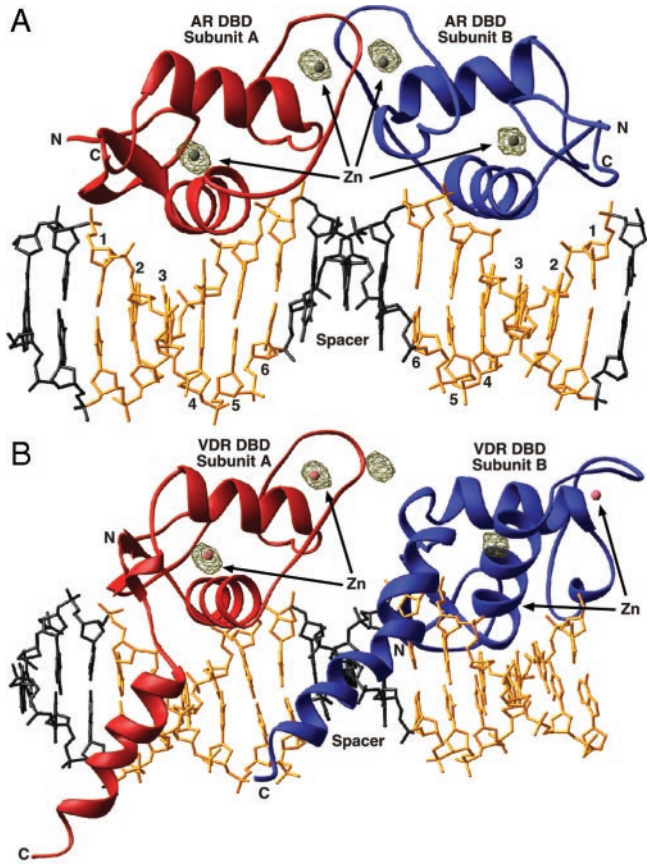

Fig. 3. Overall architecture of the AR DBD–ADR3 and VDR DBD–DR3 complexes. (*A*) The AR DBD–ADR3 complex. The two protomers are in red and blue, the hexameric half-site DNA is gold, and the spacer and flanking base pairs are black. In brown is a 20-σ contour of the experimental anomalous Fourier difference map. (*B*) The VDR DBD–DR3 complex. VDR DBD protomer A is shown in the same orientation as the AR DBD subunit A in *A*. The zincs of subunit B fail to occupy the peaks in the anomalous difference Fourier map in this dimeric arrangement, indicating the AR DBD does not form a head-to-tail dimer.

to increase the relative strength of the dimer interface of the AR DBD.

The AR DBD also makes an additional pair of symmetrical contacts between Thr-585[44] and the carbonyl oxygen of Ala-579[38] in the opposing protomer. In the GR DBD the residue at this position is an isoleucine, and replacement with a threonine as seen in the AR is likely to increase the stability of the dimer because of the enthalpic contribution of the additional two hydrogen bonds. In addition, the change from Ile in GR to Thr in AR removes a nonpolar residue from the solvent-exposed surface of the DBD, thus entropically stabilizing the AR as well.

The AR DBD (P.L.S. and D.T.G., unpublished work) and GR DBD (33) are monomers in solution. Because cooperative dimerization greatly increases the affinity of receptors for their bipartite response elements, these two changes should also increase the relative affinity of the AR for a given response element compared with GR. In support of this hypothesis, GR DBD mutants containing a serine in place of Gly-478[39] in the D box or a threonine in place of GR Ile-483[44] show increased affinity for both palindromic and direct-repeat response elements compared with wild type (34), confirming the importance of these interactions for dimer stability.

Protein–DNA Interactions. The DNA used for cocrystallization has a DR3 arrangement of hexameric half-sites, with the sense strand

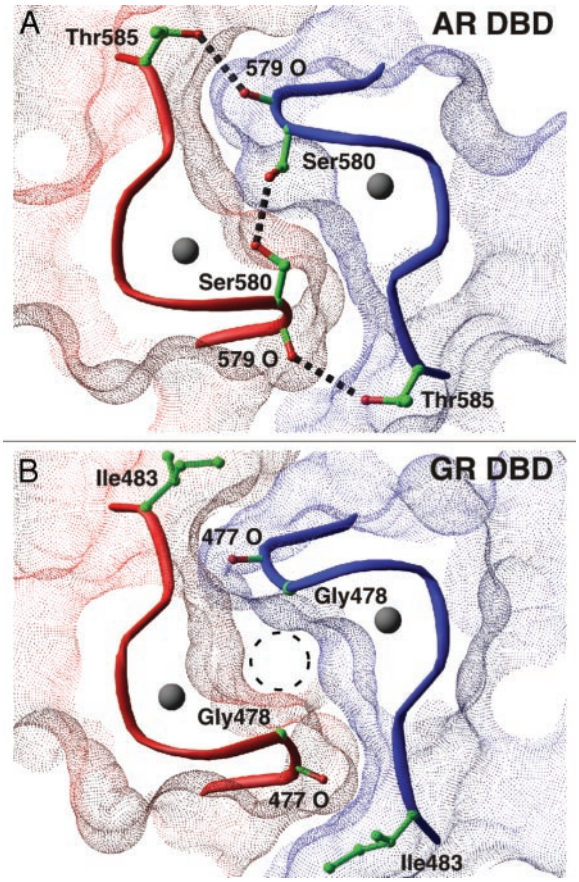

Fig. 4. (*A*) The AR DBD dimer interface. The molecular surfaces of the AR subunits are shown in red and blue. Dashed black lines are hydrogen bonds. (*B*) A similar view of the GR DBD dimer interface. The ''glycine hole'' is noted by the dashed circle.

sequence 5′-CC **AGAACA** TCA **AGAACA** G-3′. However, the AR proteins were observed to bind in a symmetric, head-to-head arrangement, as was seen with steroid receptors bound to an IR3 response element (symmetrized consensus sequence of 5′-**AGAACA** NNN **TGTTCT**-3′). One half-site, bound by protomer A and shown here as upstream, is common to both DR3 and IR3 elements and is a high-affinity, consensus-binding site for steroid DBDs. Protomer B, on the other hand, binds to the downstream half-site that contains the consensus IR3-type bases at only the second and fifth positions. Experimentally phased electron density maps were used to identify the length of the asymmetric flanking sequences and unambiguously assign the orientation of the DNA. Within the limitations imposed by the diffraction resolution, the DNA does not exhibit significant deviations from B form.

Backbone DNA contacts are similar for both AR protomers (Fig. 5) and show the pattern seen previously in structures of steroid receptor–DNA complexes (4, 35). The base-specific contacts between the AR DBD and the consensus half-site are also nearly identical with those of the GR DBD to its cognate half-site and are shown in Fig. 5*A*. In addition to these previously described interactions, we also note that the aliphatic portion of the Arg-568[27] side chain makes additional van der Waals contacts with Val-564[23] and the C5 methyl group of the thymine at the sixth position of the consensus half-site. Thymine is the only base that can form the second half of this van der Waals ''sandwich,'' and this specific contact likely explains why an A:T base pair is commonly observed at the sixth position of AR-specific half-sites (Fig. 6). Because the interaction between the conserved arginine and thymine is also present in consensus half-sites in the GR, ER, 9-*cis*-retinoic acid receptor, and other steroid and nuclear hormone receptor DBD structures, this can explain the preference for the A:T base pair at the sixth position in these protein–DNA complexes as well.

The nonconsensus half-site interaction seen in the AR DBD–ADR3 structure contains the top strand sequence 5′-AGAACA-3′, with the two bases that match the consensus for a downstream IR3 half-site underlined. These two bases lie at the correct IR3 positions because they are symmetric within the hexameric half-site. This serendipitous match to the consensus IR3 half-site allows Lys-563[22] and Arg-568[27] of protomer B to recapitulate the hydrogen bonds to the GC base pairs at positions 2 and 5 of the hexameric half-site, as seen in the upstream element. These two ''hooks'' are common elements that position the recognition helix within the major groove of the hexameric half-site (36).

In the cognate AR DBD half-complex, the side chain of Val-564[23] makes van der Waals contact with the 5-methyl group of the T4 of the antisense strand. This interaction between the two nonpolar substituents is the discriminating feature of specific steroid receptor–DNA interfaces, and the resulting dehydration of the protein–DNA interface contributes entropic stabilization to the binding (35, 37). In the nonconsensus AR half-complex, A replaces the T at position 4 of the sense strand, resulting in the loss of the Val-564[23]-T4 contact. Although this replacement reduces the number of specific, stabilizing, interactions with the DNA half-site, the substitution of an A base for the consensus T does not cause a steric clash that might disfavor binding to this element. As befits the reduced complementarity between the AR DBD and the nonconsensus half-site, the cognate half-complex buries slightly more surface area from solvent (1,230 Å^2) than the noncognate one (960 Å^2).

AR Mutations. Mutations in the AR DBD associated with partial or complete androgen insensitivity (see ww2.mcgill.ca/androgendb) can be understood mechanistically in light of the structure determined here. Many of these were correctly analyzed earlier based on the structure of the GR DBD (38). More recently, within the D box, Ala579Thr (39–41) and Ser580Thr (42) mutations have been reported to lead to loss of AR dimerization. Modeling the Ser580Thr mutation on the AR DBD dimer leads to bad steric clashes in any possible Thr conformation, forcing backbone shifts that presumably disfavor dimerization. Modeling of the Ala579Thr substitution is more problematic, because the Thr side chains can each be accommodated with modest steric overlaps of 0.3–0.4 Å. However, that may be enough to force structural changes in the interface, and the imprecision of low resolution may underestimate the problem. The Ala579Thr mutation can be relieved by a compensatory change in Thr-585 to Ala (43), close to residue 579 across the dimer interface. This further change may relieve strains in the dimer interface or in the Zn ligand geometry caused by the Ala579Thr mutation.

Discussion

We have determined the structure of the AR DBD bound to an idealized steroid DR3 response element. Based on studies of the VDR DBD (6), which also binds to a DR3-type response element, we expected the tandem arrangement of half-sites to direct head-to-tail binding of the AR DBD to the DNA. Surprisingly, however, the AR DBDs bind to the direct-repeat response element as head-to-head symmetrical dimers. This mismatch between receptor dimer- and response element-arrangement results in one AR DBD bound to a high-affinity cognate half-site, and the partner DBD bound to a lower-affinity half-site. This finding indicates that the energetic penalty in-

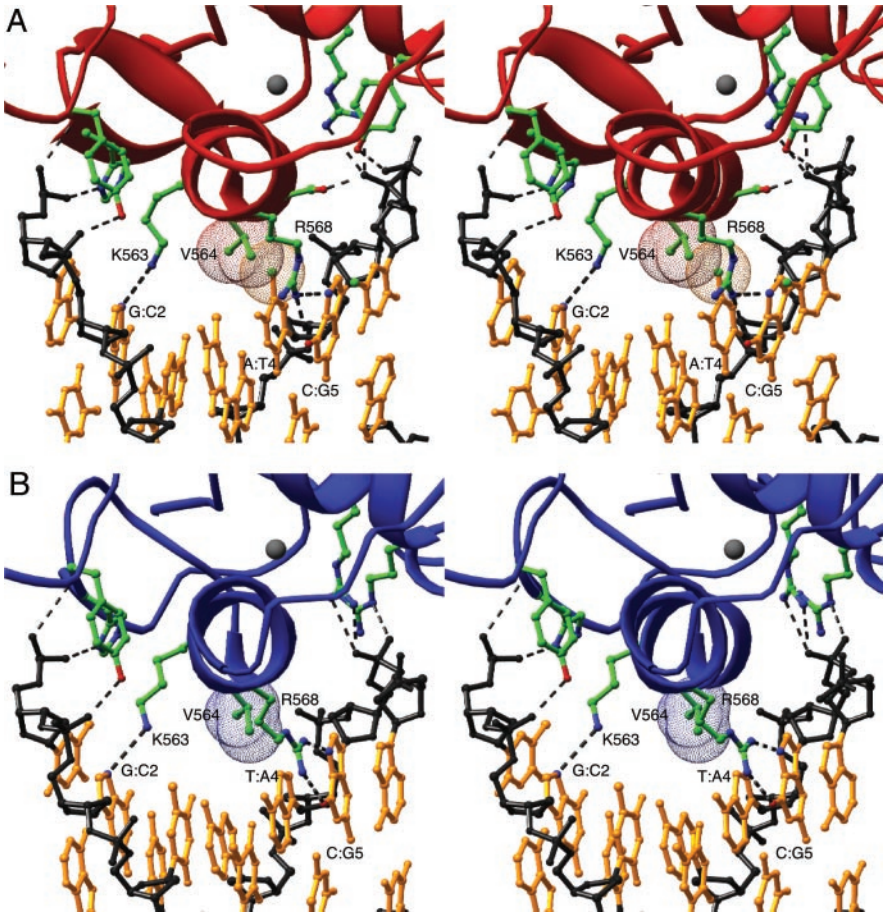

Fig. 5. Stereoview of the AR DBD–DNA interfaces. (*A*) The upstream, cognate, protein–DNA interface. (*B*) The downstream, noncognate interface. The protein is shown in the same orientation as in *A*.

curred by binding to a less favored half-site sequence is more than offset by maintaining the preferred IR3-type dimer interface. This finding is analogous to an earlier observation that the GR DBD maintains the IR3 dimer interface and spacing even when challenged with an IR4 response element (4).

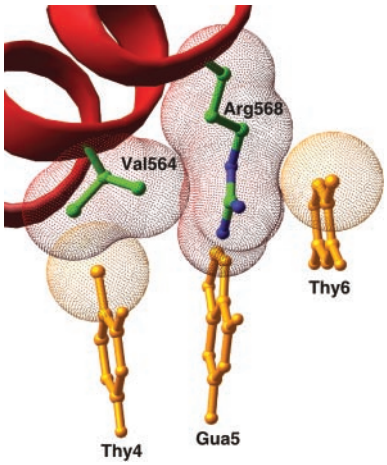

Fig. 6. The arginine ''sandwich.'' Val-564 and Arg-568 of the AR DBD subunit along with bases T4, G5, and T6 of the antisense strand of the upstream, cognate half-site are shown. The C5 methyl group of T6 forms van der Waals interactions with one face of Arg-568, whereas the other side packs against Val-564.

Both the AR and the GR exhibit similar interactions with steroid response elements, yet the AR exhibits consistently stronger binding to direct repeat-type response elements than does the GR. Some of this difference in affinity may be attributable to differences in the C-terminal extension of each DBD, although in both GR and AR these regions were disordered in the crystal structure and may contribute only general electrostatic interactions without affecting selectivity or discrimination. Within the core of the DBD, however, the protein–DNA interactions are nearly identical for both receptor DBDs, and much of the difference in response element affinity is therefore likely to reside in the ability of each receptor to cooperatively form head-to-head dimers on bipartite response elements where the interaction with one or both hexameric half-sites is nonoptimal.

The second zinc module has been shown to be necessary for AR to bind cooperatively to ADR3s (44). The steroid receptor DBD dimerization interface is contained within this module, and between AR and GR it differs at just four positions. The increased AR dimer affinity can be explained by two of these four substitutions, one in the D box, and the other two residues beyond. In the D box, AR is the only steroid receptor that has a Ser residue at the second position, Ser-580[39], and this serine packs into the core of the dimer interface, making both van der Waals interactions and a cross-subunit hydrogen bond. All other steroid receptors have a Gly at this position, which lacks this additional hydrogen bond and leaves a void in the interface. Two residues beyond the D box, an Ile-to-Thr substitution in AR allows both a favorable cross-subunit side chain-to-backbone

hydrogen bond and removes the nonpolar Ile side chain from exposure to solvent. Together these two substitutions appear to account for the stronger AR dimer interface. These substitutions in turn allow the receptor to bind to a more diverse set of response elements with higher affinity and cooperativity than the GR.

Biochemical evidence for the increased cooperativity of the AR DBD dimer correlates with these structural observations. All the steroid receptors (MR, PR, GR, and AR) show a 5- to 10-fold lower affinity for the naturally occurring PB-ARE-2 DR3-type element than the C3 (1) IR3-type element (34). However, the AR DBD binds 3- to 10-fold better to both elements relative to the other steroid receptors. Thus, the binding constant for AR on an apparent DR3 target (23 $\pm$ 5 nM) is the same as that of the other receptors for the more optimal IR3 element (the average of the other three is 23 $\pm$ 9 nM) (44). Because the concentration of individual steroid receptors in the cell is approximately nanomolar, differences in binding constants of this order are likely to be significant. AR substitutions in the GR dimerization interface, including Gly483Ser and Ile483Thr, show higher affinity binding to both DR3 and IR3 response elements (34), thus mimicking the behavior of the AR. Together with the structural data, these observations suggest a model where, because of the increased strength of the AR dimer interface, AR-selective gene activation arises from the ability of the AR to bind to IR3 response elements that have a greater deviation from the consensus half-site sequence. The reverse cross-activation of GR-responsive genes by the AR would likely be disfavored by the highly tissue-specific expression pattern of the AR compared with the GR.

The structure of the AR DBD bound as an inverted repeat to a direct-repeat response element highlights the fact that DNA target recognition by hormone receptors is strongly governed by the dimerization behavior of the two interacting protomers, even at the cost of losing specific interactions with the target DNA. With the exception of the Ecdysone receptor, which binds to IR1 rather than IR3 targets consisting of AGGTCA rather than AGAACA half-sites (45), no physiologically relevant dimerization interface within the classical steroid receptor DBDs, other than the primary one, has been observed to date in structural studies. Moreover, attempts to capture such potential alternative interfaces, as described in this report, and previously for GR (4), have been unfruitful. This in turn implies that selective hormone response elements that appear to have alternative arrangements of their hexameric half-sites, such as the pemARE with a proposed 5-bp spacer between half-sites (46), may instead simply be further examples of the ability of these receptors to exploit the strength of their DBD dimerization interfaces to accommodate suboptimal protein–half-site interactions. This ability is likely to be not only a mechanism of response element discrimination, but also an effective way of modulating transcription from different hormone-responsive genes.

We thank Nikki Fetter and Jenna Vanliere for help with crystallization and Karen Soldano for preparative assistance. This work was supported by U.S. Army Prostate Cancer Research Program grants (to D.T.G. and F.C.).

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The Hinge Region of the Androgen Receptor Plays a Role in Proteasome-Mediated Transcriptional Activation

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ABSTRACT: To investigate the function of the hinge region in transcriptional activation by the androgen receptor, we compared the actions of the wild-type receptor with a mutant receptor, deleted of amino acids 628–646 of the hinge. The role of the proteasome on the expression and activity of these two proteins was investigated. The deletion mutant demonstrated a threefold increase in transcriptional activity when compared to the wild-type receptor protein. Furthermore, we found that hormone-dependent stabilization of the receptor protein was more enhanced for the deletion mutant. In addition, experiments using the proteasome inhibitor, MG132, demonstrated that the deletion mutant is more sensitive to proteasome-mediated degradation than the wild-type receptor. However, inhibition of the proteasome had a negative effect on the transcriptional activity of the deletion mutant. Taken together, our results suggest that the hinge region not only plays an important role in controlling the transactivation potential of the androgen receptor but also in determining the influence of the proteasome on androgen receptor-mediated transcriptional activation.

KEYWORDS: androgen receptor; hinge region; proteasome; PEST sequence; transcription regulation

INTRODUCTION

The human androgen receptor (AR) is a member of the nuclear receptor superfamily. It has a modular structure and comprises 919 amino acids. It is composed of a long N-terminal domain with a transactivation function AF-1, a central DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD) with an additional transactivation function AF-2.[1] Initially, the hinge region was considered to be a nonfunctional linker between the DBD and the LBD. However, recent results indicate that the hinge region is a multifunctional region involved in DNA binding,[2,3] nuclear localization,[4] and modulation of transactivation.[5,6] The hinge region is also a phosphorylation[7] and acetylation[8,9] target site and is an interaction domain for several proteins.[10]

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Ann. N.Y. Acad. Sci. 1030: 587–592 (2004).
doi: 10.1196/annals.1329.068

RESULTS AND DISCUSSION

In this study, we investigated in more detail the role of the hinge region (amino acids 628–669) in the transactivation of the AR. Transfection experiments using COS-7 or HeLa cells and androgen-responsive reporter constructs were performed. We found that the AR deleted of amino acids 628–646 (ARΔH) is at least three times more potent than the wild-type AR (wtAR) when tested in both COS-7 and HeLa cells (FIG. 1). This is in agreement with a previous report.[6] Our data indicate that the hinge region plays an important role in controlling the transactivation potential of the AR. These results are surprising in that the nuclear localization signal and a region involved in DNA binding are partially deleted in ARΔH. However, the mutated

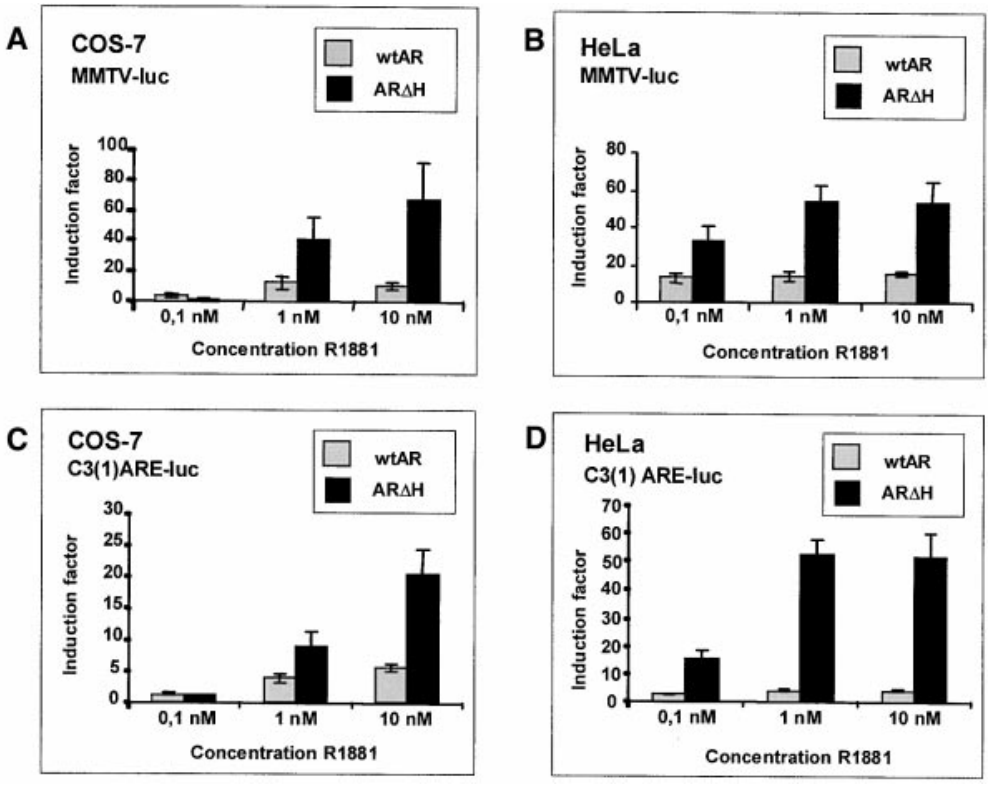

FIGURE 1. The hinge region limits the transactivation potential of the AR at androgen-responsive reporters. COS-7 and HeLa cells were seeded onto 96-well plates at 10^4 cells/well and 1.5×10^4 cells/well, respectively. Twenty-four hours later, cells were transfected with 100 ng of reporter, 10 ng of appropriate receptor, and 10 ng of β-galactosidase expression vectors using FuGENE6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were refreshed with medium with or without androgen (R1881) at the indicated concentrations and incubated for another 24 h, after which cells were lysed with Passive Lysis Buffer (Promega) and assayed for luciferase (Luciferase Assay Reagent; Promega) and β-galactosidase (Galacto Reaction Buffer; Tropix, Inc.) activities. The graphs depict induction factors for increasing concentrations of R1881 at two androgen-responsive reporters in both COS-7 cells (*left panels*) and HeLa cells (*right panels*). Reporters used were a luciferase reporter gene driven by the mouse mammary virus long-terminal repeat (MMTV-luc; **A** and **B**) and a luciferase reporter driven by an androgen response element (ARE) of the C3(1) gene of prostate-binding protein [C3(1)ARE-luc; **C** and **D**].[2] Results represent pooled data from at least three independent experiments with each condition in duplicate.

receptor can still activate transcription, so both of these functions must still be present to some extent.

Analysis of the amino acid sequence of the AR hinge region reveals the presence of a putative PEST sequence, between amino acids 638 and 658, that is conserved between different species.[11] PEST sequences are amino acid stretches (rich in proline, glutamate, serine, and threonine residues) that are involved in targeting proteins for degradation by the 26S proteasome.[12] For the AR, degradation has been shown to be regulated by the ubiquitin-proteasome pathway.[13] As the PEST sequence is partially deleted in ARΔH, degradation by the proteasome may be hindered, resulting in an increase in protein levels that would explain the increased potency observed in transfection experiments. To study the effect of the proteasome on the expression and activity of the wtAR and ARΔH proteins, we made use of the proteasome inhibitor MG132.

Western analysis revealed that in the presence of androgen, 1 nM R1881, both wtAR and ARΔH expression levels increased ([FIG. 2A, lanes 1 and 2) and that this hormone-dependent stabilization was more pronounced for the latter. Addition of MG132 did not result in a substantially higher wtAR level, but positively affected the level of ARΔH (FIG. 2A, lanes 3–8). Surprisingly, at higher concentrations (10 μM MG132), this effect was much lower. Our data suggest that the hormone-induced stabilization of the wtAR is mildly proteasome-dependent, since the addition of MG132 had only a weak influence on the hormone effect. On the other hand, it seems that the deletion of the hinge region induces an increased sensitivity of the AR to proteasome-mediated degradation (FIG. 2A, compare lane 2 with lanes 3 and 6).

Transfection experiments were carried out to determine whether or not the changes detected at the protein level have any influence at the transcriptional level, as shown for the estrogen receptor α, in which transcriptional activity is interdependent on proteasome-mediated degradation.[14] For wtAR, MG132 had no net effect on its transactivation properties (FIG. 2B, stippled bars). However, in the presence of MG132, the potency of ARΔH was reduced to the levels of wtAR (FIG. 2B, black bars). At 10 μM MG132, the level of ARΔH expression was very similar to the condition without MG132 (FIG. 2A), whereas its transactivation potential was much lower (FIG. 2B). Similar results were obtained when cells were stimulated with 1 nM (FIG. 2B) or 10 nM (data not shown) R1881. Collectively, these results imply that there are at least two interdependent effects of MG132 on ARΔH. We suggest that not only is the proteasome involved in maintaining the steady state of ARΔH but that it also affects the transactivating potential of this protein. With regard to transcriptional activation, it has previously been demonstrated that proteasome function is involved in the recruitment of the AR, in complex with coactivators, to enhancers.[15] In that system, it was further demonstrated that when proteasome function is inhibited by treatment with MG132, the release of the receptor from the promoter is inhibited, which correlates with a suppressed AR activity. Similarly, MG132 has been shown to suppress AR transactivation in two prostate cancer cell lines, LNCaP and PC-3.[16] Therefore, our results obtained with ARΔH could be explained by a proteasome function in AR transcription complex formation; in our assays, however, treatment with MG132 does not suppress wtAR transcriptional activity. Although we cannot explain these differences, our data indicate that the hinge region plays a crucial role in proteasome-mediated transactivation by the AR.

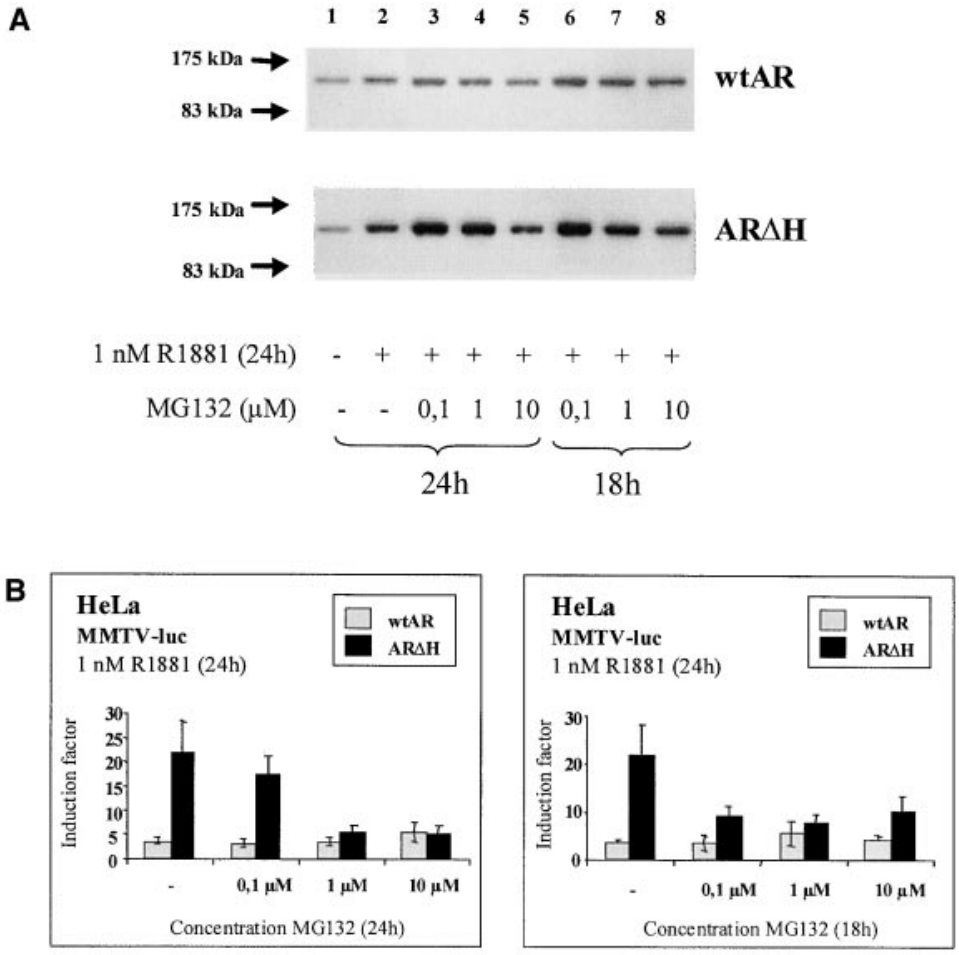

FIGURE 2. Amino acids 628–646 of the hinge region play a role in proteasome-mediated transcriptional activation by the AR. (**A**) Western blot. HeLa cells were seeded onto 24-well plates at 10^5 cells/well. After 24 h, cells were transfected with 200 ng of the appropriate Flag-tagged receptor expression vector using FuGENE6 reagent. Twenty-four hours after transfection, cells were stimulated with or without androgen (1 nM R1881) in the absence or presence of increasing concentrations of MG132 (Calbiochem). MG132 was added either together with the androgen or 6 h after addition of androgens, resulting in incubation times of 24 and 18 h, respectively. Total cell extracts were obtained by lysing cells, after 24 h of hormone stimulation, with lysis buffer (5% NP-40, 1:200 protease inhibitor mix in 1× PBS), and equal amounts of extracts were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and blotted onto Hybond-P nitrocellulose membrane (Amersham Biosciences). Membranes were probed with anti-Flag antibody, receptor proteins were detected by ECL, and autoradiography was performed. (**B**) Transfection. HeLa cells were seeded onto 96-well plates at 1.5×10^4 cells/well. Cells were transfected as described in FIGURE 1 with 100 ng of MMTV-luc reporter, 10 ng of appropriate receptor, and 10 ng of β-galactosidase expression vectors. Cells were stimulated as described for the Western blot (**A**). The graphs depict induction factors. The *left panel* shows results for when MG132 was added together with the androgen (24-h incubation), and the *right panel* depicts MG132 added 6 h after androgen stimulation (18-h incubation). Results represent pooled data from at least three independent experiments with each condition in duplicate.

The precise manner in which the proteasome regulates AR activity is still unclear and may involve multiple mechanisms and different cofactors.[14] Identification of some of these processes will involve a more detailed structure–function analysis of the hinge region of the AR as well as a clear mapping of its interacting proteins.

ACKNOWLEDGMENTS

We thank R. Bollen, K. Bosmans, and H. Debruyn for their excellent technical assistance. This work was supported in part by grants from the Fonds voor Wetenschappelijk Onderzoek, Vlaanderen (F.W.O.), the Geconcerteerde Onderzoekactie van de Vlaamse Gemeenschap, the Congressionally Directed Medical Research Program: Prostate Cancer Research Program award DAMD17-02-1-0082, and the Interuniversity Poles of Attraction Program, Belgium State, Prime Minister's Office, Federal Office for Scientific, Technical, and Cultural Affairs. A.H. is a postdoctoral fellow of the F.W.O.

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INVITED LECTURE

Molecular biology of the androgen responses

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The androgen receptor is a ligand-inducible transcription factor with very specific target genes. This definition implies the activation by the cognate ligand through the ligand-binding domain, the recognition of the target genes by means of the DNA-binding domain and the transcriptional activation through different activation functions.

When the first androgen-responsive genes were cloned, we identified receptor-binding sites by means of a DNA-cellulose competition assay with partially purified androgen receptor from rat prostate (Claessens *et al.*, 1990). Once the receptor cDNA was cloned, the separate DNA-binding domain was expressed and shown to have similar, if not identical DNA recognition properties as the full size receptor. The binding sites were proven functional in transient transfection experiments with reporter genes cloned downstream of these sites (Claessens *et al.*, 1993). The motifs which are recognized by the receptor are called androgen response elements (ARE), and a consensus of the first identified AREs pointed out that it is very similar to the glucocorticoid/progesterone response element (GRE/PRE) consensus 5′-GGTACAnnnTGTTCT-3′. Not surprisingly, these AREs also act as GRE/PRE in transient transfections.

The probasin promoter region also contains two AR-binding sites, but in contrast to what was observed for the earlier AREs, these are not recognized by the glucocorticoid receptor. Later on, several other selective AREs were characterized in the slp and sc enhancers (Verrijdt *et al.*, 2000). A comparison of the DNA-binding domains of the androgen and glucocorticoid receptors revealed specific residues which are involved in the recognition of these selective AREs, but not in the recognition of the classical AREs. These residues are not situated within the first zinc-coordinated module or zinc finger, but rather in the second one, as well as in a carboxy-terminal extension of the DNA-binding motif (Schoenmakers *et al.*, 2000). This hinted to us that the recognition of the selective AREs occurs through an alternative dimerization of the DNA-binding domain that would be specific for the androgen receptor. Indeed, when the direct repeat nature of the selective AREs was changed into inverted repeat nature, the selectivity of the AREs and of the enhancers, of which they form part, was lost (Verrijdt *et al.*, 2000). The silico screening of human genome has led to the definition of several additional selective AREs.

In collaboration with the group of Daniel Gewirth, we were able to solve a crystal structure of the DNA-binding domain of the androgen receptor complexed to a perfect direct repeat of the 5′TGTTCT-3′ hexamer (Shaffer *et al.*, 2004). This revealed that the domain is folded into two zinc-coordinated modules very similar to what has been described for other nuclear receptors. The two monomers are organized in a head-to-head configuration. Specific for the androgen receptor is the increased strength of the dimerisation interface due to an enlarged contact surface as well as to three additional hydrogen bonds.

A functional analysis of the carboxyterminal extension of the DBD, which is part of the hinge region, revealed that it has more functions besides contributing to selective DNA binding. It overlaps with part of a nuclear localization signal and it is involved in the control of transactivation. Indeed, opposite to what is expected, deletions within this region result in a superactive androgen receptor, even when DNA binding in band shifts becomes difficult to demonstrate.

The transcription activation by the androgen receptor is complex in the sense that different domains are contributing to it. For all steroid receptors, two activation functions have been described: the activation function 1 (AF1) in the amino-terminal domain and activation function 2 (AF2) in the ligand-binding domain. The androgen receptor is an exception since the AF2 is weak and in most experiments difficult to demonstrate. A possible explanation for this was found in a strong interaction between the ligand-binding domain and the amino-terminal domain of the androgen receptor. This occurs through a motif at the amino-terminal end of the receptor that interacts with AF2, described as a hydrophobic cleft on the surface of the ligand-binding domain. This interaction seems to prevent recruitment of the known p160 co-activators to

AF2. Instead, the p160 co-activators have a higher affinity for Tau-5, a region in the amino-terminal domain of the androgen receptor, through a glutamine-rich domain (Callewaert *et al.*, (2005); Bevan *et al.*, 1999; Christiaens *et al.*, 2002).

The importance of the N/C interaction for transactivation was first illustrated on the mouse mammary tumour viral enhancer. Surprisingly, the effect was much less pronounced when tested on selective AREs (Callewaert *et al.*, 2003). This changed when the selective AREs were mutated into canonical GRE/PRE/AREs, indicating an allosteric effect of the bound DNA on the transactivation outcome. Another example of such an effect was seen when studying sumoylation of the receptor. Mutations in the sumoylation sites affected cooperativity on canonical AREs, but not on selective AREs (Callewaert *et al.*, 2004).

In conclusion, the androgen receptor is classically considered a ligand-induced transcription factor. We propose that besides the steroid ligand, the DNA could also be regarded as a ligand with allosteric effects on different functions of the androgen receptor. A detailed study of the molecular biology of the androgen receptor should lead to a better understanding of its role in prostate cancer and other androgen-related pathologies, and eventually to better therapeutic strategies.

Acknowledgements

This work was supported in part by the 'Geconcerteerde Onderzoeksactie van de Vlaamse Gemeenschap', by grants from the 'Fonds voor Wetenschappelijk Onderzoek, Vlaanderen', Association for International Cancer Research and Congressionally Directed Medical Research Program: Prostate Cancer Research Program award DAMD17-02-1-0082.

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Interplay between Two Hormone-Independent Activation Domains in the Androgen Receptor

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Abstract

The androgen receptor (AR) plays a key role in prostate cancer development, as well as its treatments, even for the hormone-refractory state. Here, we report that an earlier described lysine-to-arginine mutation at position 179 in AR leads to a more potent AR. We show that two activation domains (Tau-1 and Tau-5) are necessary and sufficient for the full activity of AR and the intrinsic activity of the AR-NTD. Two α-helices surrounding the Lys^{179} define the core of Tau-1, which can act as an autonomous activation function, independent of p160 coactivators. Furthermore, we show that although the recruitment of p160 coactivators is mediated through Tau-5, this event is attenuated by core Tau-1. This better definition of the mechanisms of action of both Tau-1 and Tau-5 is instrumental for the design of alternative therapeutic strategies against prostate cancer. (Cancer Res 2006; 66(1): 543-53)

Introduction

Prostate cancer is the second leading cause of cancer-related death in men (1). Treatment for prostate cancer relies on eliminating androgen receptor (AR) activation, achieved by reducing circulating androgens to castrate levels and/or blocking ligand binding by AR antagonists. However, in most of the cases, androgen ablation therapy results in prostate cancer relapse (2–4). Recent evidence shows that AR continues to be essential for tumor progression, even in this hormone-refractory state. One possible mechanism whereby tumor cells may adapt to a reduced androgen environment is the development of a hypersensitive AR (5). Thus, understanding the mechanisms that regulate AR function is of critical importance.

AR is a member of the nuclear receptor superfamily, consisting of three functional domains: the NH_2-terminal domain (NTD), the central DNA-binding domain (DBD), and the COOH-terminal ligand-binding domain (LBD). Three-dimensional structures are available for the isolated DBD and LBD from both steroid and nonsteroid receptors (6). The NTDs, however, which contain activation function-1 (AF-1), are the least conserved among nuclear receptors, both in size and in amino acid composition.

Although AR seems to play a key role in all aspects of prostate and prostate cancer development, many fundamental aspects of its function and interaction with other molecules are ill defined (5). An important finding is that after combined androgen blockade, AR mutations predominantly colocalize in the NTD (7). Such mutations are useful in dissecting aspects of AR function in prostate cancer.

The p160 coactivators (e.g., SRC1, TIF2, and AIB1) are known to be recruited to the AF-2 of most nuclear receptors via their highly conserved α-helical LxxLL motifs (8). However, we and others have shown that for AR, the p160 recruitment occurs through the AR-NTD (9–12). Indeed, the hydrophobic cleft on AR-AF-2 is implicated in a strong NH_2-COOH terminal (*N:C*) interaction with a FQNLF-motif in the NTD (9, 13–16), and peptides that bind with high affinity to AF-2 do not block transcription activation, suggesting that AF-2 is dispensable for cell proliferation (17). These observations leave the coactivation mechanisms of transactivation by AR largely unexplained.

Jenster et al. (18) has identified two transcription activation units (Tau) in the NTD of AR. Tau-1 is responsible for wtAR transactivating capacity and resides between amino acids (aa) 100 and 370, whereas Tau-5 is responsible for the transactivating capacity of the constitutively active AR, devoided from the LBD and lies between aa 360 and 529.

The characterization of a mutation in a primary prostate cancer biopsy at position 179 in AR indicated that this residue is important for Tau-1 functioning (19). In this article, we describe a first in-depth analysis of this region in the functionality of AR. Two α-helices in the hAR-NTD are necessary for the full activity of AR and constitute the core of the Tau-1 activation function. We hypothesize the existence of a non-p160 coactivator acting through core Tau-1. Furthermore, we show that like Tau-5, core Tau-1 can also act as an autonomous activation function, independent of the LBD, and that Tau-1 affects Tau-5/p160 interactions.

Materials and Methods

Recombinant plasmids and site-directed mutagenesis. The expression vectors pSG5AR [expressing Flag-tagged full-length human AR (hAR)], pSG5AR$_{538\text{-}919}$ (encoding the hAR-DBD-LBD), pSG5SRC1e, and pSG5SRC1eM123 (expressing full-length SRC1e or mutated SRC1e); the vectors SRC1-Qr and AR-NTD (expressing the Q-rich domain of SRC1) and the AR-NTD [fused to the Gal4DBD or the etoposide (VP-16) activation domain] are described elsewhere (9, 11, 15, 20). Expression vectors for the AR-NTD, carrying the mutations of the AF-1 domain, were made by site-directed mutagenesis using the PCR-based method. As template, the expression vector for the full-length hAR (pSV-AR), a kind gift of Dr. A.O. Brinkmann (Erasmus University of Nijmegen, the Netherlands), was used. These PCR-generated hAR-NTD fragments were inserted in frame with the Gal4DBD in the *Bam*HI restriction site of pABGal4 and in frame with the VP-16-activating domain in the *Bgl*II site of pSNATCH-II (9). Expression vectors for the full-size hAR carrying those mutations were made by insertion of a *Afl*II/*Eco*47III fragment of the PCR products in the pSG5AR construct, which was cut with the same restriction enzymes. The pABGal4 fusions with core Tau-1 and its mutants were done by the PCR-based method, using the full-length AR and its mutants as templates. Insertion of multiple copies of the core Tau-1 domain in the pABGal4 vector was done by ligation of the *Bgl*II/*BamH*I fragment of core Tau-1 into the vector followed by sequencing to determine the number of copies. The same was

Note: L. Callewaert is a holder of a postdoctoral fellowship of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.

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doi:10.1158/0008-5472.CAN-05-2389

done for the Tau-5-DBD constructs containing several copies of core Tau-1. Expression vectors for the AR-Tau-5-DBD, carrying deletions in the Tau-5 domain, were made using the PCR-based method. The same deletions in the full-length AR-NTD-DBD were made by restriction digestion of the Tau-5 deletion mutants with *Bsp*68I and *Hin*dIII, ligated into the AR-NTD-DBD construct, which was digested with the same restriction enzymes. The same procedure was followed for the AR-ΔTau-1 construct. The ARΔTau-5 constructs were made by the PCR-based method. Restriction and modifying enzymes were obtained from MBI Fermentas GmbH (St. Leon-Rot, Germany). The luciferase reporter construct driven by the E1b promoter and containing two copies of the rTAT-GRE was described previously (21). TK minimal promoter-driven reporter construct containing the *slp* and *sc* upstream enhancers as well as the *pb* promoter-driven construct have also been described (22). The reporter construct $(Gal4)_5$TATA-Luc and the GST-SRC1-Qr (aa 989-1240) construct was a kind gift from Dr. M.G. Parker (Imperial Cancer Research Fund, London, United Kingdom) and was used for measuring the intrinsic AR-NTD/core Tau-1/Tau-5 activities, for the two-hybrid assays and glutathione *S*-transferase (GST) pull-down experiments with SRC1-Qr. The reporter plasmid pMMTV-luc was obtained from Dr. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). The pCMV-βGal vector was obtained from Stratagene (La Jolla, CA).

Transfection assays. All transfections and reporter assays were done in COS-7, HeLa, U-2OS, HEK293, CHO-K1, and PC3 cells (American Type Culture Collection, Manassas, VA). For transactivation and two-hybrid assays, the cells were seeded in 96-well culture plates in DMEM or DMEM/F12 (for PC3 cells only; Life Technologies, Gaithersburg, MD) containing 5% dextran-coated, charcoal-stripped (DCC) fetal bovine serum at a density of 10^4 per well. They were transfected using FuGENE6 transfection reagent (Roche, Indianapolis, IN) as described by the manufacturer. The amount of luciferase reporter construct was fixed at 100 ng/well, and the amount of pCMV-β-Gal was fixed at 5 ng/well. After transfection, the cells were incubated for 24 hours with medium containing 5% DCC and supplemented or not with R1881 (10^{-8} mol/L; Dupont/New England Nuclear, Boston, MA). Finally, the cells were lysed in 25 μL of passive lysis buffer (Promega, Madison, WI). The luciferase and β-galactosidase activities in 2.5 μL of the extracts were measured using the assay systems from Promega and Tropix (Westburg, the Netherlands), respectively. The luciferase activity in cell extracts was corrected for transfection efficiency by normalizing it according to the corresponding β-galactosidase activity. The values shown are the averages of at least three independent experiments done in triplicate. Error bars indicate the SE values.

Immunoblotting. COS-7 cells were plated in six-well culture plates and transiently transfected with 0.5 μg of expression vectors. At 24 hours after transfection, cells were stimulated with or without R1881 (10^{-8} mol/L). The medium was removed, and the cells were washed twice with ice-cold PBS. The cells were collected in 1.5 mL of ice-cold PBS per dish and pelleted by centrifugation (1 minute). The PBS was removed, and the cells were resuspended in 60 μL of ice-cold passive lysis buffer (Promega). The pellet was collected, and the supernatant was stored at −80°C. For Western blotting, equal amounts of protein were separated by SDS-PAGE on a 6% or 8% gel and blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were probed with a monoclonal M2 anti-Flag antibody (Stratagene) or the rabbit polyclonal antibody Gal4DBD (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoreactive proteins were visualized with the chemiluminescence reagent plus (NEN Life Science, Boston, MA) or with the chromogenic reagent for horseradish peroxidase detection (4CN reagent, NEN Life Science).

GST pull down. *In vitro* transcription and translation of AR-NTD-DBD or fragments were done in rabbit reticulocyte lysate in the presence of [^{35}S]methionine in a total volume of 50 μL as described by the manufacturer (Promega). The *in vitro* translated proteins were diluted to 500 μL with binding buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20]. GST or GST-SRC1-Qr (aa 989-1240) were expressed in the BL21 bacterial strain and bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech). Nonspecific protein-binding sites were blocked by incubation with 2% bovine serum albumin for 1 hour at 4°C. Fifty microliters of each *in vitro* translated protein was incubated with the beads in 250 μL of binding buffer for 30 minutes at room temperature. Beads were washed thrice with binding buffer. Bound proteins were eluted with 2× SDS sample buffer. After SDS-PAGE electrophoresis, the gel was fixed in 10% acetic acid/25% isopropanol for 30 minutes, incubated in Amplify NAMP 100 (Amersham Pharmacia Biotech) for another 30 minutes, and dried, and finally, labeled proteins were visualized by exposure to autoradiographic film (Hyperfilm Enhanced Chemiluminescence, Amersham Pharmacia Biotech).

Immunocytofluorescence staining. COS-7 cells at 6×10^4 were seeded on four-well Labtek II slides (Nalge, Rochester, NY) 24 hours before transfection with 400 ng of AR or AR mutants using GeneJuice transfection reagent (Novagen, Madison, WI). After 24 hours of transfection, cells were treated with or without 10^{-8} mol/L R1881. Immunostaining was done using M2 anti-Flag antibody (Stratagene) followed by incubation with TRITC-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO).

Results

Tilley et al. (19) described a mutation residing in the AR-NTD in a primary prostate cancer biopsy, which consists of the substitution of the Lys^{179} into arginine. We tested several luciferase reporter constructs in transient transfections of COS-7 cells or PC3 cells (Fig. 1*A* and *B*, respectively). One construct is driven by the E1b promoter and contains a tandem repeat of the rTAT-GRE. Another construct is driven by the probasin proximal promoter. Two constructs are driven by the thymidine kinase minimal promoter and contain either the *slp* enhancer or the *sc* enhancer. In addition, AR activity in PC3 cells was tested on the prostate-specific antigen promoter. We show here that the Lys^{179} mutation into arginine leads to an increased AR activity for all reporter constructs tested, and mutation into glutamine or alanine has the same outcome. The differences are not due to altered expression levels (Fig. 1*A*). The effect of the Lys^{179} mutations on the activity of the full-size receptor prompted us to evaluate its role on both Tau-1 and Tau-5.

Tau-5 region is necessary for both intrinsic AR-NTD activity and SRC1-Qr recruitment. The Tau-5 region of AR, between aa 360 and 529, is considered a constitutive activation domain (18). In an attempt to analyze which residues of Tau-5 are important for this intrinsic activity, the activity of the AR-Tau-5-DBD region (aa 360-622) was compared with that of deletion constructs AR-Tau-5_{D1}-DBD (Δaa 360-420), AR-Tau-5_{D2}-DBD (Δaa 421-480), and AR-Tau-5_{D3}-DBD (Δaa 481-529; Fig. 2*A*). Compared with the wild-type AR, the transcription activation capacity of the Tau-5-AR-DBD construct was ∼50% or 80% on the rTAT-GRE(E1b)-Luc or MMTV-Luc reporters, respectively. For all three truncations of Tau-5, there is a decreased activity. To test the ability of Tau-5 for its interaction with SRC1-Qr, we did a mammalian two-hybrid assay (Fig. 2*B*). A clear interaction with SRC1-Qr is seen for both the AR-NTD-DBD and the Tau-5-DBD constructs. However, deleting any part of Tau-5 leads to an impaired interaction.

We repeated this assay after transferring the deletions within Tau-5 to the AR-NTD-DBD (Fig. 2*C*). From those experiments, we can conclude that, indeed, the full Tau-5 region is necessary for the transactivation properties of the AR-NTD (Fig. 2*B* and *C, white columns*), as well as for its interaction with SRC1-Qr (Fig. 2*B* and *C, black columns*). Western blot analysis of extracts containing AR-NTD/Tau-5-DBD was done using a monoclonal M2 anti-Flag antibody. No difference in protein expression levels was observed (Fig. 2*B* and *C*).

To validate the interaction of Tau-5 with SRC1-Qr, we did GST pull-down experiments. Bacterially expressed GST or GST-SRC1-Qr, immobilized on glutathione-Sepharose beads, were incubated with

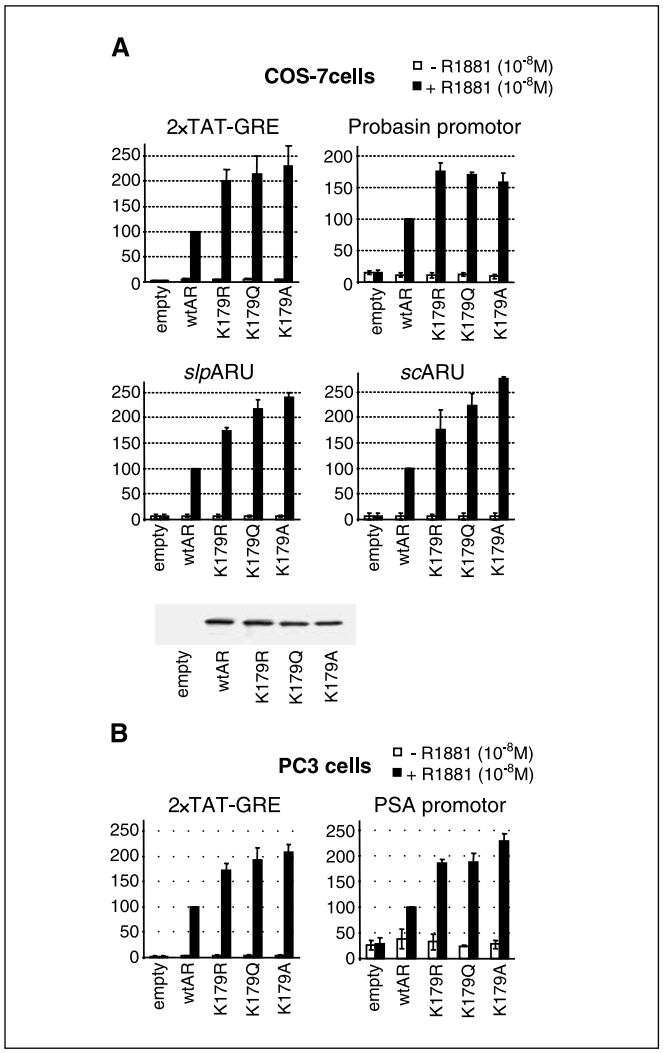

Figure 1. Prostate cancer mutation in the hAR at position 179. *A-B,* COS-7 (*A*) or PC3 cells (*B*) were transfected with an expression vector containing the wild-type or the indicated mutated ARs (10 ng/well). Assays were done using different Luciferase constructs (100 ng/well, *top*) and the CMV-β-Gal reporter (5 ng/well) in the presence or absence of 10^{-8} mol/L R1881 (*open* and *black columns*, respectively). Activities are depicted relative to the activity of the wtAR construct in the presence of hormone, which was set on 100. *Columns,* mean; *bars,* SE. Western blot analyses of the cell extracts containing wtAR or AR mutations using anti-Flag antibody were done as described in Materials and Methods (*A, bottom*).

in vitro translated and [^{35}S]methionine-labeled wtAR-NTD-DBD, a fragment containing only the Tau-5 region and the AR-DBD (AR-Tau-5-DBD) and a AR-NTD-DBD construct lacking the Tau-5 domain (AR-NTDΔTau-5-DBD; Fig. 2*D*). Besides the wtAR-NTD-DBD, only the fragment of the NTD encompassing the Tau-5 domain showed an interaction with SRC1-Qr, whereas the deletion mutant AR-NTDΔTau-5-DBD is not able to show an interaction with SRC1-Qr.

To correlate the interaction of SRC1-Qr with AR-Tau-5 with the coactivation ability of SRC1e, the expression constructs pSG5, pSG5wtAR, pSG5AR-ΔTau-5, pSG5AR-ΔTau-1, and the rTAT-GRE(E1b)-Luc reporter construct were transiently cotransfected into COS-7 cells (Fig. 2*E, top*). The wtAR is clearly coactivated by SRC1e, but when Tau-5 is deleted (AR-ΔTau-5), no coactivation is seen. However, SRC1e coactivation is possible when the first part of the AR-NTD is deleted, resulting in an AR fragment still containing the Tau-5 region (AR-ΔTau-1, aa 360-919). Moreover, cotransfection of wtAR and AR-ΔTau-1 with an SRC-1e construct containing mutated LxxLL motifs in its nuclear receptor interaction domain (SRC-1e M123) shows that the observed increase in activity is due to the Tau-5 interaction and not to the AF-2 in their LBD (Fig. 2*E*). Western blot analysis of AR constructs shows no difference in expression level.

Comparison of Tau-1 of the hAR with τ1 core of the hGR. Simental et al. (23) and Palvimo et al. (24) described the regions between aa 141 and 338 or aa 147 and 296 in human and rat AR-NTD, respectively, as essential for transcriptional activity. In the human GR, the major transactivation domain, called τ1, has been identified as a 185-amino-acid long region, containing a τ1 core region, which retains 60% to 70% of the activity of the intact domain (25, 26). This τ1 core domain contains three putative α-helical segments with the first two as the most important ones. In the AR-NTD, three segments form putative α-helices: helix 1 at position aa 177 to 185, helix 2 at position aa 187 to 199, and helix 3 at position aa 231 to 238 (Fig. 3*A*).

We have studied the effect of mutations in the three helices on the transactivation activity of the full-length AR in transient transfection experiments (Fig. 3*B*). A first construct contains the double mutation I_{181} and L_{182} to alanine (AR_{M1}). In the second mutant AR, L_{190} and L_{191} are mutated into an alanine (AR_{M2}). The third mutant is the combination of these four substitutions (AR_{M3}). In the fourth construct, K_{234} and E_{235} are mutated to glycine, leading to the loss of the predicted α-helical structure (AR_{M4}). Western blot analysis of extracts containing wtAR or AR mutants shows no difference in protein expression levels. The separate mutations in helices 1 and 2 lead to a >2-fold decrease in AR activity on the TAT-GRE elements, whereas AR_{M4} shows even a slight increase in activity. Furthermore, the combination of the mutated helices 1 and 2 leads to a 5-fold reduced activation potential. This region is conserved among AR from different species (Fig. 3*C*). We will now call the two first helices the core Tau-1 activation domain of AR.

Mutation analysis of core Tau-1 of the hAR. To assess the relative importance of the core Tau-1 in AR activity, we did a mutation analysis of helices 1 and 2 in the full-size AR (Fig. 3*D*). Helix 1 has been predicted to form an acidic amphipathic α-helix (9, 27, 28). From Fig. 3*B and D*, it is obvious that the hydrophobicity and the helical structure correlate with activity because alanine and asparagine substitutions at positions L_{178}, I_{181}, L_{182}, and L_{191} (Fig. 3*D*) have decreased AR activity. Alanine substitutions of K_{179} and S_{183} (AR_{M7}, AR_{M9}, and AR_{M23}) lead to an increased AR activity. Although single mutations of the charged amino acids D_{177}, D_{180}, and E_{184} (AR_{M5}, AR_{M8}, and AR_{M10}) do not have an effect on AR activity, triple mutation of these charged residues into alanines (AR_{M22}) lead to a 2-fold decrease. The overall results obtained in HeLa cells are similar to what is shown for COS-7 cells in Fig. 3*D* (data not shown).

Western blot analysis revealed no difference in expression levels for wtAR or any of AR mutants. Furthermore, gel shift assays showed similar DNA binding for all constructs to the TAT-GRE element, and we could not detect changes in cellular localization and hormone binding (data not shown).

Core Tau-1 is an autonomous transcriptional activation domain. Because the core Tau-1 is involved in the transactivation by AR, we tested whether this region might contain an autonomous activation function by fusing it to the Gal4DBD and testing its

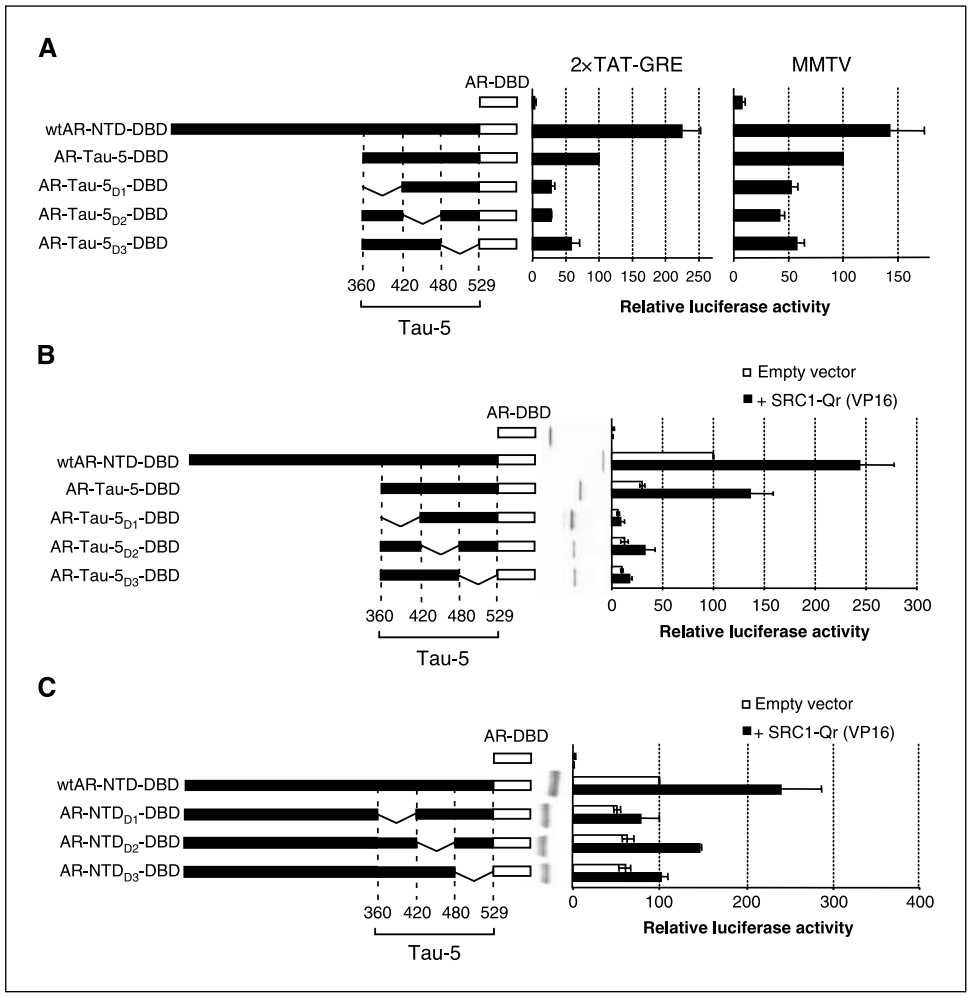

Figure 2. Deletion analysis of the transcription activation function Tau-5. *A,* Tau-5 as an autonomous transcription activation function. *Left,* COS-7 cells were transfected with either an expression vector for the AR-DBD, AR-NTD-DBD, or AR-Tau-5-DBD fragments (50 ng/well). As reporter constructs, 2×TAT-GRE(E1b)-Luc (100 ng/well) and the CMV-βGal (5 ng/well) were used. Activities are depicted relative to the activity of the AR-Tau-5-DBD construct, which was set on 100. *Columns,* mean; *bars,* SE. For Western blot analysis of the cell extracts, see (*B*). *B-C,* two-hybrid assays for the interaction between AR-Tau-5-DBD (*B*) or AR-NTD-DBD (*C*) deletion fragments and SRC1-Qr. COS-7 cells were transfected with the expression constructs (*left*), together with either the empty pSNATCH-II expression vector encoding the VP-16 activation domain (*open columns*) or the same expression vector encoding SRC1-Qr-VP-16 fusion (50 ng/well) (*black columns*). Assays were done using the 2×TAT-GRE(E1b)-Luc reporter (100 ng/well) and the CMV-βGal reporter (5 ng/well). Activities are depicted relative to the activity of the AR-NTD-DBD construct in the absence of SRC1-Qr-VP-16, which was set on 100. *Columns,* mean; *bars,* SE. Western blot analysis of the cell extracts containing AR-NTD-DBD and fragments using anti-Flag antibody were done as described in Materials and Methods.

activity on a luciferase reporter gene controlled by Gal4 response elements (Fig. 4*A* and *B*). The core Tau-1_{M1-3}, core Tau-1_{M17}, core Tau-1_{M20}, and core Tau-1_{M21} mutations corrupt transactivation, whereas the core Tau-1_{M23} mutation enhances the transactivation potential of core Tau-1 by >3-fold. This correlates very well with the effect of these substitutions in the full-size AR (Fig. 3*D*). Fusion of more than one copies of core Tau-1 to the Gal4DBD leads to a gradual increase in intrinsic activity when tested in the mammalian one-hybrid assay (Fig. 4*C*).

Involvement of core Tau-1 in the interaction of the NTD with AR-LBD. It has been proposed earlier that an LKDIL-motif, here helix 1 of core Tau-1, also plays a role in the *N:C* interaction (9). To test the involvement of core Tau-1 in these interactions, a two-hybrid assay was done with mutant AR-NTDs fused to the VP-16 activation domain (Fig. 5*A*). Clearly, all AR constructs with mutated hydrophobic residues (AR-NTD_{M1-3}, AR-NTD_{M17}, and AR-NTD_{M20-21}) show a decreased interaction (~50%). Furthermore, AR-NTD_{M22} and AR-NTD_{M24} show an increase in *N:C* interaction.

Core Tau-1 controls the interaction of SRC1-Qr with the Tau-5 domain of AR. We subsequently tested whether the core Tau-1 activity is due to a possible interaction with SRC1-Qr in a mammalian two-hybrid assay. Wild-type AR-NTD and fragments were fused to the VP-16 transactivation domain and coexpressed together with the SRC1-Qr fragment fused to the Gal4DBD domain (Fig. 5*B*). As expected, the AR-NTD interacts well with SRC1-Qr, whereas the first part of the AR-NTD (AR-NTD aa 1-360) and core Tau-1 do not interact. However, deletion of core Tau-1 positively influences the interaction of Tau-5 with SRC1-Qr. Western Blot analysis of the constructs revealed equal expression of the different constructs.

To analyze this interplay in more detail, we tested mutations in the core Tau-1 region for their effect on binding of AR-NTD with SRC1-Qr (Fig. 5*C*). The luciferase expression in the two-hybrid assay clearly indicates a comparable interaction between the AR-NTDs with substituted hydrophobic residues (AR-NTD_{M1-3}, AR-NTD_{M6}, AR-NTD_{M17}, AR-NTD_{M20}, and AR-NTD_{M21}) and SRC1-Qr compared with wtAR-NTD. There is only a small increase when E_{184} was substituted in AR-NTD (AR-NTD_{M10}). Strikingly, the constructs AR-NTD_{M5} and AR-NTD_{M8} show a 2-fold stronger interaction. These effects seem synergistic because substitution of all negatively charged residues in helix 1 (AR-NTD_{M22}) into alanines leads to a striking 13-fold induction compared with the wild-type fragment. The observed effects can not be explained by differences in protein levels.

Both core Tau-1 and Tau-5 are indispensable for intrinsic activity of the AR-NTD and for full AR activity. We already showed a strong autonomous function for Tau-5 (Fig. 2*A*) and core Tau-1 (Fig. 4*B*). From Fig. 6*A*, it is clear that both core Tau-1 and Tau-5 domains contribute to the full intrinsic activity of the AR-NTD. A fusion of the AR-NTD to the Gal4DBD domain shows a

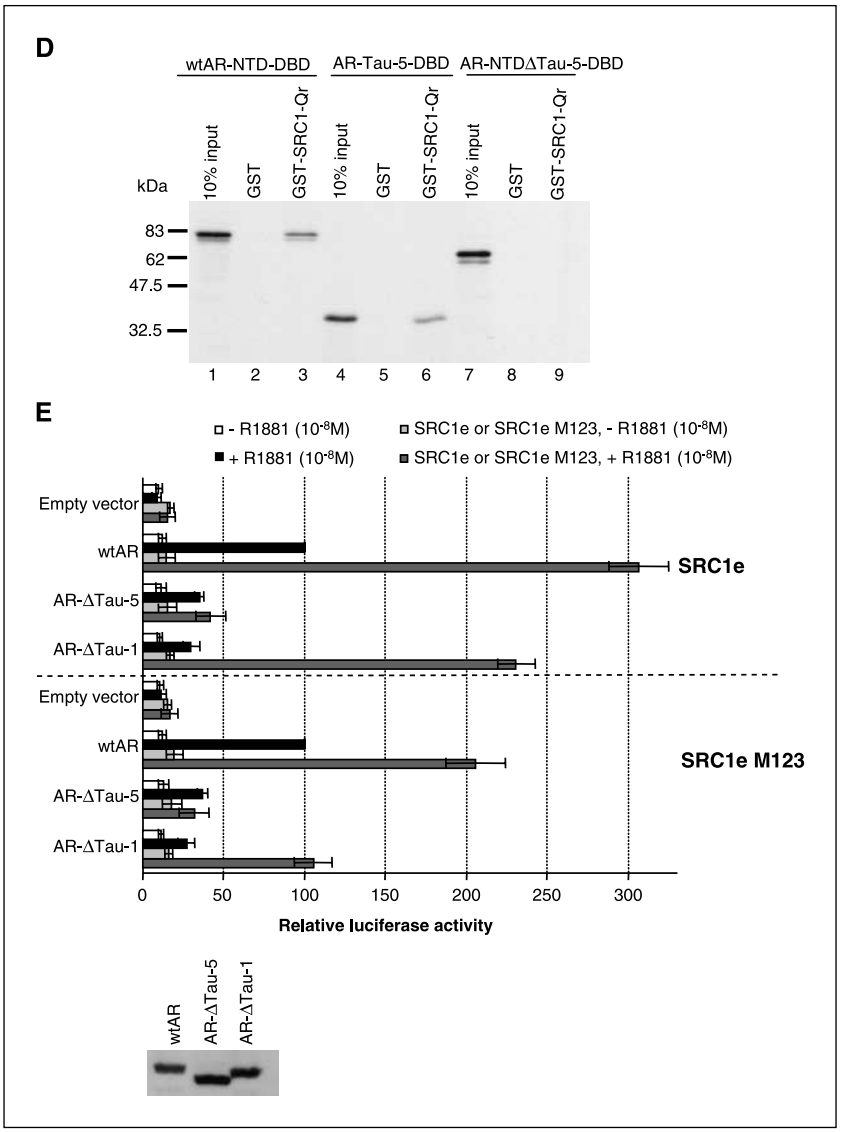

Figure 2 *Continued. D,* GST pull-down assay. wtAR-NTD-DBD (*lanes 1-3*) and the AR fragments AR-Tau-5-DBD and AR-NTDΔTau-5-DBD (*lanes 4-6* and *lanes 7-9*, respectively) were transcribed and translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and incubated with GST or GST-SRC1-Qr beads. Elution was done with SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. The amount of protein loaded in the input lane is equivalent to 10% of the amount of protein assayed in each binding experiment. *E,* coactivation of wtAR and deletion mutants with SRC1e or SRC1e M123. Ten nanograms of expression vector for the wtAR, AR-ΔTau-5 or AR-ΔTau-1 were transfected in COS-7 cells, together with 100 ng of MMTV-Luc and 5 ng CMV-βGal reporter constructs (*top*). *Open* and *black columns,* activity of the AR in the absence and presence of hormone, respectively. *Light gray* and *dark gray columns,* values with SRC1e or SRC1e M123 coactivation, in the absence or presence of hormone, respectively. Activities are depicted relative to the activity of the wtAR in the presence of hormone, which was set on 100. *Columns,* mean; *bars,* SE. Western blot analysis of the cell extracts containing wtAR and AR deletion mutants using anti-Flag antibody were done as described in Materials and Methods (*bottom*).

strong constitutive transcription activation property, and deletion of Tau-5 (AR-NTDΔTau-5) does not abolish activation potency completely. Interestingly, an almost 2-fold decrease in luciferase activity is seen when helices 1 and 2 of core Tau-1 are mutated (AR-NTD$_{M3}$). Combining both (AR-NTD$_{M3}$ΔTau-5) leads to a construct lacking any intrinsic activation potency. Again, the observed effects are not due to differences in protein levels (Fig. 6*A*).

That core Tau-1 and Tau-5 cooperate for the AR-NTD intrinsic activity is also reflected in Fig. 6*B*. Fusion of both Tau-1 and Tau-5 to the DBD of AR leads to a luciferase activity higher than observed for the Tau-5-DBD construct. In addition, the more copies of the core Tau-1 domain fused to the Tau-5-DBD protein, the higher the activity observed. More importantly, also in the full-length AR, both an intact core Tau-1 and an intact Tau-5 are necessary (Fig. 6*C*). Indeed, mutation of core Tau-1 (AR$_{M3}$) or deletion of Tau-5 (ARΔTau-5) each lead to a 5-fold decrease in AR activity. Combining both mutations (AR$_{M3}$ΔTau-5) inactivates AR completely (Fig. 6*C*). Western blot analysis revealed no difference in expression levels for wtAR or any of AR mutants. Furthermore, the mutations did not result in changes in cellular localization or nuclear translocation (Fig. 6*D*).

Discussion

Prostate cancer mutations in the AR gene. Somatic mutations in the *AR* gene have been described in prostate cancer.[1] Here, we report that the lysine-to-arginine mutation at position 179 described in a primary prostate cancer biopsy (19) results in a more potent AR. This is not due to a defect in acetylation because mimicking acetylation by the introduction of a glutamate also results in a more potent AR. Substitution by alanine has a similar potentiating effect (Fig. 1). Therefore, other posttranslational modifications, like methylation or ubiquitylation, or changed interactions with coactivators, or between the NTD and the LBD, could explain this observation. We have analyzed in more detail the activation domains within the NTD in an attempt to unravel its mechanisms of action.

Transcription activation function Tau-5. The large NTD of AR (529 amino acids long) contains a ligand-dependent activation function, called Tau-1, and a constitutively active activation domain, called Tau-5 (18). From a deletion analysis, we conclude

[1] http://www.androgendb.mcgill.ca.

that the integrity of the complete Tau-5 extending from position 360 to 529 is required for its optimal autonomous activation function (Fig. 2*A*). The relative importance of Tau-5 for the AR-NTD activity depends on the response element or promoter used in the assay, pointing to a changing relative importance of Tau-1 and other activation functions within the AR-NTD. Similarly, the importance of the *N:C* interaction and of the SUMOylation of AR at position aa 385 varies according to the enhancer tested (15, 29).

It was described earlier that AR recruits p160 coactivators through an interaction between the AR-NTD and a glutamine-rich domain within the p160s (9–12). Here, we show that the integrity of Tau-5 is a prerequisite for p160 recruitment (Fig. 2*B*). Indeed, a deletion of Tau-5 in the AR or AR-NTD prevented coactivation by SRC1, whereas coactivation is maintained for AR constructs still containing the Tau-5 domain (Fig. 2*C-E*).

Defining core Tau-1 in the hAR. The molecular mechanism of action of Tau-1, the ligand-dependent activation function of the AR-NTD, and that of other nuclear receptors remains obscure. The NH_2-terminal transactivation domains of steroid receptors are the least conserved domains; hence, each is believed to act through alternative mechanisms. For several receptors, putative helices have been suggested as important for the NTD AF-1 function

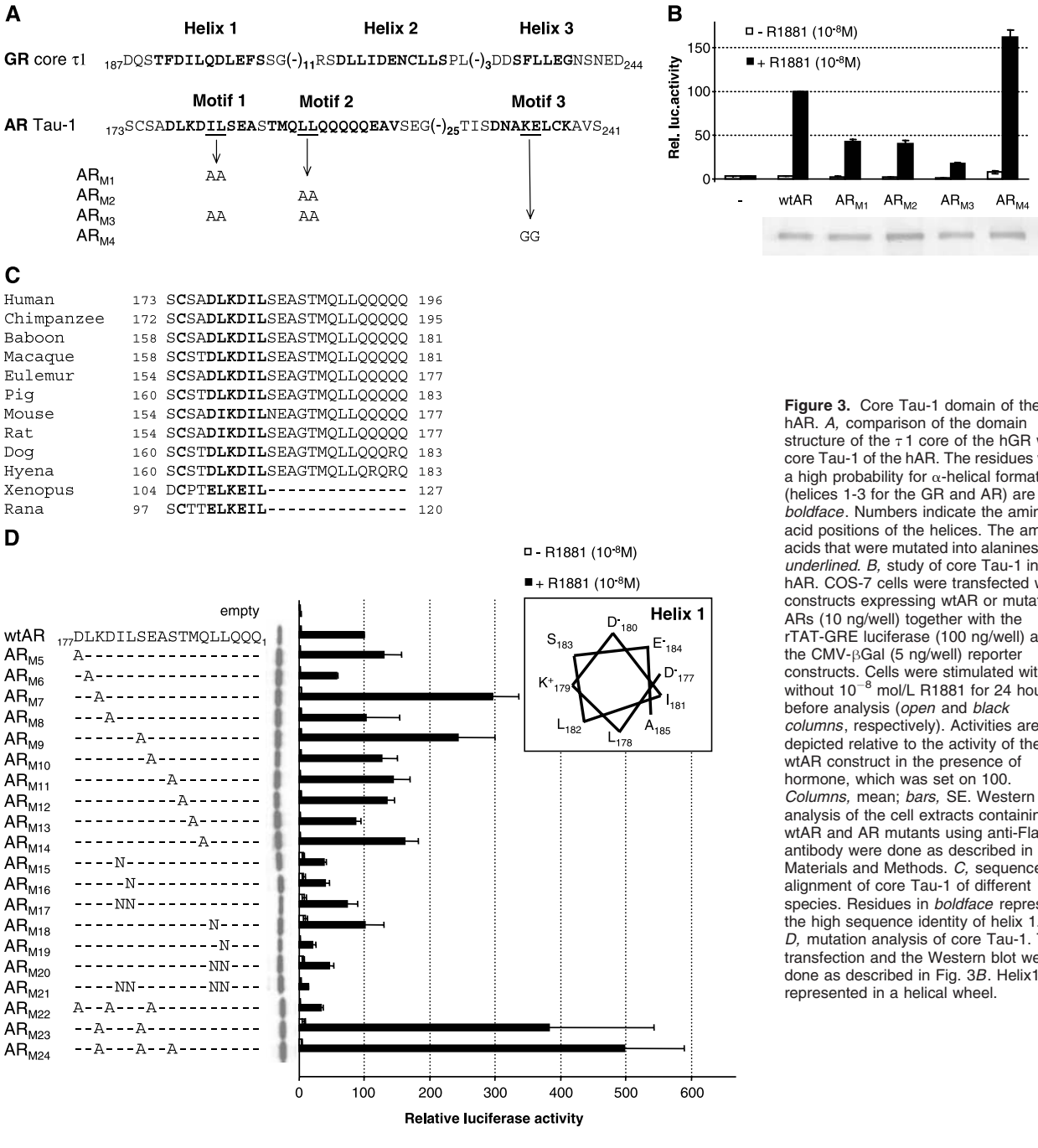

Figure 3. Core Tau-1 domain of the hAR. *A,* comparison of the domain structure of the τ1 core of the hGR with core Tau-1 of the hAR. The residues with a high probability for α-helical formation (helices 1-3 for the GR and AR) are in *boldface*. Numbers indicate the amino acid positions of the helices. The amino acids that were mutated into alanines are *underlined*. *B,* study of core Tau-1 in the hAR. COS-7 cells were transfected with constructs expressing wtAR or mutated ARs (10 ng/well) together with the rTAT-GRE luciferase (100 ng/well) and the CMV-βGal (5 ng/well) reporter constructs. Cells were stimulated with or without 10^{-8} mol/L R1881 for 24 hours before analysis (*open* and *black columns*, respectively). Activities are depicted relative to the activity of the wtAR construct in the presence of hormone, which was set on 100. *Columns,* mean; *bars,* SE. Western blot analysis of the cell extracts containing wtAR and AR mutants using anti-Flag antibody were done as described in Materials and Methods. *C,* sequence alignment of core Tau-1 of different species. Residues in *boldface* represent the high sequence identity of helix 1. *D,* mutation analysis of core Tau-1. The transfection and the Western blot were done as described in Fig. 3*B*. Helix1 is represented in a helical wheel.

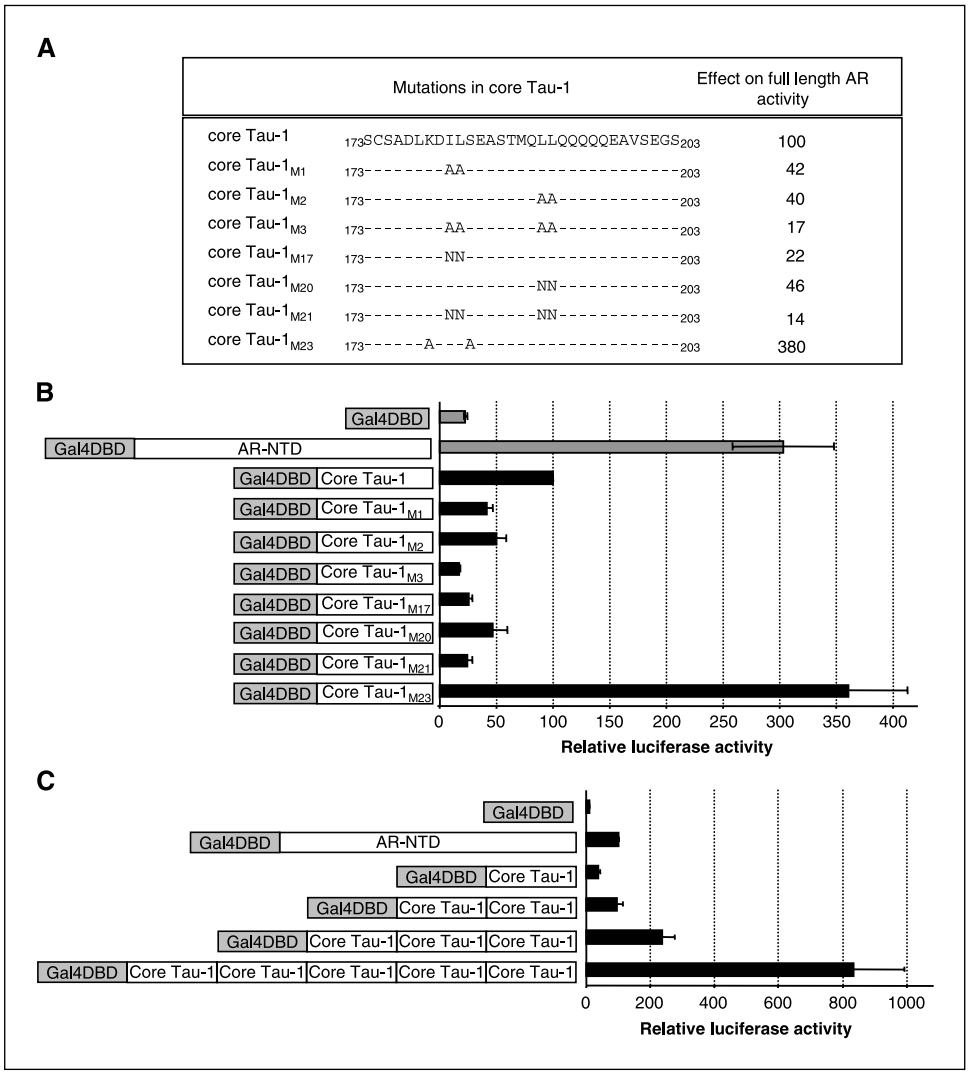

Figure 4. Core Tau-1 of the AR as a strong autonomous transcription activation function. *A,* schematic representation of the core Tau-1 (aa 173-203) mutants. *Left,* mutated amino acids in the core Tau-1 region. *Right,* effect of those mutations on AR activity in the full-length AR, according to the wtAR activity in the presence of hormone (set on 100). *B,* one-hybrid assay. COS-7 cells were transfected with 50 ng of empty pABGal4 (encoding the Gal4DBD), pABGal4AR-NTD (*gray columns*), or pABGal4core Tau-1 (wild type or mutated core Tau-1s, *black columns*), together with the luciferase reporter construct $(Gal4)_5$-TATA-Luc (100 ng/well) and the CMV-βGal reporter (5 ng/well). Activities are depicted relative to the activity of the wild-type core Tau-1-Gal4DBD construct, which was set to 100. *Columns,* mean; *bars,* SE. *C,* one-hybrid assay of multiple copies of core Tau-1. COS-7 cells were transfected and activities were depicted as described in Fig. 4*B* with the constructs used (*left*).

(26, 27, 30, 31). Because the Lys^{179} mutation into arginine resulted in a more active AR and because it is situated in a conserved domain in the AR-NTD (Fig. 3*C*), we did a mutation analysis of the putative α-helices surrounding this lysine. This led to the definition of a two-helical core Tau-1, which is mandatory for proper AR functioning (Fig. 3*A* and *B*).

Mutation analysis of Tau-1. Three α-helices are predicted near Lys^{179} (Fig. 3*A*). In the rat AR, helix 1 was called AF-1a by Chamberlain et al. (27). Besides AF-1a, Chamberlain et al. identified a second transactivation region called AF-1b, which resembles an acidic activation domain. Although this sequence is highly conserved among AR of different species, a deletion of the corresponding fragment (292-351) in the hAR did not alter its transactivation capacity in transient transfections (data not shown). Mutation analysis of helix 1, however, revealed the importance of this region for AR functioning (Fig. 3*D*).

The mutation analysis of core Tau-1 reveals that both the hydrophobic side chains and the negatively charged side of the amphipathic helix 1 are important. Surprisingly, the residues K_{179} and S_{183} (M23) seem to have a repressive effect because their mutation resulted in a much more potent AR (Fig. 3*D*). We also showed the importance of L_{190}/L_{191} in helix 2, as well as its α-helical structure for the Tau-1 function (Fig. 3). We observed a small increased AR activity when helix 3 is mutated (Fig. 3*A*). The latter is in agreement with studies of the group of Greenberg, who observed an enhanced prostate cancer development in transgenic mice expressing an AR-E_{231}G mutant (32).

Taken together, we define the core Tau-1 within the NTD of AR as a two-α-helix-containing fragment with a central role in the proper functioning of the hAR. Core Tau-1 is well conserved among mammalians, but in *Xenopus laevis* and *Rana catesbeiana* AR, only the first α-helix of core Tau-1 is conserved, and in fish AR, core Tau-1 seems completely absent (Fig. 3*C*).

Although Tau-1 was initially described as a ligand-dependent activation function within the AR-NTD, a chimerical protein consisting of core Tau-1 fused to the Gal4DBD activates transcription up to 40% of that observed for a AR-NTD fused to Gal4DBD (Fig. 4). This shows that, next to the autonomous core Tau-5, also core Tau-1 is an important autonomous activation function. The structure-function relationships within this autonomous function is identical to that in the ligand-dependent function, because mutation analysis of core Tau-1 resorted in nearly identical effects in the full-length AR and in the Tau-1 Gal4DBD fusion (Fig. 4*B*). The mutation of K_{179} and S_{183} results in an almost 4-fold more active AR (Fig. 3*A*) and a 3- to 4-fold more active autonomous Tau-1 (Fig. 4*B*). Furthermore, the transactivating properties of the fusion of

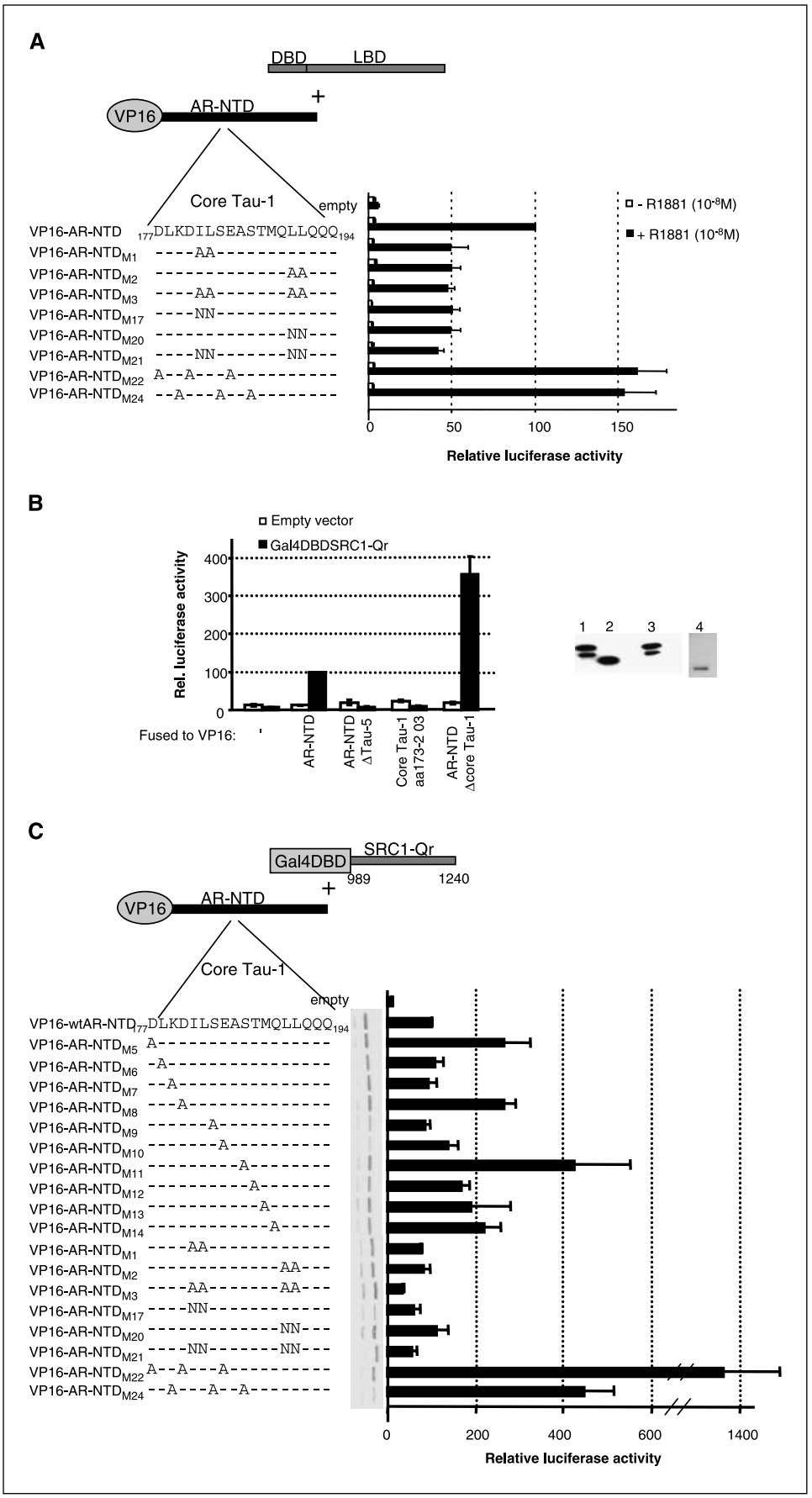

Figure 5. *A,* two-hybrid assay for the interaction between AR-NTD and AR-LBD. pSG5AR-DBD-LBD (aa 538-919; 50 ng/well) was coexpressed in COS-7 cells with either the empty pSNATCH-II expression vector encoding the activation domain of VP16 or the same expression construct containing the wild-type NTD or mutant NTDs fused to it (50 ng/well). Assays were done using the 2×TAT-GRE(E1b)-Luc reporter (100 ng/well) and the CMV-βGal reporter (5 ng/well) in the presence or absence of 10^{-8} mol/L R1881. Activities are depicted relative to the activity of the wtAR-NTD construct, which was set to 100. *Columns,* mean; *bars,* SE. *B-C,* effect of core Tau-1 on the interaction of SRC1-Qr with Tau-5. Deletion mutants in the hAR-NTD and their interaction with SRC1-Qr. pABGal4-DBDSRC1-Qr (989-1240; 50 ng/well) was coexpressed in COS-7 cells with 50 ng of either the empty pSNATCH-II expression vector (*open columns*) or the same expression vector containing the wild-type AR-NTD or the indicated deletion mutants (50 ng/well; *black columns*). Assays were done using the $(Gal4)_5$-TATA-Luciferase reporter (100 ng/well) and the CMV-βGal reporter (5 ng/well). *Columns,* mean; *bars,* SE. Western blot analysis of the cell extracts using anti-Flag antibody were done as described in Materials and Methods [*lane 1,* AR-NTD; *lane 2,* AR-NTDΔTau-5; *lane 3,* AR-NTD ΔcoreTau-1; *lane 4,* core Tau-1 (aa 173-203)]. Since core Tau-1 fused to the Gal4DBD is a small fragment, a longer exposure was necessary. *C,* the same analysis for mutated AR-NTDs as done in (*B*).

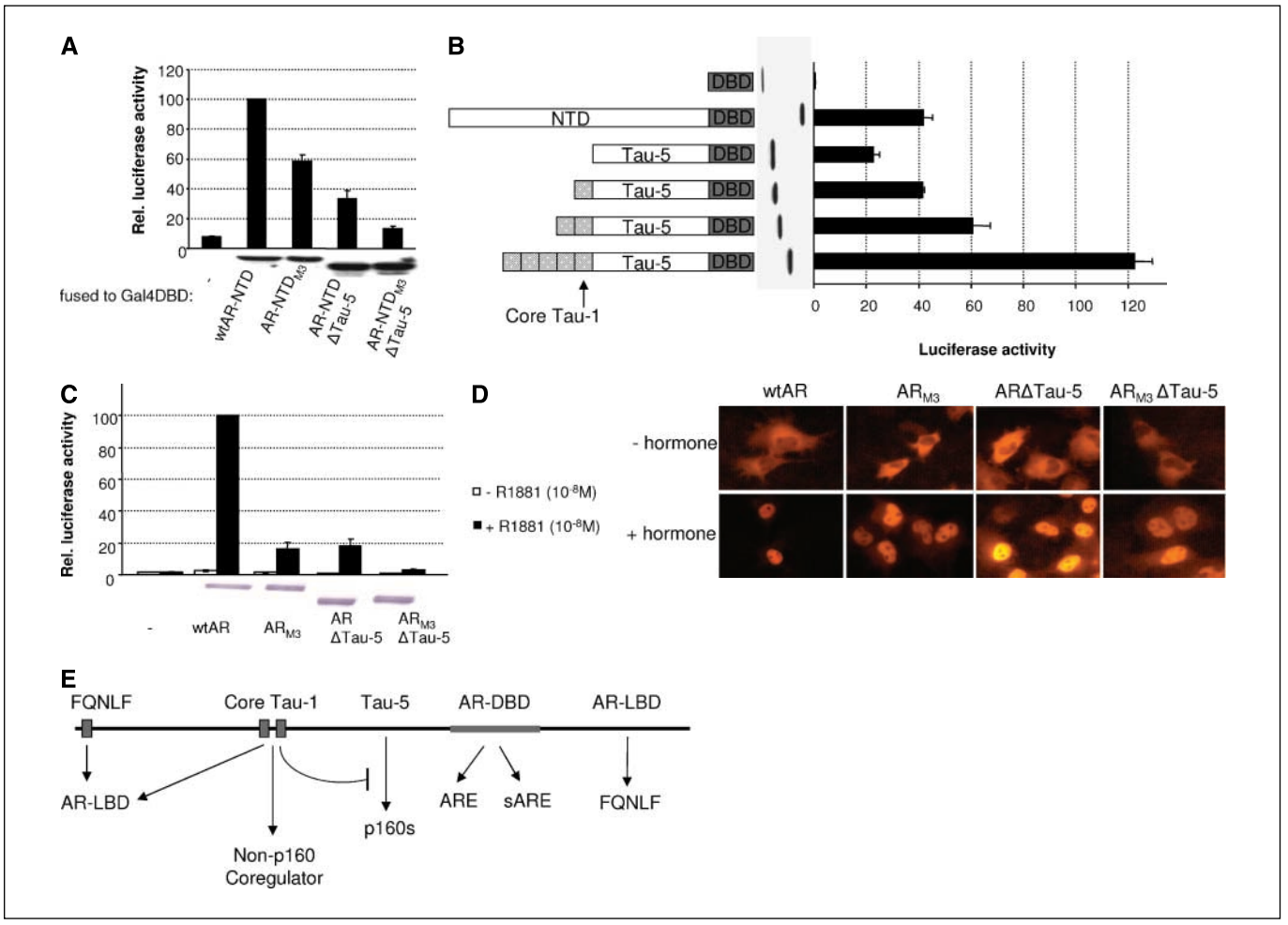

Figure 6. *A,* abrogating core Tau-1 activity and/or Tau-5 activity leads to a decreased intrinsic activity of the AR-NTD. COS-7 cells were transfected with 50 ng of empty pABGal4, pABGal4AR-NTD or its (deletion) mutants, together with the luciferase reporter construct $(Gal4)_5$-TATA-Luc (100 ng/well) and the CMV-βGal reporter (5 ng/well). Activities are depicted relative to the activity of the wtAR-NTD construct, which was set to 100. *Columns,* mean; *bars,* SE. Western blot analysis of the cell extracts containing Gal4AR-NTD and fragments using antiGal4DBD antibody was done as described in Materials and Methods. *B,* core Tau-1 and Tau-5 are both necessary for the full intrinsic activity of the hAR-NTD. *Left,* COS-7 cells were transfected with either an expression vector for the AR-DBD, the AR-NTD-DBD, Tau-5-DBD or with an increasing number of core Tau-1 copies fused to the Tau-5-DBD (50 ng/well). Assays were done using the 2×TAT-GRE(E1b)-Luc reporter (100 ng/well) and the CMV-βGal reporter (5 ng/well). *Columns,* mean; *bars,* SE. Western blot of the cell extracts was done as described in Materials and Methods. *C,* both core Tau-1 and Tau-5 are necessary for the activity of the full-length AR in the presence of hormone. COS-7 cells were expressed with an expression vector containing the wild-type or the indicated mutated ARs (10 ng/well). Assays were done using the 2×TAT-GRE(E1b)-Luc reporter (100 ng) and the CMV-β-Gal reporter (5 ng/well) in the presence or absence of 10^{-8} mol/L R1881 (*open* or *black columns*, respectively). *Columns,* mean; *bars,* SE. Western blot of the cell extracts was done as described in Materials and Methods. *D,* nuclear localization. COS-7 cells were transfected with the indicated ARs. After 1 hour of stimulation, cells were fixed with 3.7% of formaldehyde, permeabilized with Triton X-100 and incubated with the M2 anti-Flag antibody. Immunocytochemical signals were detected by incubation with a TRITC-labeled secondary antibody. *E,* model of the complex interplay of several domains in the AR. See text for details.

multiple copies of Tau-1 to the Gal4DBD increased with the number of Tau-1 copies, indicating its independent function (Fig. 4*C*).

Functional relations between different domains of AR and SRC1-Qr. A strong *N:C* interaction has been correlated with AR function, mainly through the FQNLF-motif at the NH_2-terminal end of AR, and the hydrophobic cleft in the AR-LBD (9, 13–16, 33). Surprisingly, some mutations of helix 1 and 2 of core Tau-1 led to a 2-fold decreased *N:C* interaction, even when the FQNLF motif is present in the construct (Fig. 5*A*; ref. 9). Mutations enhancing the hydrophobic nature of the surroundings of the LKDIL motif enhanced the *N:C* interactions (Fig. 5*A*). This means that not only the LKDIL motif but also residues in core Tau-1 are involved in this interaction. However, because the deletion of the FQNLF-motif abolished the *N:C* interaction (15), core Tau-1 can only be a secondary interaction site for the LBD.

Deletion of Tau-5 abolished the p160 coactivation of the full-length AR (Fig. 2*E*) or the AR-NTD (Fig. 5*B*) almost completely. Although the isolated Tau-1 does not interact with the glutamine-rich region of SRC1 (Fig. 5*B*), the deletion of core Tau-1 in the AR-NTD enhanced the SRC1-Qr recruitment to the NTD >3-fold. Mutation analysis of the core Tau-1 for changes in the interactions between the NTD and SRC1-Qr led to the observation that some mutations indeed increased this interaction (Fig. 5*C*). The mutation of the three negatively charged residues in helix 1 (ARM_{22}) increased the interaction 13-fold. This mutation in the full-length receptor did not increase the androgen response in COS-7, CHO-K1, and HEK293 cells, but a 2-fold increase in AR activity of ARM_{22} was seen in HeLa and U-2OS cells (data not shown), indicating that cell-specific factors may be involved. Mutation of the positively charged amino acids (ARM_{24}) led

to both an increased SRC interaction and an increased AR activity.

In conclusion, although Tau-1 is not a primary interaction site for p160 coactivators, it does affect the p160/Tau-5 interactions. Because there is no direct interaction of core Tau-1 with SRC1, and no interdomain interactions between Tau-1 and Tau-5 (data not shown), this effect must be indirect (e.g., via induction of a conformational change), or the recruitment of a secondary interaction partner. If the role of core Tau-1 would only be the induction of an activating change of conformation in Tau-5, adding multiple copies of core Tau-1 should not have an additional effect on Tau-5 fused to Gal4DBD. However, the transactivating properties of these fusions increased with the number of core Tau-1 copies. These results might, therefore, be explained by the involvement of another coactivating partner for Tau-1. Although several candidate coactivators for AR AF-1 have been reported (34), they should now be tested against the mutations in core Tau-1 described here.

Tau-1 and Tau-5 explain the transactivating properties of AR. The AR-NTD is a very potent activation domain. When core Tau-1 or Tau-5 are mutated, its potency is diminished, and when both mutations are combined, the NTD is inactivated (Fig. 6*A*). Interestingly, the fusion of one copy of each Tau-1 and Tau-5 led to a transcription factor more potent as the Tau-5 alone, indicating that the two Taus are the major players in the transactivation by this domain (Fig. 6*B*).

When Tau-1 and Tau-5 mutations are introduced in the full-length AR, it is almost completely inactivated (Fig. 6*C*), indicating their important role in transactivation and also illustrating the weakness of the AR AF-2.

In conclusion, we propose the following steps in AR induction of transcription (Fig. 6*E*). AR-LBD recognizes ligand and induces the nuclear translocation and DNA-binding of AR to either classic or selective AREs (35). The absence of an activation function in the AR-LBD is probably explained by the strong intramolecular *N:C* interaction, mediated via the FQNLF motif and enhanced by the LKDIL motif. This interaction prevents the recruitment of LxxLL-containing coactivators to the AR-LBD but stabilizes the interaction of the agonist with AR (36, 37). It is a new finding that for the full activity of AR, a concerted interplay between Tau-1 and Tau-5 is necessary and sufficient to explain the transactivation. Tau-5 is the recruitment surface for the p160s. We have assigned an autonomous transactivation function to a core Tau-1, which is also indirectly involved in the recruitment of p160s to Tau-5. This model explains the enhanced transactivating properties of mutant ARs described in some prostate cancer biopsies and indicates the existence of an additional non-p160 coactivator, which acts through Tau-1.

Acknowledgments

Received 7/8/2005; revised 9/21/2005; accepted 10/19/2005.

Grant support: Geconcerteerde Onderzoeksactie van de Vlaamse Gemeenschap, Fonds voor Wetenschappelijk Onderzoek, Vlaanderen, Association for International Cancer Research, and Congressionally Directed Medical Research Program: Prostate Cancer Research Program award DAMD17-02-1-0082.

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We thank R. Bollen, H. Debruyn, and K. Bosmans for their excellent technical assistance and our colleagues of the Molecular Endocrinology Laboratory for helpful discussions.

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