The presence of a transcription activation function in the hormone-binding domain of androgen receptor is revealed by studies in yeast cells

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Abstract To assess the importance of various regions of the androgen receptor (AR) in transcriptional regulation, we have compared its activation functions (AFs) in yeast and mammalian cells. The receptor's amino-terminal region contains a major transcriptional activator (AF-1) in both cell types, whereas AF-2 in the ligand-binding domain (LBD) is very weak in mammalian cells but clearly functional in the yeast. Hormone-binding ability of LBD is mandatory for AF-2 to operate, as illustrated by mutated LBD constructs. The activity of AF-2 in yeast is severely attenuated when the hinge region is attached to LBD, suggesting that the former region modulates AF-2 in vivo, probably by presenting an interface for interacting proteins.
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Key words: Androgen receptor; Transcription; Ligand-binding domain; Yeast

1. Introduction

Androgen receptor (AR) is a ligand-inducible transcription factor that mediates the effects of male sex steroids to the transcription machinery [1]. AR is a member of the nuclear receptor superfamily that comprises receptors for steroids, thyroid hormones, and retinoids [2,3]. AR, like other nuclear receptors, is a modular protein that encompasses three separate and interchangeable domains with specific functions in transactivation, DNA recognition and hormone binding [2,3]. Previous studies from this and other laboratories have shown that the amino-terminal half of AR contains sequences mandatory for both activation and repression of transcription [1,4,5]. Very little is known about functional interactions between activation regions of transcription factors in general, and there is no clear sequence similarity in the amino-terminal transactivation domains (AF-1s) amongst the members of the nuclear receptor family [2,3]. Besides AF-1, another weaker transcription activation function (AF-2) has been identified in the ligand-binding domain (LBD) of other steroid receptors [3], but the presence of a comparable function in AR has not been demonstrated. Moreover, the functional role of the hinge region residing between the DNA-binding domain (DBD) and LBD has remained elusive.

The basic mechanisms controlling RNA polymerase II-catalyzed transcription are conserved throughout the eukaryotic kingdom, and many mammalian transcriptional activators also function in yeast and vice versa. For example, various nuclear receptors behave as ligand-inducible transcriptional activators of reporter genes when expressed in the yeast *Saccharomyces cerevisiae* [6–11]. To gain better understanding of AR function, we have compared its transcription activation functions in yeast and mammalian cells. Our results indicate that the amino-terminal AF-1 is the major activating region of this receptor in both cell types, whereas AF-2 located in LBD functions poorly in mammalian cells, but is quite active in the yeast. The activity of AF-2 is severely attenuated by the presence of the hinge region, suggesting that this latter region modulates – probably via protein–protein interactions – the activity of LBD.

2. Materials and methods

2.1. Materials

[^{3}H]Mibolerone and [^{3}H]acetyl-CoA were from New England Nuclear Corp. (Boston, MA). Casodex and hydroxyflutamide were gifts from Zeneca Pharmaceuticals (Macclesfield, UK). Restriction endonucleases and other DNA-modifying enzymes were purchased from Pharmacia Biotech (Uppsala, Sweden). pLex-a vector encoding bacterial LexA and yeast strain L40 [*MATa trpl-901 his3D200 leu2-3,112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ*] were gifts from Dr. S. Hollenberg [12] (Vollum Institute, Oregon Health Sciences University, Portland, OR). Yeast construct pGBT9 expressing *Saccharomyces cerevisiae* GAL4 DBD fusion proteins and the corresponding mammalian construct pM, pVP16 expressing fusion proteins with the transcriptional activation domain of herpes simplex virus (VP16), pG5CAT containing five GAL4-binding sites in front of the adenovirus E1b TATA box sequence driving the chloramphenicol acetyltransferase (CAT) gene, and the yeast strain SFY526 were purchased from Clontech (Palo Alto, CA).

2.2. Construction of expression plasmids

LexA expression vectors encoding LexA fused to parts of LBD of human (h) AR [pLexA-LBD(657–919), pLexA-LBD(668–919), pLexA-LBD(657–909), amino acid residues in parentheses], to a hinge region-containing LBD [pLexA-HLBD(624–919)] and to a rat (r) AR lacking only the two amino-terminal residues [pLexA-rAR] were constructed by polymerase chain (PCR) reaction using Vent polymerase (New England Biolabs, Beverly, MA) and cloned in-frame to the carboxyl-terminus of LexA (Fig. 1A). Gal4 expression vectors encoding GAL4 DBD fused to two amino-terminal (NT) transactivation regions of hAR [pGAL4-NT(141–407) and pGAL4-NT(141–340)] and to LBD of hAR [pGAL4-LBD(657–919)] were also made by PCR and cloned in-frame to pGBT9 expression vector (Fig. 1B). Mammalian expression vector pM-LBD(657–919) was constructed by inserting the indicated residues of hAR in-frame to GAL4 DBD. Correct nucleotide sequences of all constructs were verified by DNA sequencing using the Pharmacia ALF Express system (Pharmacia, Uppsala, Sweden).

2.3. Reporter gene activity assays

Yeast strains L40 and SFY526 were transformed with LexA and GAL4 fusion protein expression vectors, respectively, to generate a series of strains expressing the AR constructs. These strains were used to inoculate liquid media without ura and trp (only trp for GAL4 constructs) in the presence or absence of 50 nM testosterone ± 5 μM antiandrogen, and the cultures were grown overnight at 30°C. One-ml

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aliquots of the cultures were used to inoculate 8 ml of YPD media, and the cells were grown at 30°C with shaking until $OD_{600\ nm}$ reached a value of 0.5–1.0. β-Galactosidase activity in yeast cells was measured according to the instructions of the Clontech Matchmaker Two-Hybrid System. Chinese hamster ovary (CHO) or green monkey kidney (CV-1) cells (both from ATCC) were maintained in Dulbecco's minimal essential medium containing penicillin (25 U/ml), streptomycin (25 U/ml), 10% (v/v) fetal bovine serum (FBS) and, for CHO cells, also non-essential amino acids. Cells were transfected using the calcium phosphate precipitation method as described previously [4,5]. β-Galactosidase expression plasmid pSVβ-gal served as an internal control for transfection efficiency. Eighteen hours after transfection, the medium was changed to one containing charcoal-stripped 2% (v/v) FBS in the presence or absence of androgen. CAT and β-galactosidase activities were assayed as previously described [4,5].

2.4. Ligand-binding assays in yeast cells

Selective media were inoculated with a single colony of yeast, and the cultures were incubated by shaking overnight at 30°C. Yeast cells were then diluted to $OD_{600\ nm}$ of 0.2 using YPD medium, and [^{3}H]mibolerone was added to a final concentration of 50 nM in the presence or absence of a 100-fold molar excess of non-radioactive testosterone. After 2 h at 30°C, cells were harvested by centrifugation, washed twice with cold phosphate-buffered saline and once with distilled water. Cell pellets were resuspended in 0.2 ml of lysis buffer [20 mM Tris-HCl (pH 7.5), 1% $NaDodSO_4$] containing 80 mg of glass beads (Sigma, St. Louis, MO) and vortexed vigorously for 2 min. After centrifugation at $16000 \times g$ for 10 min, the radioactivity in the supernatant was measured by liquid scintillation counting.

3. Results

Transcriptional activation and ligand-binding properties of AR were first studied in yeast cells. Full-length AR or various parts of LBD were fused to LexA, and the resulting expression vectors were used to transform L40 yeast cells, in which transcription of the β-galactosidase gene is under the control of LexA-binding sites. A fusion construct encompassing full-length rAR activated the reporter gene by 140-fold in the presence of 50 nM testosterone (Table 1). The construct with LBD alone [pLexA-LBD(657–919)] was almost as active as full-length rAR, indicating that LBD contains indeed a transcription activation function termed AF-2. Transcriptional activation occurred only in the presence of testosterone. Activation of the reporter gene by androgen was diminished to one-fifth, when residues containing the hinge region (624–656) were present in the construct [pLexA-HLBD(624–919)]. No significant differences in androgen-binding capacity between the three receptor forms were observed: 700–800 receptors per cell were determined for each construct. Interestingly, a 100-fold molar excess of two non-steroidal antiandrogens (casodex or hydroxyflutamide), which abolish the agonist-dependent transcriptional activity of wild-type AR in mammalian cells [13,14], did not inhibit androgen-induced transcriptional activity of either AR LBD or full-length rAR fused to LexA (Table 1).

Transcription-activating and ligand-binding properties of several LBD mutants were next examined. pLexA-LBD(657–919) was truncated by 10 amino acids at each end, yielding pLexA-LBD(668–919) and pLexA-LBD(657–909). Deletion of the first 10 amino acid residues from either the NH_2-terminus or the COOH-terminus of LBD resulted in a complete loss of ligand binding that was accompanied by the loss of AF-2 (Table 1). Additional truncation at either end of LBD restored neither one of the two activities (data not shown).

To study the function of AF-2 of AR in mammalian cells and to compare its transactivation properties with those observed in yeast, LBD of hAR was cloned in-frame to the COOH-terminus of *S. cerevisiae* GAL4 DBD. The chimeric protein was expressed in CV-1 and CHO cells and tested for its ability to activate a reporter gene driven by GAL4-binding sites as *cis*-acting elements. The fold activation in the presence of testosterone in the culture medium was not clearly measurable in CV-1 cells, as the LBD construct appeared to act as a repressor of transcription in the absence of androgen, whereas in CHO cells, transfected pGAL4-LBD brought about a modest 5-fold activation (over GAL4 DBD alone) of the reporter gene in the presence of steroid (Table 2). A 100-fold molar excess of casodex or hydroxyflutamide inhibited AF-2 function activated by androgen by 50% (Table 3). In SFY526 yeast cells, which carry a GAL4-responsive β-galactosidase reporter gene, pGAL4-LBD(657–919) exhibited transcriptional activity comparable to that of pLexA-LBD(657–919), indicating that expression of AF-2 of AR did not depend on the partner to which LBD was fused.

We next compared the transcriptional activity of the major AF-1 of hAR [15] to that of AF-2 as GAL4 DBD fusion proteins in yeast cells. GAL4-NT(141–407) was about 80-fold more efficient in activating GAL4-responsive β-galactosidase reporter gene than GAL4-LBD(657–919) in the presence of testosterone (Table 2). Activation by pGAL4-NT(141–340) was significantly lower than that by pGAL4-NT(141–407) and close to that achieved by ligand-bound pGAL4-LBD(657–919). The N-terminal AF-1 is thus the major transcription activation domain in both mammalian and yeast cells. Moreover, tethering of full-length AR through its amino-terminus to LexA severely restrains the activity of AF-1 (cf. Tables 1 and 2).

Table 1
Functional analysis of LexA-AR fusion proteins in *Saccharomyces cerevisiae*

Chimeric protein construct	Androgen binding	β-Galactosidase activity					
		−Test	+Test	+Cdx	+Flu	+Test +Cdx	+Test +Flu
LexA-rAR	+	≤1	140 ± 1	≤1	≤1	175 ± 34	115 ± 15
LexA-LBD(624–919)	+	≤1	100 ± 1	≤1	≤1	152 ± 6	89 ± 1
LexA-LBD(657–919)	+	≤1	21 ± 0.5	ND	ND	ND	ND
LexA-LBD(668–919)	−	≤1	≤1	ND	ND	ND	ND
LexA-LBD(657–909)	−	≤1	≤1	ND	ND	ND	ND
LexA	−	1	1	1	1	1	1

The numbers depict the amino acids of hAR or rAR fused in-frame to the COOH-terminus of LexA. Fusion proteins were expressed in yeast cells and ligand binding and transactivation properties were analyzed in the presence and absence of 50 nM testosterone (Test), 5 μM casodex (Cdx) or 5 μM hydroxyflutamide (Flu). ND = not determined. β-Galactosidase activities are expressed relative to LexA alone. Mean ± S.E.M. values of three independent experiments are shown.

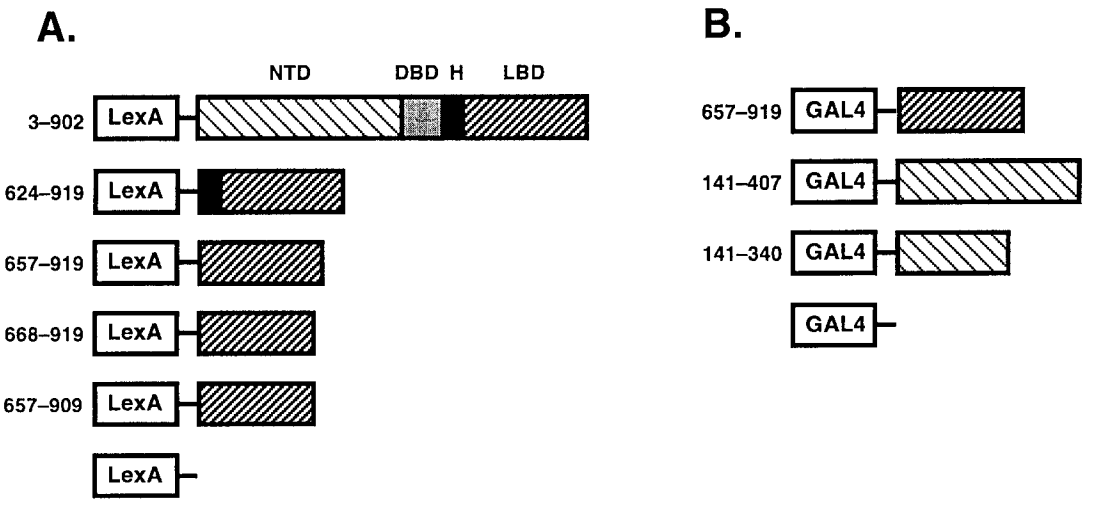

Fig. 1. Schematic representation of LexA-AR and GAL4-AR fusion proteins used for transactivation studies in yeast and mammalian cells. (A) LexA fusion constructs. (B) GAL4 DBD fusion constructs. The numbers depict the amino acids of hAR or rAR (3–902) fused in-frame to the COOH-terminus of LexA or GAL4 DBD. NTD = amino-terminal domain; H = hinge region.

4. Discussion

We have investigated transactivation and ligand-binding properties of AR in yeast and mammalian cells. Our findings demonstrate, for the first time, that LBD of AR harbors a ligand-inducible activation function. Deletion of only 10 amino acids from either end of LBD abolished both transcription activation and androgen binding, indicating that these two functions are connected. It is of note, however, that AF-2 in LBD as a separate domain is considerably weaker than the amino-terminal AF-1 both in yeast and mammalian cells, emphasizing the role of AF-1 as the major transactivation domain. A plausible explanation for the finding that extension of the LBD construct to include the hinge region (LexA-HLBD) lowers in yeast cells steroid-dependent AF-2 activity, but not steroid binding, is that the hinge region provides an interaction interface for inhibitory proteins.

Androgen-induced transcriptional activity of full-length AR fused to heterologous DBD was only marginally higher in yeast cells than that of LBD alone. Given the high inherent activity of AF-1 fusion protein, it is not likely that the attachment of full-length AR through its amino-terminus to a heterologous DBD would cause incorrect folding of the AF-1 region. A plausible explanation for the low activity of full-length AR in this context is that its anchorage to another protein interferes with ligand-dependent interaction between the amino-terminal region and LBD [16] and/or retains the receptor in a conformation that cannot make proper contacts with auxiliary proteins.

It was somewhat unexpected that two non-steroidal antiandrogens, casodex and hydroxyflutamide, were not able to inhibit agonist-induced transactivation by LexA-LBD or LexA-rAR in yeast cells or did so poorly in mammalian cells. These results may reflect the fact that antiandrogens bind very weakly to LBD and that this property, rather than expression of the full complement of biological actions of antiandrogens, was examined under the present experimental conditions. It is worth pointing out, however, that Kralli et al. have recently shown that an ATP-binding cassette transporter is capable of selectively decreasing intracellular levels and thereby biological potency of some synthetic steroids in yeast [17]. In view of this, antiandrogen resistance in yeast cells may be, at least in part, due to selective efflux of these compounds by transporter protein(s).

In conclusion, transactivation properties of AR in yeast cells are in many respects similar to those in mammalian cells, suggesting that principal coactivators or auxiliary proteins mediating the contacts between AF-1 or AR and general transcription apparatus are conserved in these cells. However, the activity of AF-2 in the LBD can be demonstrated much better in yeast than in mammalian cell lines (CV-1 and CHO cells) examined in this work, possibly due to the presence of core-

Table 2
Functional analysis of GAL4-AR fusion proteins in yeast cells and comparison of GAL4-LBD function in yeast and mammalian cells

Chimeric protein construct	Androgen binding	Reporter gene activity (arbitrary units)					
		Yeast		CV-1		CHO	
		−Test	+Test	−Test	+Test	−Test	+Test
GAL4-LBD(657–919)	+	≤1	70±4	0.1	1.3	0.3	4.6
GAL4-NTD(141–407)	−	5510±707	ND	≤1	≤1	ND	ND
GAL4-NTD(141–340)	−	131±13	ND	ND	ND	ND	ND
GAL4	−	1	1	1	1	1	1

The numbers depict the amino acids of hAR fused in-frame to the COOH-terminus of GAL4 DBD. Fusion proteins were expressed in yeast cells or in CV-1 and CHO cells, and assays for ligand binding in yeast cells and transactivation ability ± 50 nM testosterone (Test) in yeast and mammalian cells performed as described in Section 2. The reporter gene activities are expressed relative to GAL4 DBD (GAL4) alone. Mean ± S.E.M. values of three experiments in yeast cells and the mean of two experiments in mammalian cells are depicted.

Table 3
Influence of antiandrogens on transcriptional activation mediated by pGAL4-LBD(657–919) in CHO cells

Androgen or antiandrogen (nM)	Relative CAT activity
Testosterone (50)	100 ± 22
Casodex (5000)	13 ± 3
Hydroxyflutamide (5000)	15 ± 7
Testosterone (50)+casodex (5000)	55 ± 12
Testosterone (50)+hydroxyflutamide (5000)	55 ± 11

GAL4-LBD(657–919) fusion protein was expressed in CHO cells and its transactivation ability measured in the presence of testosterone ± antiandrogen as described in Section 2. For the relative CAT activity, the value for GAL4-LBD(657–919) in the presence of 50 nM testosterone was set as 100%. Mean ± S.E.M. values of three independent experiments are shown.

pressor proteins in the latter. It remains to be elucidated whether these putative inhibitory proteins play any role in cell type- and/or development stage-specific expression of androgen action.

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