The Androgen-specific Probasin Response Element 2 Interacts Differentially with Androgen and Glucocorticoid Receptors*

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The nuclear receptors constitute a large family of transcription factors characterized by a well conserved DNA-binding domain. The receptors for glucocorticoids, progestins, mineralocorticoids, and androgens constitute a subgroup because they bind in vitro with high affinity to DNA elements containing a partial palindrome of the core sequence 5' -TGTCT-3'. In vivo, however, the corresponding steroids differentially regulate members with similar relative affinities. In contrast, one glucocorticoid receptor binds most androgen response elements with similar relative affinities. In contrast, one element (5'-GGTTCTGGAGTACT-3') which was recently described in the promoter region of the probasin gene selectively interacts with the DNA-binding domain of the androgen receptor and not with that of the glucocorticoid receptor. From studies with chimeric elements, it can be deduced that it is the left subsequence 5'-GGTTCT-3' which excludes the glucocorticoid receptor domain from binding.

In co-transfection experiments where the ARE of the C3(1) gene is responsive to both androgens and glucocorticoids, the probasin element is induced only by androgens and not by glucocorticoids. The existence of response elements which are recognized preferentially by the androgen receptor provides yet another possible mechanism to explain the differences of the in vivo effects between androgens and other steroids of the subgroup.

Nuclear receptors are transcription factors which mediate signals of a variety of hormones. Upon ligand binding, the receptors activate transcription by interacting with specific DNA sequences located within or near gene promoters. All members of the nuclear receptor superfamily bind with high affinity to directly or inversely repeated DNA sequences (1) by the DNA-binding domain (DBD) which contains two zinc-finger motifs. Mader et al. (2) have demonstrated that differences between the glucocorticoid receptor (GR) and the estrogen receptor (ER) involving three amino acids located in the so-called P-box, are responsible for the difference in sequence recognition. The GR recognizes the sequence 5'-TGTCT-3', while the ER interacts with the 5'-TGACCT-3' element.

Within the subfamily of steroid receptors, the glucocorticoid, progesterone (PR), mineralocorticoid (MR), and androgen receptors (AR) are closely related (3). Several observations indicate that the GR, PR, MR, and AR recognize the same DNA elements. Firstly, the P- and D-boxes of the GR, PR, MR, and AR are conserved (4); secondly, all four receptors have been shown to bind to different glucocorticoid response elements (GREs) (5–7); and thirdly, in transient transfection experiments, all four receptors can transactivate transcription of reporter genes through these elements (5, 8). Indeed, most GREs are imperfect palindromes of the core sequence 5'-TGTCT-3', and they have been shown to act as progesterone, mineralocorticoid, or androgen response elements (PREs, MREs, or ARs) (5–7). In conclusion, it has been stated that GRE, PRE, MRE, and AREs are not receptor-specific (1, 10). This is, however, in contrast with the observation that in a specific cell type, different steroids evoke different specific effects. The molecular mechanisms underlying these cell-specific steroid responses are of particular interest in cell types which co-express related receptors. Several mechanisms might account for these specificities, including tissue-specific ligand metabolism, regulation of receptor level, or receptor-specific interactions with auxiliary factors which modulate receptor function (11, 12).

The transcription of the probasin gene (pb) and the C3(1) gene in rat prostate is androgen-inducible in vivo. In both genes, AREs have been described: the C3(1) gene contains an ARE (C3(1)ARE) as part of an androgen-responsive unit in the first intron (13, 14), and in the probasin gene promoter two AREs have been found (PB-ARE-1 and -2 (15)).

Here we describe that the DBDs of the AR and the GR (6, 16) bind DNA in a sequence-specific manner. Most AREs or AR-binding sequences display a similar relative affinity for both DBDs. The C3(1)ARE, for example, has a high affinity for both the AR-DBD and the GR-DBD. In contrast, the GR-DBD does not recognize the PB-ARE-2 nor several other derived artificial sequences. The difference in affinity is reflected in functional
assays, in which the PB-ARE-2 element acts as ARE and not GRE in conditions were the C3(1)ARE construct is responsive to both androgens and glucocorticoids. This is the first report of a simple ARE which is specifically recognized by the AR and therefore provides evidence for one of the older hypotheses put forward to explain steroid specificity of in vivo responses, i.e. the existence of receptor-specific response elements (17).

EXPERIMENTAL PROCEDURES

Enzymes were purchased from Pharmacia Biotech Inc., Promega, and Boehringer Mannheim. [γ-32P]ATP was purchased from Amer- sham. X-Ormat S x-ray films were from Kodak. Oligonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer (Milligen Corp., Bedford, MA).

The fragment of the AR cDNA coding for amino acids 533 to 637 (numbering according to Ref. 4) and the corresponding fragment from the GR were cloned 3` to an expression cassette for protein A in pHIT2T (Pharmacia). Procedures for expression in Escherichia coli and subsequent purification of the proteins have been described elsewhere (6, 16). The only modification applied here was the addition of 1 M ZnCl2 to the lysis buffer, which resulted in an increase of the affinity of the prepared proteins for DNA. To exclude variations in quality of proteins, experiments were repeated with three independent protein preparations. No clear differences have been observed.

DNA binding was studied by gel retardation analysis. Constant amounts of the labeled oligonucleotides were incubated with increasing amounts of the fusion protein in 20 μl of binding buffer (10 mM Hapes, pH 7.9, 2.5 mM MgCl2, 0.05 mM EDTA, 10% glycerol, 50 mM NaCl, 1 μg of poly(dI-dC), 0.1% Triton, 1 μg dithiorthreitol). Subsequently, free probe was separated from bound probe by a 90-min electrophoresis at 120 V in a nondenaturing 4% polyacrylamide gel.

The African green monkey kidney cell line CV-1 was obtained from the American Type Culture Collection and maintained at 37°C under 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 4.9 g/liter glucose, 10 mM Hepes, 10 units/ml penicillin, and 10 μg/ml streptomycin (Life Technologies, Inc.), and 10% fetal calf serum (Se- ralab). Transient transfections were done as described (14). Oligonu- cleotides (Table II) were cloned as dimers in the Sal site of a pBLCAT2-derived vector. To lower background activity, the RsrII-BamHI fragment, containing part of the thymidine kinase promoter, as well as the NdeI-HindIII fragment, containing a forskolin-responsive element, were deleted from the original pBLCAT2 (18).

RESULTS AND DISCUSSION

DNA Binding by AR-DBD and GR-DBD Is Very Similar—A receptor fragment containing the DNA-binding domain and the first 30 amino acids from the hinge region of the AR was expressed in E. coli as part of a fusion product with protein A. Sequence-specific DNA binding by this protein has been well documented for the AREs of the MMTV LTR promoter and the C3(1) intron (6, 16). Since then, we and others have described several new sequences that bind the AR or act as AREs (Table I and references therein). These AR-binding sequences were tested for their relative affinity for AR-DBD in comparative gel retardation experiments (Fig. 1). Based on our results, we can classify these sequences as either high or low affinity binding elements (Table I).

The low affinity binding group contains elements which do not display any retardation. Nevertheless, we either found them to bind AR-DBD in footprinting experiments (19–21) or we considered them as candidate AREs in genomic fragments with in vitro affinity for the AR (22, 23). Additional binding sites in the vicinity of these AREs in the genomic context seem to be necessary for the functionality of these elements in transfection experiments (20, 21).

Elements displaying obvious AR binding in our gel retardation experiments (Fig. 1) were classified as high affinity binding. The consensus derived from these motifs (Table I) was identical to the GRE consensus (10), except for a A/G difference at position –7 relative to the central nucleotide. This difference, when introduced in the distal GRE of the MMTV LTR, has no dramatic effect on the recognition by GR or PR (7, 9). The high resemblance to the GRE consensus is in agreement with the results from a DNA-binding site selection assay performed with the AR-DBD fusion protein used in this study (24). Furthermore, when the elements described in Table I were examined for GR-DBD binding, it became clear that all sequences with high affinity for AR-DBD also have a high affinity for GR-DBD (results not shown). Similar observations have been reported for the Slp, TAT, and C3(1) elements (25).

AR-DBD, but Not GR-DBD, Binds to the ARE-2 from the Probasin Gene Promoter—In the second part of this study, we compared the relative affinities of the AR-DBD as well as the GR-DBD for the C3(1)ARE and the PB-ARE-2 (Table I and Fig. 2). The C3(1)ARE has a high affinity for both DBDs, in agreement with the results of transfection experiments in which this ARE can act as a GRE as well (13). The PB-ARE-2 is recognized by the AR-DBD with high affinity, and, when aligned with the C3(1)ARE as in Table I, its partial palin-
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Table II: Sequences of the PB-ARE-2 and C3(1)ARE derived oligonucleotides

| Code     | Sequence                  |
|----------|---------------------------|
| C3(1)ARE | AGCTACAT AGTACG TGA TGGTTCT CAAGGTCGA |
| PB-ARE-2 | AGCTTACAAT GGTTCCT TCC AGTACT TTACGTCGA |
| PB/C3    | AGCTTACAAT GGTTCCT TCC AGTACT TTACGTCGA |
| IR1      | AGCTTACAAT AGTACT TGA AGTACGCA |
| IR2      | AGCTTACAAT GGTTCCT TCC AGTACT TTACGTCGA |
| DR2      | AGCTTACAAT GGTTCCT TCC AGTACT TTACGTCGA |
| 4N       | AGCTTACAAT GGTTCCT TCC AGTACT TTACGTCGA |
| DR3      | AGCTTACAAT GGTTCCT TCC AGTACT TTACGTCGA |

Fig. 2: Comparative gel retardation with AR-DBD versus GR-DBD. Identical amounts of oligonucleotides from Table II were incubated without proteins (lanes 1) or with 5 pmol of AR-DBD (lanes 2), 10 pmol of AR-DBD (lanes 3), 5 pmol of GR-DBD (lanes 4), 10 pmol of GR-DBD (lanes 5). Gels were dried and exposed to X-Omat S films.

Fig. 3: Induction of PB-ARE-2 and C3(1)ARE containing constructs by androgens and glucocorticoids. 10^6 CV-1 cells were transfected with the empty reporter vector (tkcat) or with constructs containing two C3(1)AREs (C3(1)ARE) or two PB-ARE-2 (PB-ARE-2), and CAT assays were performed as described (19). The amount of acetylated chloramphenicol was determined on a Molecular Dynamics PhosphorImager device and expressed as the sum of all pixel values in the area of the acetylated chloramphenicol minus background. Cells were grown in the absence of hormone (white bars), or induced with 10^-9 M R1881 (as androgen; black bars) or dexamethasone (as glucocorticoid; striped bars). All numbers are the mean of three independent experimental values.

PB-ARE-2

Which Characteristics of PB-ARE-2 Are Important for the AR-DBD-specific Binding?—To verify whether the selectivity of the AR-DBD for the PB-ARE-2 was due to the partial palindromic element, and not to the surrounding nucleotides, the underscored elements in the sequence 5'-AGTTCTnnnAGTACT-3' were inserted in the C3(1)ARE environment (PB/C3; Table II). This new sequence, when compared to the PB-ARE-2, has slightly decreased affinity for the AR-DBD (Fig. 2), which indicates that the stabilization of the DNA-protein interaction (28) by the surrounding nucleotides is sequence-dependent. Similar observations have also been made for other AREs (24, 29). However, the PB/C3 element has no affinity for the GR-DBD as measured in our gel retardation experiments (Fig. 2), indicating that it is the imperfect palindrome (as underlined) of the PB-ARE-2 which is involved in the specific receptor interactions.

Gel retardation experiments with other chimeric oligonu-
cleotides (Table II) revealed that an inverted repeat (IR1; in this case identical to a direct repeat) of the 5′-AGTACT-3′ subsequence is recognized by both the GR-DBD and AR-DBD (Fig. 2), while the inverted repeat (IR2) of the 5′-GGTTCT-3′ motif retains the AR-DBD specificity. Much to our surprise, a direct repeat (DR2) of this element, which has a G at position +2, is also bound by the AR-DBD, but not by the GR-DBD. In conclusion, it seems to be the 5′-GGTTCT-3′ motif which excludes the GR-DBD from binding.

The importance of the length of the spacer was illustrated by the absence of retardation of oligomers containing a four- instead of three-nucleotide spacer. Adding a third copy of the 5′-GGTTCT-3′ core to the PB-ARE-2 sequence (DR3) does not increase the amount dramatically, nor does it change the position of retarded oligonucleotide (Fig. 2).

All these data indicate that the AR-DBD is binding the PB-ARE-2 in a classical way (1, 10), as two molecules oriented head to head interacting with the two parts of an imperfect palindrome separated by a three-nucleotide spacer.

Which Characteristics of the Receptor Fragments Could Explain This Difference in Sequence Specificity?—In the DNA-binding domain, defined as the 75-amino acid stretch starting at the first Cys of the first zinc-finger, the GR, PR, and MR differ only at 7 positions, but the AR differs in 17 residues from the less conserved hinge region were included. Mader et al. (30) reported that the affinity of the ER-DBD increases significantly when parts of the hinge region were included in the receptor fragments as used in this study, 30 amino acids from the less conserved hinge region were included. Mader et al. (30) reported that the affinity of the ER-DBD increases significantly when parts of the hinge region were included in the proteins, but changes in sequence specificity have not been reported.

In summary, the AR-DBD has a similar but not identical sequence specificity when compared to the PB-DBD. With the PB-ARE-2 element, we have a first example of a selective ARE. Further investigation of the many recently described androgen-regulated genes (summarized in Ref. 31) may reveal whether other examples of AR-specific elements exist.

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