Heterodimerization is mainly responsible for the dominant negative activity of amino-terminally truncated rat androgen receptor forms

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Abstract Rat androgen receptor (rAR) mutants devoid of the amino-terminal transactivation domain are able to behave as dominant negative regulators of wild-type rAR. To address the underlying mechanisms of the *trans*-dominant negative action, we have examined the roles of the DNA-binding domain (DBD) and the ligand-binding domain (LBD) in this process. Transactivation experiments in CV-1 cells complemented by electrophoretic mobility shift assays revealed that the dominant negative receptor forms repress the function of wild-type rAR mainly through heterodimer formation, rather than through competition for binding to cognate DNA elements. Heterodimerization of receptor forms containing LBDs may take place even in the absence of specific DNA binding.

Key words: Androgen receptor; Dominant negative mutant; Heterodimer formation; Transactivation; DNA binding

1. Introduction

Androgen receptor (AR) is a ligand-inducible transactivating protein that regulates the differentiation, development and maintenance of male reproductive functions, in addition to supporting sexually dimorphic functions of non-reproductive tissues [1]. AR is a member of the nuclear receptor superfamily that comprises receptors for steroids, thyroid hormones and retinoids [2,3]. Nuclear receptors have three major functional regions: an amino-terminal transactivation domain (TAD), a central domain for DNA binding (DBD) composed of two zinc finger motifs, and a carboxyl-terminal domain for ligand binding (LBD). By and large, nuclear receptors bind to DNA as dimeric proteins. Loss of function in any of the three AR domains may generate a receptor form that exhibits no transcriptional activity but attenuates the function of wild-type protein, i.e. the mutant receptor possesses dominant negative activity. In the case of human thyroid hormone receptors, many naturally occurring, functionally deficient receptor forms have been reported to be capable of repressing the function of their normal counterparts in a *trans*-dominant negative fashion, which results in resistance to thyroid hormone (RTH) syndrome [4–6]. In most patients, the mutations leading to RTH are located outside the dimerization interface of the thyroid hormone receptor β, implying that receptor heterodimerization plays an important role in this context [4–6]. Potentially dominant negative forms of estrogen receptors may have some bearing on the progression of breast cancer [7].

We have previously shown that transcriptionally inactive rat AR mutants devoid of the main activation function 1 (AF1) region located in TAD can behave as dominant negative regulators of the transactivation elicited by the wild-type protein [8]. In this study, the underlying mechanism was examined in more detail by using a constitutively active rAR lacking the LBD and a double mutant, rARΔ46–408/C562G, devoid of TAD and deficient in DNA binding due to an amino acid substitution in the DBD. Our results show that heterodimerization, rather than competition for DNA binding, is mainly responsible for the dominant negative behavior of rAR forms devoid of TAD. The rARΔ46–408/C562G mutant inhibited the transactivation function of wild-type rAR more strongly than that of an LBD-deficient, constitutively active rAR form, indicating that LBD plays a role also in androgen receptor dimerization.

2. Materials and methods

2.1. Expression vectors and other materials

pARE$_2$tk-CAT contains two copies of the rat tyrosine aminotransferase (TAT) gene androgen response elements (AREs) inserted upstream of the thymidine kinase (tk) promoter driving the bacterial chloramphenicol acetyltransferase (CAT) gene. The cryptic AP-1 site was deleted from the vector backbone of this construct [9]. The β-galactosidase (β-gal) expression plasmid pSVβ-gal was obtained from Promega (Madison, WI, USA). Rat AR expression vector pSGrAR and the deletion mutant vectors pSGrARΔ46–408 and pSGrARΔ641–902 (numbers refer to the first and the last amino acid deleted) and the double mutant vector pSGrARΔ46–408/C562G were constructed as previously described [8,10,11]. Testosterone was obtained from Makor Chemicals, Ltd. (Jerusalem, Israel). Restriction endonucleases and DNA-modifying enzymes were purchased from Pharmacia Biotech (Uppsala, Sweden). [^{3}H]Acetyl-coenzyme A was obtained from New England Nuclear (Boston, MA, USA). Anti-receptor antiserum raised against AR peptide 3 corresponding to residues 14–32 of rAR has been described previously [12].

2.2. Cell culture and transfections

CV-1 and COS-1 cells (from ATCC) were maintained in Dulbecco's minimal essential medium containing penicillin (25 U/ml), streptomycin (25 U/ml), and 10% (v/v) fetal bovine serum (FBS). Cells were transfected using the calcium phosphate precipitation method as described previously [8,13]. In short, the cells (1.5×10^6) were plated on a 10-cm dish 24 h before adding the precipitate with indicated amounts of expression and reporter vectors. β-Galactosidase expression plasmid, pSVβ-gal (4 μg/10-cm plate), was used as an internal control for transfection efficiency. For the preparation of whole cell extracts, indicated amounts of expression vectors/10-cm dish were transfected by electroporation into COS-1 cells [13]. An electrical discharge was applied by a Bio-Rad Gene Pulser Transfection Apparatus (settings: 300 V and 500 μF). 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-gal activities were assayed as described [8,14,15]. Protein concentration was determined using Bio-Rad reagents according to the manufacturer's instructions. Statistical analyses of the data were carried out using two-tailed Student's *t*-test.

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2.3. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) with whole cell extracts was carried out as previously described [12,13]. Harvested cells were resuspended in a buffer containing 400 mM KCl, 1 mM EDTA, 15% glycerol, 10 mM Na-phosphate (pH 7.4), 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml antipain and 5 μg/ml leupeptin. Cells were lysed by five cycles of freeze-thaw (freezing in liquid nitrogen and brief thawing in 10°C water bath). Cell lysates were centrifuged at $16\,000\times g$ for 30 min. Fifteen μg cell extract were used per assay.

2.4. Immunoblotting

Whole cell extracts from COS-1 cells were resolved by electrophoresis on polyacrylamide gels under denaturing conditions. Proteins were transferred onto Immobilon-P membrane (Millipore, Bedford, MA, USA) and processed as previously described [8].

3. Results

We have previously shown that coexpression of an excess of the deletion mutants rARΔ46–408 (Fig. 1A) and rARΔ38–296, which lack large parts of the amino-terminal region of rAR, abolished almost completely the transactivation elicited by the native androgen receptor, at least in part through formation of receptor heterodimers [8]. These experiments could not, however, distinguish between the formation of functionally inactive AR heterodimers prior to or after the interaction with specific DNA elements. To determine more precisely the mechanism by which this dominant negative function is exerted, interference of the rARΔ46–408/C562G mutant with the function of wild-type rAR and a constitutively active rAR form (rARΔ641–902), devoid of the ligand-binding domain (LBD), was studied. The rARΔ46–408/C562G mutant, in which the fourth cysteine (Cys^{562}) in the first zinc finger is converted to a glycine, does not bind to androgen response elements (AREs) either in vitro or in vivo, as judged by EMSA experiments and promoter interference assays, and has no transcriptional activity of its own (see Fig. 2, Palvimo, J.J., unpublished observations) [16]. Coexpression of a 10-fold excess of rARΔ46–408/C562G attenuated transcriptional activity of the native receptor almost completely in CV-1 cells (Fig. 1B) and thus behaved as a negative regulator almost as efficiently as rARΔ46–408 that binds to DNA with high affinity [8,12,16]. The same 10-fold excess of rARΔ46–408/C562G suppressed the function of rARΔ641–902, which lacks the LBD, only by one-third (Fig. 1). This relatively minor effect was dependent on the conformation of the LBD, as ligand-free rARΔ46–408/C562G (TEST –) did not influence the activity of rARΔ641–902 at all (Fig. 1C, black bars). It is of further note that the receptor form rARC562G with an intact transactivation domain (TAD, Fig. 1A) did not act as a repressor in our assays (data not shown), implying that impaired activation function 1 (AF1) residing in the amino-terminal region of rAR is important in the generation of *trans*-dominant behavior.

To address the underlying mechanisms of the dominant negative behavior in more detail, transfection studies were complemented by EMSA experiments. Native rAR and rARΔ641–902 proteins were expressed alone or together with the dominant negative mutants rARΔ46–408/C562G or rARΔ46–408 in COS-1 cells. Immunoblot analysis of the cell extracts revealed that expression of either rARΔ46–408/C562G or rARΔ46–408 did not modify dramatically the expression of transcriptionally active rAR forms, at least not to

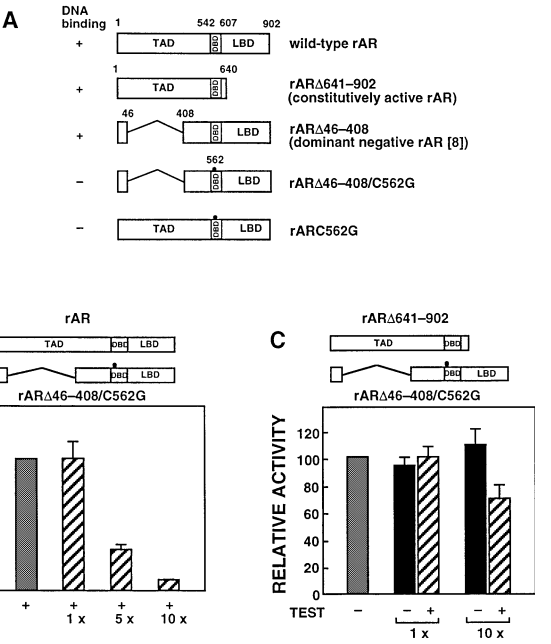

Fig. 1. Influence of the rARΔ46–408/C562G mutant on the function of wild-type rAR and a constitutively active rAR form (rARΔ641–902). A: Schematic structures of rAR forms relevant to this work. Abbreviations: TAD, transactivation domain; DBD, DNA-binding domain; and LBD, ligand-binding domain. The ability of the receptor forms to interact with specific DNA elements (AREs) is depicted by + and − signs. B: Influence of rARΔ46–408/C562G on the function of the native androgen receptor. C: Modulation of the function of rARΔ641–902 by rARΔ46–408/C562G in the presence (+) and absence (−) of androgen. Transactivation ability of the native and constitutively active rAR forms was examined by coexpressing 1–10-fold excess of the rARΔ46–408/C562G mutant (0.2–2 μg of DNA) in a transient expression system. Testosterone (TEST, 10 nM) was included in the culture medium when indicated. Mean ± S.E.M. values for a minimum of three separate experiments are shown. Reporter gene ($pARE_2tk$-CAT) activities are normalized by pSVβ-gal activity and expressed relative to that of wild-type rAR (B) or rARΔ641–902 (C) in the absence of cotransfected rARΔ46–408/C562G (= 100, shown as the shaded bar in both panels).

such an extent as to explain the resulting dominant negative action (Fig. 2A). All receptor forms with intact DNA-binding domains (DBDs) formed specific complexes with ARE as revealed by EMSA (Fig. 2B, lanes 2, 3, and 5). The ARE-rARΔ46–408 complexes migrated clearly faster than AREs complexed with native rAR. A new retarded complex of intermediate mobility was observed, when the native and rARΔ46–408 receptor forms were coexpressed (Fig. 2B, lanes 6 and 7). Heterodimer formation on hormone response elements was dependent on the integrity of the DBD of the dominant negative rAR, as the DNA-binding-deficient rARΔ46–408/C562G mutant failed both to bind to ARE and to form heterodimers that were detectable by EMSA analysis (Fig. 2B, lanes 4, 8, and 9). It is of particular note, however, that cotransfection of native rAR with a 4-fold excess of rARΔ46–408/C562G abolished the formation of ARE-native rAR complexes. By contrast, rARΔ46–408/C562G was not able to perturb significantly the interaction by the LBD-deficient rARΔ641–902 with AREs, which, in agreement with the transactivation data, speaks for an important role of the LBD in the heterodimer formation.

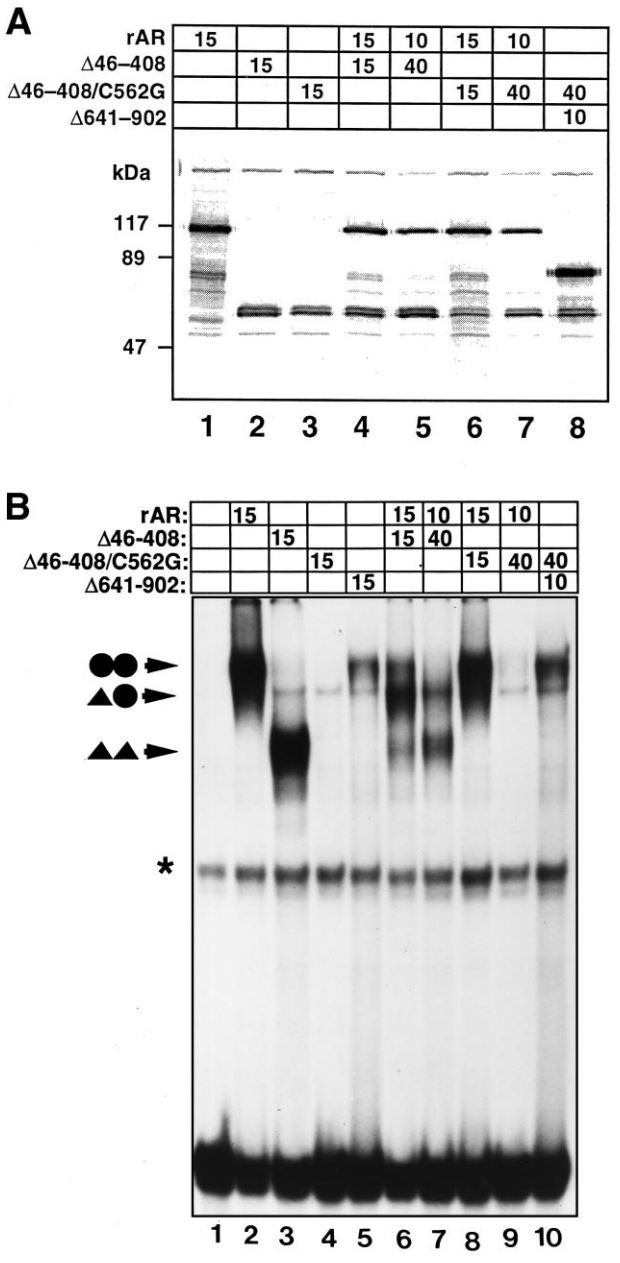

Fig. 2. Immunoblot and electrophoretic mobility shift analyses of rAR forms coexpressed in COS-1 cells. A: Immunoblot analysis of native rAR and its mutants coexpressed in COS-1 cells. Indicated amounts of wild-type and mutant rAR expression vector DNA (in μg) were transfected by electroporation into COS-1 cells. Cells were cultured for 30 h in the presence of 10 nM testosterone. Whole cell extracts were prepared and immunoblot analysis was performed as described in Section 2. Rabbit antiserum raised against a synthetic peptide corresponding to amino acid residues 14–32 of rAR (ARp3) was used. Each lane contained 15 μg protein. Weakly stained bands smaller than native rAR (molecular size ~110 kDa) may originate from proteolytic fragmentation of the receptor. AR deletion mutants rARΔ46–408 and rARΔ46–408/C562G migrate as double bands at ~60 kDa, and the apparent molecular size of rARΔ641–902 is ~70 kDa. B: Detection of receptor homo- and heterodimers by EMSA using cell extracts from the same experiment as in the immunoblot analysis. The samples (15 μg protein) were incubated with double-stranded ^{32}P-labeled C3(1)-ARE oligomer before separating protein-bound from free oligonucleotide by gel electrophoresis under non-denaturing conditions. Symbols: ●●, homodimeric rAR-ARE complex; ▲▲, homodimeric rARΔ46–408-ARE complex; ▲●, heterodimeric rAR-rARΔ46–408-ARE complex. Asterisk depicts non-specific complexes. In addition, a weak band seen in all lanes (e.g. lanes 4 and 9) represents non-specific binding of the [^{32}P]ARE oligonucleotide to an unknown macromolecule.

4. Discussion

The dominant negative action of mutant nuclear receptors on their wild-type counterparts is potentially mediated by at least three different mechanisms, which need not be mutually exclusive: (i) formation of transcriptionally inactive receptor heterodimers; (ii) competition for binding to a cognate DNA element; and (iii) titration of transcriptional coactivators present in limiting amounts in nuclei [17]. Comparison of the *trans*-dominant activity of the rARΔ46–408 mutant devoid of AF1 in the amino-terminal region to a corresponding mutant with no specific DNA binding activity (rARΔ46–408/C562G) demonstrated that, in the case of wild-type rAR, heterodimer formation appears to suffice for the dominant negative action. Transactivation assays along with EMSA experiments indicated indeed that dominant negative rAR forms repress the function of native rAR mainly through heterodimer formation – possibly already in solution – rather than through direct competition for binding to AREs. In our previous experiments, AR mutants were shown to attenuate also the function of the glucocorticoid receptor (GR) [18]. In this latter case, however, competition for interaction with shared hormone response elements, that is DNA binding, as opposed to AR-GR heterodimer formation appeared to play a principal role.

There are other examples to indicate that heterodimerization of steroid receptors may occur even in the absence of specific DNA binding. In the case of human progesterone receptor (hPR), it has been shown that this receptor's A form (hPR-A), when it is otherwise transcriptionally inactive for context-dependent reasons, may act as a *trans*-dominant negative regulator of transcription that is activated by the B form of hPR [19]. The *trans*-dominant action of hPR-A could also be observed by using a hPR-A mutant deficient in DNA binding [19], suggesting that, similar to our findings on rAR in this work, the inhibitory function of hPR-A does not necessarily require DNA binding.

A 10-fold excess of the mutant rARΔ46–408/C562G, an amount that was sufficient to abolish almost completely the function of native rAR, suppressed the activity of the LBD-deficient rAR form (rARΔ641–902) only weakly (Fig. 1B,C). A likely reason for this marked difference in sensitivity is that the LBD is needed for homo- or heterodimerization of rAR proteins to occur in solution. In keeping with results from transactivation studies, EMSA experiments revealed that rARΔ46–408/C562G did not influence the binding of rARΔ641–902 to ARE. Although the major determinants for dimer formation of AR lie in the second zinc finger of the DBD [12], the results of this study indicate that LBD also plays a role in (hetero)dimerization of the androgen receptor.

Taken together, these and previous findings [8,12,16,18] demonstrate that, by taking advantage of the *trans*-dominant negative behavior of selected AR forms, it should be possible to develop expression vectors aimed at specific elimination of androgen action in intact cells. By targeting to specific tissues,

these *trans*-dominant negative androgen receptors may be used as alternative means to knock-out AR function, for example, as a novel therapeutic modality for prostate cancer.

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