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Dimerization Characteristics of the DNA- and Steroid-binding Domains of the Androgen Receptor

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The DNA-binding domain (DBD) of the androgen, mineralocorticoid, and glucocorticoid receptors and the steroid-binding domain (SBD) of the androgen receptor (AR) were expressed separately as fusion proteins with glutathione-*S*-transferase (GST) in *Escherichia coli*. Native polyacrylamide gel electrophoresis and gel exclusion HPLC demonstrated that the GST–ARDBD fusion protein was present as a dimer. On the other hand, the GST–ARSBD fusion protein formed a high-molecular weight oligomer, which seemed to be formed by two separate interactions, i.e. GST–GST and ARSBD–ARSBD between the fusion molecules. These findings strongly suggest that ARSBD has a potent ability to form a homodimer and that ARDBD does not. GST–ARDBD specifically interacted with the glucocorticoid response elements of the mouse mammary tumor virus long terminal repeat (GRE_{MMTV}). Cleavage of the fusion protein by thrombin abolished the binding, while the nonspecific DNA–cellulose binding ability was retained. Therefore, the dimeric configuration of GST–ARDBD, even if accomplished through the interaction with the GST moiety, is needed for high-affinity binding to the response element. The binding of GST–ARDBD to GRE_{MMTV} was strongly competed by the glucocorticoid response element of rat tyrosine aminotransferase gene, followed by the androgen response element of the rat probasin gene. A palindromic thyroid response element showed no competition. Unexpectedly, no apparent different in the binding affinity to these response elements was observed among the DBDs of androgen, mineralocorticoid and glucocorticoid receptors.

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INTRODUCTION

Steroid receptors exert their action through binding to a specific DNA sequence, called a hormone response element (HRE). The consensus sequence of the HRE for the glucocorticoid receptor (GR), i.e. glucocorticoid response element (GRE), is related to, but is distinct from, the HRE for the estrogen receptor (ER) [1]. Mouse mammary tumor virus (MMTV) long terminal repeat contains several sequentially located GREs, which also function as HREs for androgen and progesterone receptors (AR and PR, respectively) [2]. At present, it is unclear whether or not specific response elements for androgen, mineralocorticoid and progesterone, distinct from GRE, are present. Claessens *et al.*[3] reported an androgen response element (ARE) in the first intron of the C3 gene of which expression is primarily regulated by androgens *in vivo*. However, the sequence of this half site is identical to the consensus sequence of GRE, i.e. TGTTCT; and a transfection study revealed that the ARE of the C3 gene also functions as GRE or progesterone response element to a similar extent. Roche *et al.* [4] cloned several putative ARE by means of the *in vitro* DNA-binding site

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Abbreviations: AR, ER, GR, MR and PR, the androgen, estrogen, glucocorticoid, mineralocorticoid and progesterone receptors, respectively; DBD and SBD, the respective DNA- and the steroid-binding domains of the steroid receptor; GST, glutathione-*S*-transferase; MMTV, mouse mammary tumor virus; HRE, ARE, GRE and TRE, the hormone, androgen, glucocorticoid and thyroid response elements, respectively; ARE_{PROB}, ARE2 of the rat probasin gene; GRE_{TAT}, GRE of the rat tyrosine aminotransferase gene; GRE_{MMTV}, GRE of MMTV; TRE_{PAL}, the palindromic TRE; PCR, polymerase chain reaction; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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selection assay. The sequence is comprised of two 6-bp nucleotides with 3-bp spacers, 5′-GGA/TACANN-NTGTTCT-3′, which is similar to that for GREs. Very recently, Rennie *et al.* [5] reported two AREs of the rat probasin gene, which act together to mediate androgen-specific induction. The sequences with which the mineralocorticoid receptor (MR) specifically interacts have not been reported to date.

Biochemical and crystallographic studies demonstrated that one DNA-binding domain of GR and ER interacts with a half site of HRE [6, 7]. Moreover, GR, PR and ER form homodimers before binding to HRE [8–10]. However, homodimerization is not common to all members of the steroid/thyroid receptor superfamily. At least, thyroid and retinoic acid receptors interact with cognate response elements primarily as a heterodimer with 9-*cis* retinoic acid receptor (also called RXR) [11, 12]. Vitamin D_3 receptor interacts with its response element either as a heterodimer with RXR or as a homodimer, depending on the structure of the response element [13]. Recently, it has been shown that, like GR and PR, AR binds to ARE as a dimer in a ligand-dependent manner [14]. However, it has not been clear whether or not AR forms a homodimer before binding to DNA.

In this study, the DBDs of AR, MR and GR, and the SBD of AR were expressed in *E. coli* as fusion proteins with glutathione-*S*-transferase (GST). Utilizing a dimerization/oligomerization characteristic of the GST-fusion proteins, we examined the dimerization ability of ARDBD and ARSBD and showed that ARSBD has the ability to form a dimeric configuration but that ARDBD does not. We also compared the DNA-binding activity of the three DBDs by the gel retardation assay.

EXPERIMENTAL

Materials

[^{3}H]R1881 (80–86 Ci/mmol) was purchased from DuPont-New-England Nuclear (Boston, MA). [α-^{32}P]-dCTP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Restriction enzymes and DNA-modifying enzymes were obtained from Boehringer Mannheim, and Takara (Tokyo, Japan). An expression vector, pGEX-2T, and glutathione–Sepharose 4B were from Pharmacia Biotech (Uppsala, Sweden). Thrombin (54 U/mg protein) and calf thymus double-stranded DNA–cellulose were from Sigma (St Louis, MO). All other reagents were of analytical grade.

Methods

Plasmid construction. The DNA fragments encoding DBDs of human AR, MR, and GR were amplified by the polymerase chain reaction (PCR). Sense primers for DBDs were designed to possess a *Bam*HI site (italics), and the antisense primers were prepared with an *Eco*RI site (underlined) as follows: ARDBD, 5′-GTT*GGATCC*ATTGACTATTACTTTC-3′ and 5′-TCTGCAGAATTCGGCTCCCAGAGTCATC-3′; MRDBD, 5′-ACT*GGATCC*TCAAGACCTTC-3′ and 5′-GACTGCAGAATTCCTAAATTCA-TTC-3′; GRDBD, 5′-TCC*GGATCC*TCAACAG-CAACAAC-3′ and 5′-TCTGCAGAATTCCAG-GTTCATTCC-3′. Primers for the SBD of human AR were designed to possess a *Bam*HI site (italics). The sequences of the sense and antisense primers were 5′-AAGCA*GGATCC*ACTCTGGGAGCCCG-3′ and 5′ - AGG*GGATCC*AATGCTTCACTGGGTG - 3′, respectively. cDNA clones encoding the full length of human AR, MR and GR, generously provided by Drs S. Liao (Chicago University), J. L. Arizza (University of California) and R. L. Evans (Salk Institute), respectively, were used as templates for the PCR. PCR was done for 25 cycles with denaturation for 2 min at 92°C, annealing for 2 min at 50°C, and polymerization for 2.5 min at 70°C. The amplified fragments encoding DBDs were cut with *Bam*HI and *Eco*RI, and inserted into the *Bam*HI/*Eco*RI site of a pGEX-2T vector. The constructed plasmids were designated pGEX-ARDBD for AR, pGEX-MRDBD for MR, and pGEX-GRDBD for GR. *E. coli* Y1090 was transformed with these plasmids separately.

The amplified fragment encoding ARSBD [Fig. 1(a)] was cut with *Bam*HI and inserted into the *Bam*HI site of pGEX-2T. *E. coli* Y1090 was transformed with the ligated plasmid, designated pGEX-ARSBD.

A 139-bp fragment containing three GREs of the MMTV was amplified by PCR with synthetic primers: 5′-CCTTGC*GGATCC*CAGGGCT-3′ (*Bam*HI site represented by italics) and 5′-GATTTGGAT-*GAATTC*CAAAAG-3′ (*Eco*RI site represented by italics). MMTV5031 (Japanese Cancer Research Resources) was used as a PCR template. The PCR product was cut with *Bam*HI and *Eco*RI, and inserted into the corresponding site of pUC18, designated pUCMMTV.

Induction and purification of recombinant proteins. The transformed bacteria were cultured at 37°C in 250 ml LB medium with 50 μg/ml ampicillin. When the A_{600} of the culture had reached 0.8–1.0, the temperature was lowered to 28°C and the bacteria were further cultured for 4–5 h in the presence of 0.2 mM β-isothiogalactopyranoside. The cells were harvested and stored at −80°C until used.

The cell lysate was prepared and the recombinant proteins were purified according to the manufacturer's protocol except that protease inhibitors (20 μg/ml each of leupeptin, soybean trypsin inhibitor, antipain and pepstatin) were added as supplements to the lysate preparation buffer. In the case of GST–ARSBD, the sample eluted from glutathione–Sepharose contained a significant amount of a 27-kDa protein, presumably a proteolysed product of the fusion protein. This sample was further subjected to DEAE-5PW column

(0.75 × 5 cm, Tosoh, Japan) chromatography and eluted with a linear gradient of 0–0.6 M NaCl in 20 mM Tris–HCl (pH 7.3).

Polyacrylamide gel electrophoresis. Bacterial lysates or purified proteins were subjected to electrophoresis at 4°C on 12.5 or 15% (w/v) polyacrylamide gels under denaturing conditions according to Laemmli [15], or on 7.5% (w/v) polyacrylamide gels under nondenaturing conditions [16].

Gel exclusion HPLC on TSK-G3000SWXL. Purified proteins (0.3 ml) were subjected to gel chromatography at 4°C on a TSK-G3000SWXL column (0.78 × 30 cm, Tosoh, Japan), and eluted with 20 mM Tris–HCl (pH 7.3), 1 mM EDTA and 50 mM NaCl. Flow rate was 0.25 ml/min, and 1-min fractions were collected.

Molecular weight markers estimating molecular weights of native forms. For native polyacrylamide gel electrophoresis as well as gel exclusion HPLC analysis, ovalbumin (45-kDa), bovine serum albumin (monomer, 66-kDa; dimer, 132-kDa; trimer, 198-kDa) and catalase (240-kDa) were used as molecular weight markers. Additionally, cytochrome C (13-kDa) and sperm whale myoglobin (17-kDa) were used as markers for gel exclusion HPLC analysis.

Gel retardation analysis. Interaction of the expressed proteins with specific DNA sequences was investigated by gel retardation analysis. The 139-bp fragment excised with *Eco*RI and *Xba*I from pUCMMTV, designated GRE_{MMTV}, was labeled with [α-^{32}P]dCTP and Klenow fragment, and used as a probe for gel retardation analysis.

Purified proteins were subjected to gel retardation analysis as described previously [17]. After electrophoresis, the gels were dried and subjected to autoradiography. In some experiments, radioinert GRE_{TAT}, TRE_{PAL} and ARE_{PROB}, synthesized with a Model 381A DNA synthesizer (Applied Biosystems, CA), were used as competitors. The sequences of GRE_{TAT}, TRE_{PAL} and ARE_{PROB} were 5′-GGGTCTGCTGTACAG-GATGTTCTAGCTACGCCC-3′ [18], 5′-GATC-CAAGATTCAGGTCATGACCTGAGGAGAGC-CTAG-3′ [19] and 5′-CCGGGTAAAGTACTC-CAAGAACCTATTTGCCCGG-3′ [5], respectively.

RESULTS

Expression and purification of the recombinant proteins

Our previous expression studies in *E. coli* revealed lower expression levels of larger molecules of steroid receptors [20, 21]. Accordingly, in this study, we did not express a full-length receptor, but rather expressed functional domains of AR, i.e. DBD and SBD, as fusion proteins with GST [Fig. 1(a)]. DBDs of MR and GR were also expressed [Fig. 1(b)]. As shown in Fig. 1(c) the amino acid sequence of ARDBD is less similar to that of MRDBD or GRDBD compared with the similarity between MRDBD and GRDBD.

The bacterially-expressed GST–DBD fusion proteins, designated GST–ARDBD, GST–MRDBD and GST–GRDBD, were analyzed by SDS–PAGE [Fig. 2(a)]. A predominant band at around M_r 35,000 was found in lysates of *E. coli* Y1090 [pGEX-ARDBD, pGEX-MRDBD, and pGEX-GRDBD] [Fig. 2(a), lanes 1–3], and at M_r 27,000 in the lysate of Y1090[pGEX-2T] (lane 4). These values are in good agreement with the molecular weights calculated from the predicted amino acid sequences of cloned receptor cDNAs [22–24]. The recombinant proteins were purified to near homogeneity by a single-step purification with glutathione–Sepharose affinity chromatography (lanes 5–8).

In the lysate of Y1090[pGEX-ARSBD], specific [^{3}H]R1881-binding was detected (20,455 ± 477 dpm/0.1 ml lysate at 10 nM [^{3}H]R1881), while no specific binding was found in the lysate of Y1090[pGEX-2T]. After purification by glutathione–Sepharose chromatography, M_r 60,000 and 27,000 bands were observed [Fig. 2(b), lane 2]. From the calculation of the predicted molecular weight of GST–ARSBD (60.9-kDa), the M_r 60,000 band was assigned to the fusion protein, and the band with M_r 27,000 appeared to be GST, presumably produced by proteolysis of the fusion protein. The M_r 60,000 band was subsequently purified to homogeneity by ion-exchange DEAE–HPLC (lane 3). The purified samples were used in the following experiments.

Analysis of the molecular configuration of recombinant proteins

To characterize the molecular forms of the expressed proteins, we subjected the purified proteins to native gel electrophoresis. GST–ARDBD migrated at M_r 150,000 (Fig. 3, lane 2) on the native gel. This value was approximately four times as large as the value on SDS–PAGE (M_r 35,000), indicating a tetrameric structure of GST–ARDBD. However, we supposed that GST–ARDBD migrated slower than that expected from its real molecular weight, on native gel electrophoresis, because of the basic amino acid residues of DBD and its coordination with two zinc ions. In fact, the dimeric configuration of GST–ARDBD was ascertained by gel exclusion HPLC analysis as described below (see Fig. 4).

To the contrary, most of GST–ARSBD did not penetrate the gel, but remained concentrated at the top (Fig. 3, lane 1). This indicated that GST–ARSBD formed an oligomeric structure with a large molecular mass. A faint band observed at M_r 140,000 may correspond to a dimer of GST–ARSBD.

The molecular form of the expressed proteins was further characterized by gel exclusion HPLC analysis [Fig. 4(a)]. The native M_r was calibrated in comparison with marker proteins [Fig. 4(b)]. It has been already shown that GST exists as a homodimer [25]. In fact, Fig. 4 shows that GST (M_r 27,000) eluted at the position of a dimer (M_r 55,000). GST–ARDBD (M_r

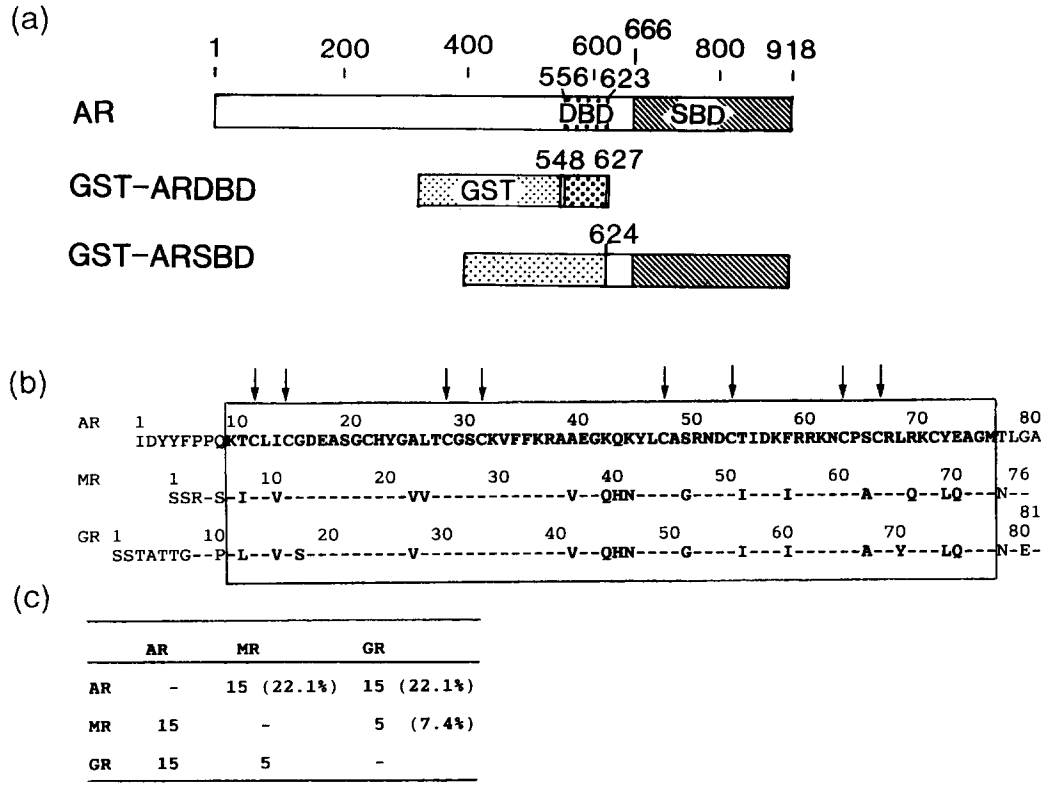

Fig. 1. Schematic illustration of recombinant AR, GST–ARDBD and GST–ARSBD. (a) Human AR, GST–ARDBD and GST–ARSBD are schematically illustrated with putative DBD (amino acid residues 556–623) and SBD (amino acid residues 666–918). (b) The amino acid sequences of AR, MR and GR expressed as GST-fusion proteins are illustrated. A box indicates the putative DBD. Arrows indicate the cysteine residues coordinated with zinc ions. (c) The number of substituted amino acids in DBD (68 amino acid residues) between the receptors and their percentages (parentheses) are represented.

35,000) also eluted at the position of a dimer (M_r 70,000). Therefore, as we postulated above, the migration of GST–ARDBD on native gel electrophoresis was retarded due to the basic nature of DBD. The phenomenon was found even on SDS–PAGE of thrombin-cleaved GST–ARDBD, i.e. ARDBD, as described below (see Fig. 6). Consistent with the results of native gel electrophoresis, GST–ARSBD, forming a high-molecular weight oligomer, eluted at the void volume fraction of the gel chromatography (Fig. 4).

Interaction of the expressed proteins with specific DNA sequences

Gel retardation analysis of GST–ARDBD, GST–MRDBD and GST–GRDBD was performed by use of a 139-bp fragment of GRE_{MMTV} which contains three GREs in its sequence. As shown in Fig. 5, all three fusion proteins bound to the probe, and gave similar shifted band patterns. The increase in input proteins resulted in heavier retardation of the DNA–DBD complexes, because several DBDs interacted with GRE_{MMTV} at higher protein concentrations. Essentially no binding was found with GST. Unexpectedly, a higher protein concentration was needed for GST–GRDBD to obtain an intensity comparable to that of GST–ARDBD and GST–MRDBD.

The junction of GST–ARDBD can be cleaved by thrombin. Following thrombin cleavage, GST–ARDBD was separated into two species, i.e. GST and ARDBD [Fig. 6(a)]. The M_r 13,000 of ARDBD obtained on SDS–PAGE (lane 2) was appreciably larger than that calculated from its cDNA seuqence (9.7-kDa), because of its basic nature. The gel retardation analysis with the cleaved protein revealed a shifted band only at the highest protein concentration examined [Fig. 6(b), lane 10]. Accordingly, a greater than 64-fold molar excess of proteins was needed for ARDBD to obtain a protein–DNA complex comparable to that of GST–ARDBD [compare Fig. 6(b), lanes 2 and 10]. However, both ARDBD and GST–ARDBD similarly bound to calf thymus DNA–cellulose [Fig. 6(c), lane 4]. These results strongly indicate that a dimeric configuration of ARDBD is required for high-affinity binding to HRE. In Fig. 6(c), although a small portion of GST was also retained by the matrix (lane 4), this species may have been derived from a heterodimer of GST and GST–ARDBD bound to the matrix.

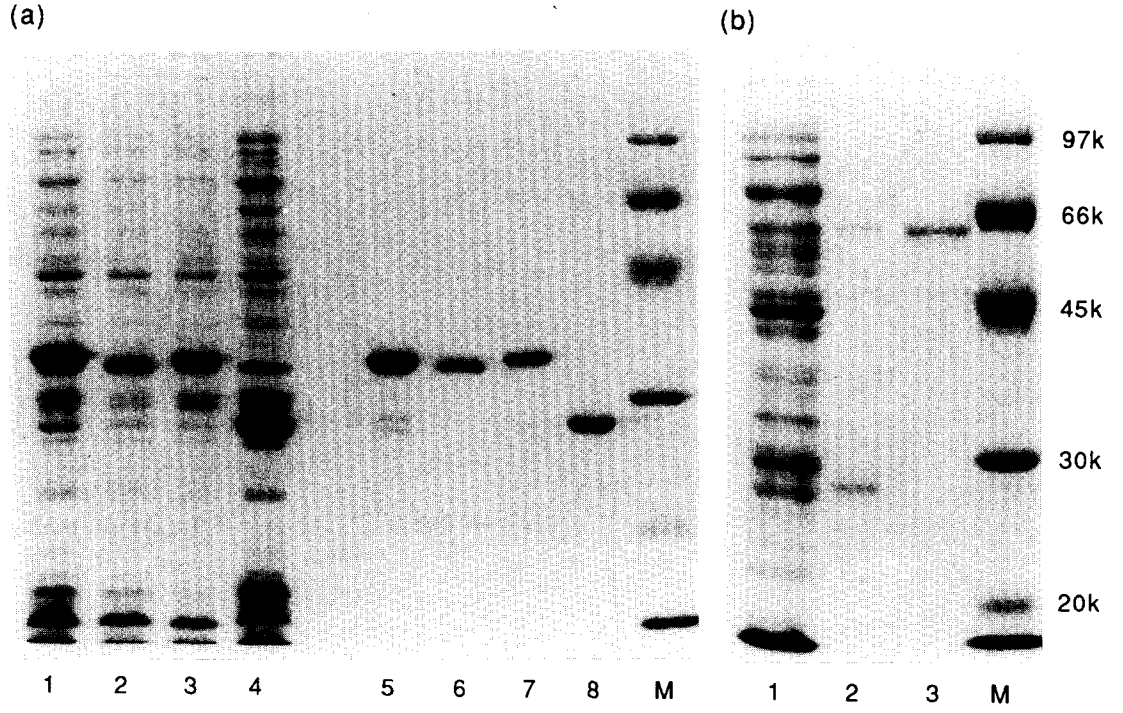

Fig. 2. SDS–PAGE of recombinant proteins. (a) The bacterial lysates (lanes 1–4) and the affinity-purified proteins (lanes 5–8) of *E. coli* Y1090[pGEX-ARDBD], [pGEX-MRDBD], [pGEX-GRDBD], and [pGEX-2T] were analyzed on SDS–PAGE. Lanes 1 and 5, Y1090[pGEXARDBD]; lanes 2 and 6, Y1090[pGEX-MRDBD]; lanes 3 and 7, Y1090[pGEX-GRDBD]; lanes 4 and 8, Y1090[pGEX-2T]. (b) The lysate (lane 1), affinity-purified (lane 2) and DEAE-HPLC purified (lane 3) samples of Y1090[pGEX-ARSBD] were analyzed on SDS–PAGE. M, molecular weight markers.

The DNA-binding specificity of GST–DBD fusion proteins of AR, MR and GR was examined. As a potent response element for androgen, i.e. ARE, Rennie *et al.* [5] recently reported two *cis*-acting DNA elements involved in androgen regulation of the rat probasin gene. Here, we analyzed one of the sequences (designated ARE_{PROB} in this report, and ARE2 in Ref.[5]), because the binding affinity of AR to this sequence is higher than that to the other. The binding affinities of GRE_{TAT} and TRE_{PAL} were also compared.

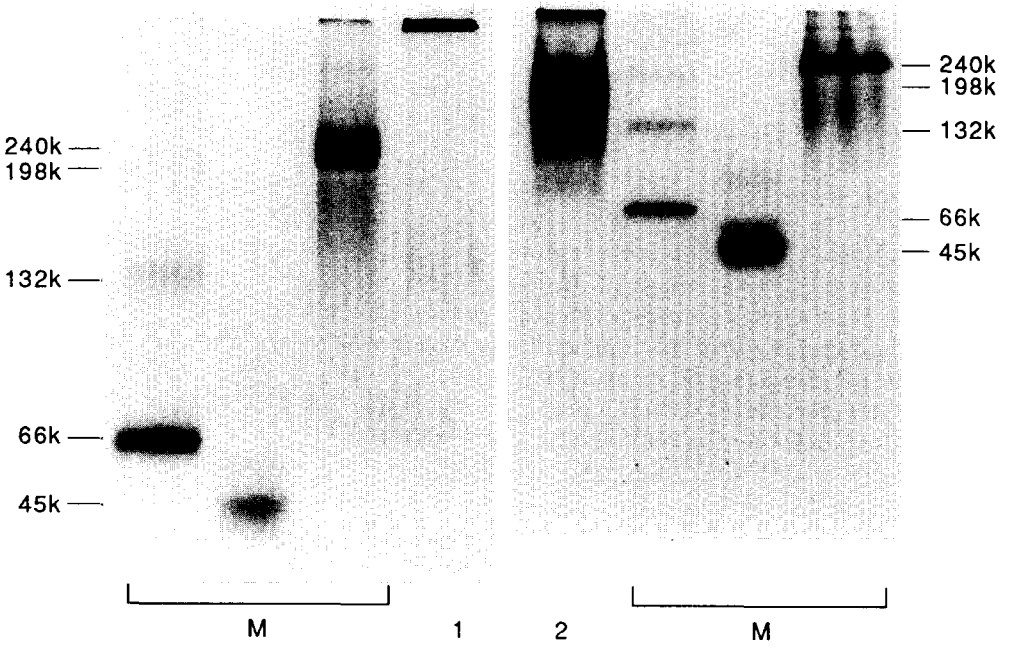

Fig. 3. Native gel electrophoresis of purified proteins. GST–ARSBD (lane 1) and GST–ARDBD (lane 2) were subjected to native gel electrophoresis. Molecular weight markers (M) of native gel electrophoresis were run in parallel.

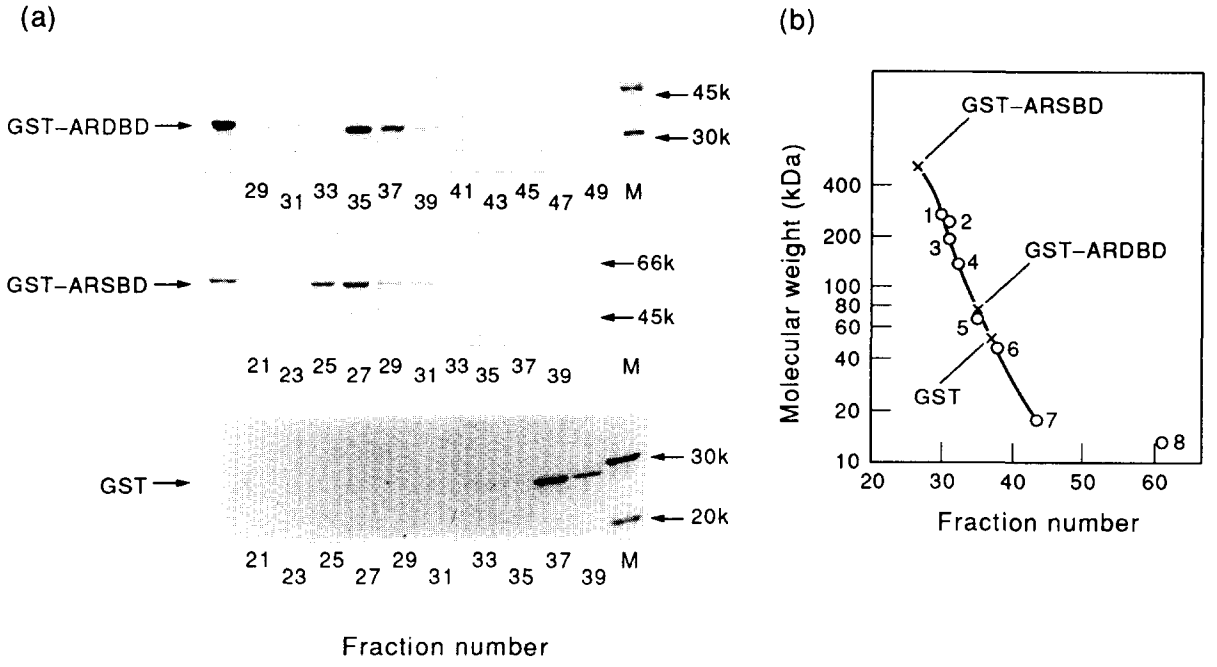

Fig. 4. Gel exclusion HPLC analysis on TSK-G3000SWXL. (a) GST–ARDBD, GST–ARSBD, and GST were analyzed on a TSK-G3000SWXL column equilibrated with 20 mM Tris–HCl (pH 7.3), 1 mM EDTA and 50 mM NaCl. Aliquots (20 μl) of the eluted samples were analyzed on SDS–PAGE. (b) The relative molecular weights of eluted samples in "a" are plotted. Markers are as follows: 1, bovine serum albumin tetramer (264-kDa); 2, catalase (240-kDa); 3, albumin trimer (198-kDa); 4, albumin dimer (132-kDa); 5, albumin monomer (66-kDa); 6, ovalbumin (45-kDA); 7, sperm whale myoglobin (17-kDa); 8, swine cytochrome C (13-kDa).

The binding of GST–ARDBD to GRE_{MMTV} was most strongly competed by GRE_{TAT} and weakly by ARE_{PROB} (Fig. 7). TRE_{PAL} showed no competition within the concentrations used in this study. Completely identical results were obtained with GST–MRDBD. In the case of GST–GRDBD, GRE_{TAT} was the strongest competitor as in the other cases, but the difference between ARE_{PROB} and TRE_{PAL} was ambiguous because of a weak binding of GST–GRDBD to the probe. In conclusion, ARE_{PROB} had a weaker affinity than GRE_{TAT} for the binding of GST–ARDBD, GST–MRDBD, or GST–GRDBD.

DISCUSSION

In this study, we expressed DBDs of three receptors and ARSBD as GST fusion proteins. The GST-fusion expression system has been already employed for numerous proteins including AR [26]. In this study, however, we note a novel characteristic of our GST-fusion proteins: The GST portion of the fusion protein possesses the property of dimer formation; and therefore, when a fused portion tends to bind each other, the GST-fusion protein results in forming a high-molecular weight oligomer. Utilizing this unique characteristic, we analyzed the dimerization abilities of ARDBD and ARSBD.

Both native gel electrophoresis and gel exclusion chromatography clearly demonstrated that GST–ARSBD existed as an oligomeric form. Taking into account the dimeric form of GST (Ref. [25] and Fig. 4) and the oligomeric one of GST–ARSBD (Fig. 4), GST–ARSBD should possess an additional interacting

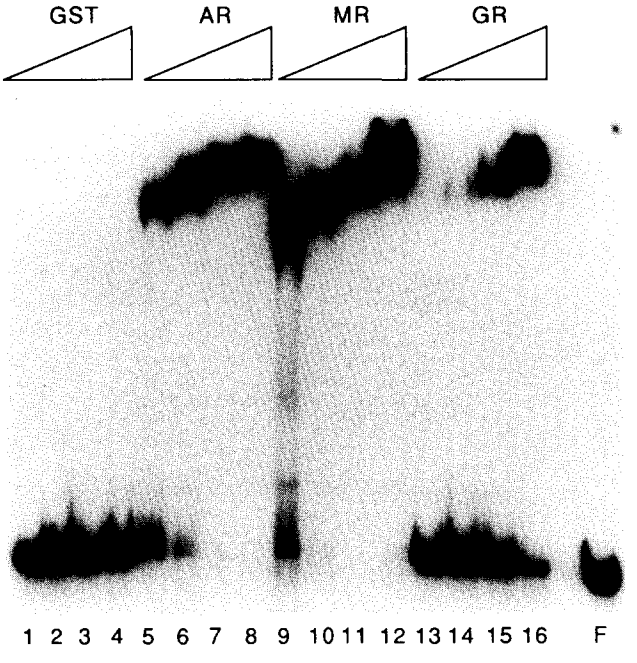

Fig. 5. Gel retardation analysis of GST–ARDBD, GST–MRDBD and GST–GRDBD. GST, GST–ARDBD, GST–MRDBD and GST–GRDBD were incubated with the ^{32}P-labeled GRE_{MMTV} probe (5000 cpm). After 20 min, DNA–protein complexes were separated on a 5% (w/v) polyacrylamide gel. Lanes 1, 5, 9 and 13, 0.5 μg proteins; lanes 2, 6, 10 and 14, 1 μg; lanes 3, 7, 11 and 15, 2 μg; lanes 4, 8, 12 and 16, 4 μg. F, free probe.

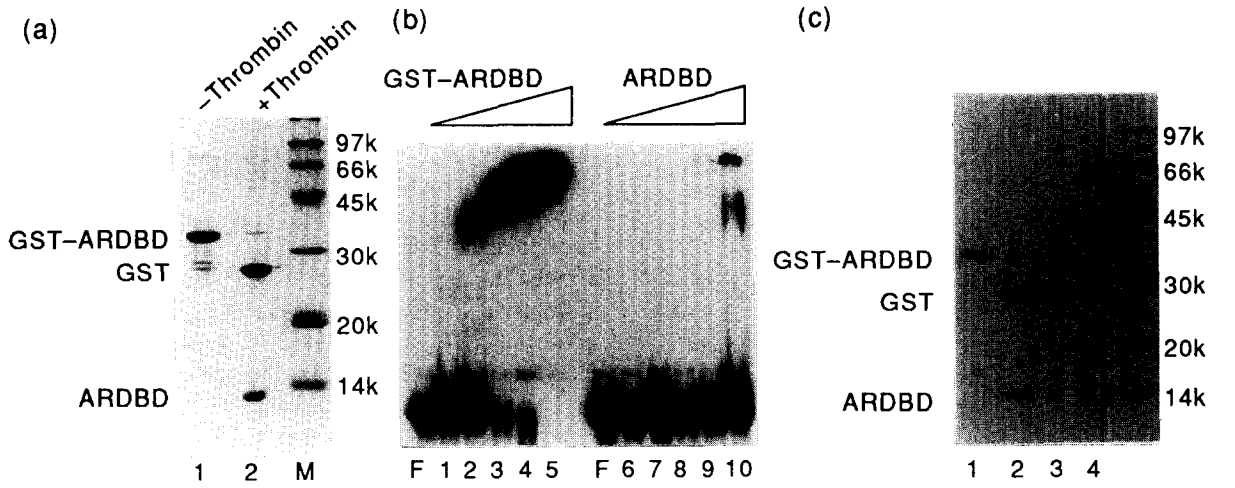

Fig. 6. Effect of thrombin cleavage on the DNA-binding of GST–ARDBD. (a) GST–ARDBD (210 μg) was incubated with or without 1.6 U thrombin at 30°C for 3 h. Aliquots of the sample uncleaved (lane 1) or cleaved (lane 2) with thrombin were analyzed on a 15% (w/v) gel of SDS–PAGE. (b) Uncleaved (GST–ARDBD) or cleaved (ARDBD and GST) sample obtained as in "a" was incubated with ^{32}P-labeled GRE_{MMTV} and separated on a 5% polyacrylamide gel. Lanes 1 and 6, 0.1 μg; lanes 2 and 7, 0.4 μg; lanes 3 and 8, 1.6 μg; lanes 4 and 9, 6.4 μg; lanes 5 and 10, 25.6 μg proteins. (c) Thrombin-treated GST–ARDBD was applied to a DNA–cellulose column (1 × 5 cm) equilibrated with 20 mM Tris–HCl (pH 7.3) containing 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, and 50 mM NaCl. The column was washed with the same buffer, and then eluted with the same buffer with 0.3 M NaCl as a final concentration. Samples were analyzed by SDS–PAGE. Lane 1, untreated GST–ARDBD; lane 2, thrombin-treated GST–ARDBD; lane 3, DNA–cellulose nonadsorbed fraction; lane 4, 0.3 M NaCl-eluted fraction. Data of "a and b" and "c" are from separate experiments.

site other than the GST moiety; and, hence, GST–ARSBD is capable of interacting with two other molecules. Finally, GST–ARSBD forms an oligomer as illustrated in Fig. 8. Thus, the oligomeric structure of GST–ARSBD implies that ARSBD (more precisely, amino acid residues 624–918) have the ability to form a homodimer. Since no ligand was added under these experimental conditions, ligand-binding is not required for the dimerization of ARSBD. Presumably, the ligand-binding is primarily needed for the liberation of an associating component of the non DNA-binding form of heteromeric AR [21].

We previously reported that bacterially expressed truncated ARs retaining DBD and SBD (AR438) or SBD (AR612) sedimented at 4–5S and 3.5–4.5S, respectively, upon glycerol gradient centrifugation [21]. Those species may represent dimeric forms. Moreover, the study showed that ARSBD itself does not form an oligomeric structure such as GST–ARSBD. To directly demonstrate a dimeric form of ARSBD, we attempted to separate GST and ARSBD by thrombin cleavage. However, GST–ARSBD was never cleaved for some unknown reason. While trypsin efficiently degraded GST–ARSBD, a stably-existing species cor-

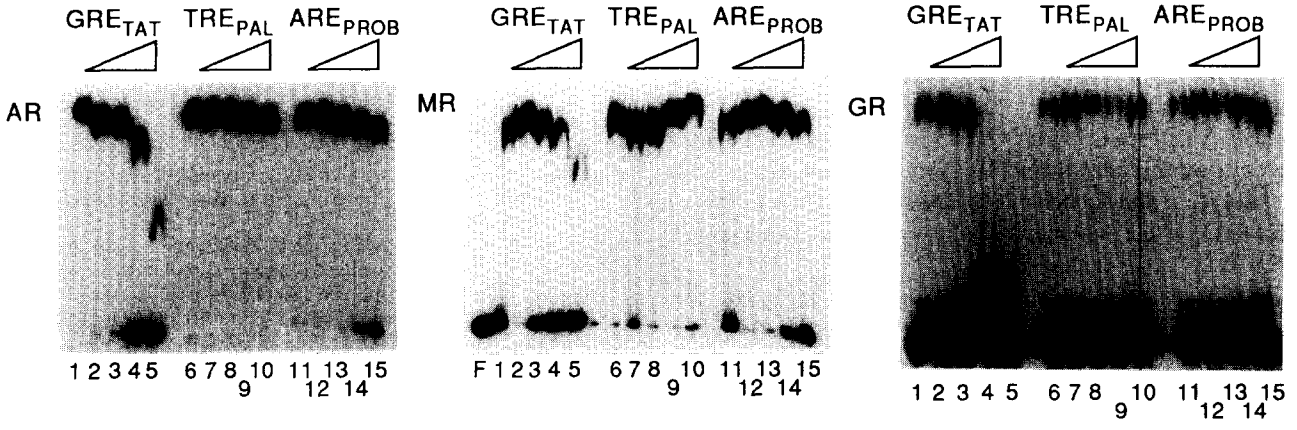

Fig. 7. DNA-binding specificity of GST–ARDBD, GST–MRDBD and GST–GRDBD. GST–ARDBD (0.75 μg), GST–MRDBD (0.75 μg), and GST–GRDBD (4 μg) were incubated with ^{32}P-labeled GRE–MMTV in the absence or presence of competitors. DNA–protein complexes were separated on a polyacrylamide gel. Lanes 1, 6 and 11, no competitor; lanes 2, 7 and 12, 0.04 μg; lanes 3, 8 and 13, 0.2 μg; lanes 4, 9 and 14, 1 μg; lanes 5, 10 and 15, 5 μg competitors. F, free probe.

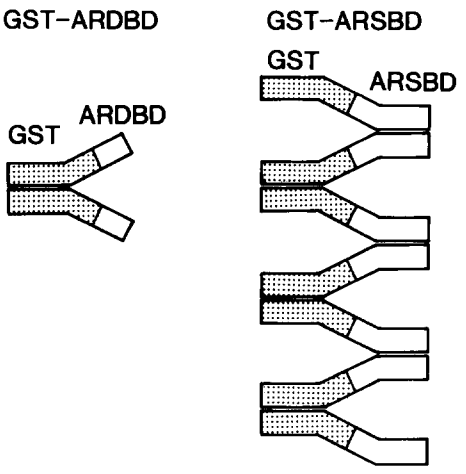

Fig. 8. Model of dimerization and oligomerization of GST fusion proteins. The model of dimerization of GST–ARDBD and oligomerization of GST–ARSBD are schematically represented.

responding to the M_r of ARSBD was not obtained (data not shown).

On the other hand, the dimeric structure of GST–ARDBD implies that ARDBD (more precisely, amino acid residues 548–627) has no interacting site. In other words, ARDBD itself does not form a homodimer. It has already been accepted that two molecules of DBD of GR and ER bind to a palindromic structure of HRE. Moreover, GR [8], ER [10] and PR [9] form homodimers after liberating binding components, such as 90-kDa heat shock protein, before binding with HRE. NMR and crystallographic studies on GR and ER clearly showed that the DBDs interact with each other primarily within the second zinc finger [6, 7]. However, this interaction appears to be insufficient to form a dimeric complex when the two are not associated with HRE [27, 28]. This study also demonstrated that ARDBD alone does not homodimerize. Moreover, our data strongly suggest that the native AR molecule homodimerizes through the interaction of SBD. Hence, one of the functions of SBD may be the stabilization of the dimeric structure of DBD. The dimeric configuration of GST–ARDBD may be appreciably different from that of an intact AR. Nevertheless, it seems important to note that, at least with respect to enhancement of DNA-binding activity of ARDBD, GST is able to substitute the function of ARSBD.

In this study, we did not analyze the N-terminal A/B domain of ARDBD, which might be involved in the dimerization of AR. In fact, Wong *et al.* [14] reported that AR dimerization requires androgen-binding only when the N-terminal A/B domain of AR is present, and suggested that the N-terminal A/B domain acts to inhibit AR dimerization and DNA-binding. On the other hand, this domain is needed for the full activity of transcription activation of GR, and is assumed to interact with receptor-specific transcription factors [29]. Further studies are needed to elucidate the function of the N-terminal A/B domain of steroid receptors.

The DNA-binding specificity of the three DBDs fused to GST did not reveal any difference with three response elements; all three DBDs possessed a higher binding affinity for GRE_{TAT}. Although the amino acid sequence of ARDBD is 22.1% different from those sequences of MR and GR [Fig. 1(b and c)], the difference seems not to affect the DNA-binding properties of AR, at least not its *in vitro* DNA-binding characteristics. Therefore, the DNA sequence of HRE and amino acid sequences of DBD do not appear the primary determinant for the specificity of the actions of androgen, mineralocorticoid and glucocorticoid.

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