JCI The Journal of Clinical Investigation

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J Clin Invest. 1997;99(4):669-675. https://doi.org/10.1172/JCI119210.

Research Article

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Vitamin D and Gonadal Steroid–resistant New World Primate Cells Express an Intracellular Protein Which Competes with the Estrogen Receptor for Binding to the Estrogen Response Element

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Abstract

New World primates (NWP) exhibit a form of compensated resistance to vitamin D and other steroid hormones, including 17β-estradiol. One postulated cause of resistance is that NWP cells overexpress one or more proteins which block hormone action by competing with hormone for its cognate hormone response element. Here we report that both nuclear and postnuclear extracts from NWP, but not Old World primate, cells contained a protein(s) capable of binding directly to the estrogen response element (ERE). This ERE binding protein(s) (ERE-BP) was dissociated from the ERE by excess of either unlabeled ERE or excess of the ERE half-site motif AGGTCAcag. DNA affinity chromatography using concatamers of the latter resulted in > 20,000-fold purification of the ERE-BP. The intensity of the ERE-BP-ERE complex in electromobility shift assay was indirectly related to the amount of wild-type Old World primate estrogen receptor (ER) but not affected when potential ligands, including 17β-estradiol (up to 100 nM), or anti-ER antibody was added to the binding reaction. We conclude that vitamin D–resistant and gonadal steroid–resistant NWP cells contain a protein(s) that may "silence" ER action by interacting directly with the ERE and interfering with ER binding. (*J. Clin. Invest.* 1997. 99:669–675.) Key words: monkey • platyrrhine • steroid receptor • transcription factor • nucleic acid regulatory sequence

Introduction

Compared with Old World primates (OWP)[1] including humans, New World primates (NWP) are resistant to adrenal and gonadal steroids as well as the vitamin D hormone, 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D). This resistance is manifested biochemically in most genera of NWP by high circulating levels of glucocorticoid (1–4), mineralocorticoid (2), progesterone (5), testosterone (5), 17β-estradiol (5), and 1,25-$(OH)_2$D (6, 7). Resistant NWP species are not clinically affected as long as there is adequate substrate available for accelerated steroid/sterol hormone synthesis (8). The underlying cause for near global resistance to steroid hormones remains poorly understood. Some previous investigations indicated that there was a decrease in the number of specific receptors for these hormones in NWP cells compared with cells from OWP (2, 4), while the results of other studies favored the hypothesis that there was a decrease in binding affinity of the various receptors for their cognate ligands (5). More recent studies using specific antireceptor antibody for detection and quantitation of receptor suggest that the results of receptor-ligand binding studies may be adversely influenced by the presence of other proteins in NWP cells which interfere with the receptor-hormone interaction (9, 10). For example, there is evidence that the cytoplasm (and to a lesser extent, the nucleus) of resistant NWP cells is enriched in a binding protein or a family of closely related binding proteins which compete with the vitamin D and estrogen receptor (ER) for ligand (9–11). We have postulated that the presence of this protein(s), which we have named intracellular vitamin D binding protein (IDBP), results in a requirement for a relatively high intracellular concentration of the vitamin D and gonadal steroid hormones in order to permit normal signal transduction initiated by the hormone-receptor interaction. However, this protein(s) does not bind other adrenal steroids (11) and therefore cannot explain the resistance to glucocorticoids and mineralocorticoids which are not bound by this protein. Moore and colleagues, studying glucocorticoid resistance in NWP, found that elevated intracellular concentrations of glucocorticoid and mineralocorticoid are maintained by diminished catabolism of cortisol via the 11β-hydroxysteroid dehydrogenase (12). The fact that there are apparently at least two different functional classes of nonreceptor proteins, one a relatively high-capacity, low-affinity cytoplasmic ligand binding protein and another a catalytically altered enzyme, associated with the hormone-resistant state suggested to us that there might be a more proximal, common cause of hormone resistance in NWP cells. Using the ER-estrogen response element (ERE) interaction as a paradigm, we describe the presence of a protein enriched in NWP cells, but not OWP cells, which binds directly to the ERE and competes with the estrogen-bound ER homodimer for ERE binding.

Methods

Cell culture. The B95-8, MLA-144, owl monkey kidney (OMK), and Vero cell lines were obtained from the American Type Culture Collection (Rockville, MD). B95-8 and MLA-144 cells were maintained

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Received for publication 6 August 1996 and accepted in revised form 22 November 1996.

1. *Abbreviations used in this paper:* EMSA, electromobility shift assay; ER, estrogen receptor; ERE, estrogen response element; ERE-BP, estrogen response element binding protein; IDBP, intracellular vitamin D binding protein; NWP, New World primates; OMK, owl monkey kidney; OWP, Old World primates; TRE, thyroid response element; VDR, vitamin D receptor; VDRE-BP, VDRE-binding protein.

J. Clin. Invest.

0021-9738/97/02/0669/07 $2.00
Volume 99, Number 4, February 1997, 669–675

in RPMI 1640 medium and OMK and Vero cells in MEM (Irvine Scientific, Irvine, CA). All cultures were routinely supplemented with 10% fetal calf serum (FCSI; Gemini Bioproducts, Calabasas, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (both from Gibco Laboratories, Grand Island, NY) and in atmosphere of 95% air, 5% CO_2. In some experiments, confluent cultures were preincubated overnight in medium containing 100 nM 17β-estradiol, 25-hydroxyvitamin D_3 (25-OHD_3) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) before harvest and extract preparation.

Preparation of cellular extracts. Postnuclear extracts of each cell line were prepared as described previously (9). Harvested cells were washed twice in ice-cold PBS and twice with ETD buffer (1 mM EDTA, 10 mM Tris-HCl, 5 mM DTT; pH 7.4) containing 1 mM PMSF. The cells' pellets were then resuspended in ETD buffer and homogenized on ice in five 10-s bursts. Nuclei, with associated nuclear steroid receptor proteins, were pelleted at 4,000 *g* for 30 min at 4°C. The supernatant of this spin was filtered through a Microcon-30 filter (molecular mass cutoff 30 kD; Amicon, Beverly, MA) for the purposes of desalting and concentrating before being aliquoted and stored at −70°C.

Nuclear extracts were prepared as described by Zerivitz and Akusjarvi (13). Cells were washed twice in ice-cold PBS, washed once in buffer A (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM spermine), resuspended in buffer A, and allowed to swell at room temperature for 5 min. Lysis of the cell membrane was achieved by addition of lysolecithin to a concentration of 400 mg/ml and gentle repetitive inversion of the cells for 90 s. Cell membrane lysis was terminated by addition of two volumes of ice-cold buffer B (buffer A containing 3% BSA). All subsequent steps were performed at 4°C. Nuclei were initially pelleted at 1,000 *g* for 30 s and then repelleted at 25,000 *g* for 60 s. The resultant nuclear pellet was resuspended in buffer C [20 mM Hepes (pH 7.9), 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% (vol/vol) glycerol] and the nuclear membrane disrupted by repetitive passage through a 23-gauge needle. The homogenate was gently stirred on ice for 30 min and then centrifuged for 30 min at 25,000 *g*. The supernatant, designated nuclear extract, was dialyzed against buffer D [20 mM Hepes (pH 7.9), 0.1 M KCl, 0.2 M EDTA, 0.5 mM DTT, 0.5 mM PMSF in 20% glycerol] for 30 h with three exchanges. The nuclear extract was cleared by centrifugation at 25,000 *g* for 20 min, aliquoted, and stored at −70°C. The protein concentrations of nuclear and postnuclear extracts were determined by the method of Bradford (14).

Electromobility shift assay (EMSA). Synthetic oligonucleotides representing various forms of steroid response elements were either prepared at the Molecular Biology Core Facility of the Cedars-Sinai Research Institute or purchased from Promega (Madison, WI) or from Santa Cruz BioTech (Santa Cruz, CA). Sequences for the various oligonucleotides were as follows: consensus estrogen response element (ERE) 5′-CTAGAAAGTCAGGTCACAGTGACCT-GATCAAT-3′; 5′ ERE half-site-type 1 (5′ EREhs1) 5′-CTAGAAAGTCAGGTCACAGGAT-CAAT-3′; 5′ ERE half-site-type 2 (5′ EREhs2) 5′-CTAGTGCTCGGGTAGAGGTCACAG-CTCGACTCGT-3′; AGGTCA half-site direct repeat with three nucleotide spacer-type 1 (DR31) 5′-CTAGTGCTCGGGTAGAGGTCACAGAGGTCA-CTCGACTCGT-3′; AGGTCA half-site direct repeat with three nucleotide spacer-type 2 (DR32) 5′-CTAGAAAGTCAGGTCACAGAGGTCAGATCAAT-3′; thyroid response element (TRE) 5′-AGCTTCAGGTCACAGG-AGGTCAGAGAG-3′; TRE palindrome (TREpal) 5′-AAGATTCAGGTCATGACTGAGGAGA-3′; retinoid X response element (RXRE) 5′-AGCTTCAGGTCAGAGGTCAGAGAGCT-3′; chicken ovalbumin upstream promoter-transcription factor-1 response element (COUP-TF1E) 5′-TTCTA-TGGTGTCAAAGGTCAAAC-3′. The single-stranded ERE oligonucleotide was used to prepare labeled probe. The ERE 32-mer was annealed with complementary sequence and radiolabeled with [^{32}P]ATP (DuPont-New England Nuclear, Boston, MA) by T4 Kinase (Gibco Laboratories) to a specific activity of 10^8 cpm/μg DNA. Postnuclear or nuclear extracts (10 μg protein), preincubated or not for 1 h at 23°C with 100 nM 17β-estradiol, 25-OHD_3, or 1,25-$(OH)_2D_3$, were incubated in the presence or absence of human ER and/or antibody with 2 μg poly-dI-dC (Boehringer-Mannheim, Indianapolis, IN) and 20 mM Hepes (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$ in 10% glycerol on ice for 15 min. Overexpressed wild-type human ER was recovered in extracts of *Saccharomyces cerevisiae* as described previously (15). Anti-ER antibodies were purchased from Santa Cruz BioTech (C-314 recognizing ER residues 120-170 and C-311 recognizing residues 495-595) and StressGen (Victorville, British Columbia, Canada; recognizing ER residues 263-302). Anti–chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) antibody was a generous gift from M.-J. and S.Y. Tsai (Baylor College of Medicine, Houston, TX). ^{32}P-Labeled probe was then added and the incubation continued at room temperature for 15 min. Samples of this reaction were subjected to 6% polyacrylamide gel electrophoresis in 0.5× TBE buffer at 100 V. The gels were dried and exposed to Kodak X-OMAT AR film. Densitometry was performed on an Helena EDC densitometer (C. Helena Laboratories, Beaumont, TX).

DNA affinity chromatography. A DNA affinity column was prepared as described by Kadondaga and Tijan (16). Two gel-purified oligodeoxynucleotides (30-mers containing 26 nucleotides of complementary sequence and having 4- bp, cohesive ends; 5′-GATCCTAGAAAGT-CAGGTCACAGGATCAAT-3′ and 5′-GATCATTGATCCTGTGACCTGACTTTC-TAG-3′) were synthesized. 220 μg of each oligonucleotide was annealed, 5′-phosphorylated with [γ-^{32}P]ATP to ascertain coupling efficiency, and ligated. The resultant DNA oligomers were then coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ). After blocking the excess remaining active groups with ethanolamine, the DNA-coupled resin was placed in a 2 ml poly-prep column (Bio-Rad, Hercules, CA) for chromatography. The column was equilibrated in elution buffer [25 mM Hepes (pH 7.6), 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, and 0.1% NP-40] containing 0.1 M KCl. The B95-8 cell extract (13–20 mg) prepared as above was solubilized in the elution buffer containing nonspecific competitor poly dI-dC (4 mg/ml) and added to the column by gravity flow (12 ml/h). Protein was then eluted from the column in a stepwise gradient fashion by the successive addition of ten 10-ml aliquots of elution buffer containing 0.1–1.0 M KCl. A fraction (2 ml) of each column eluate was concentrated and desalted through a Microcon-30 filter and ERE binding capacity was assessed by EMSA. An aliquot of each fraction was used to determine total protein concentration. The constituent proteins in each fraction were resolved on a 10% SDS-PAGE and identified by silver staining.

Results

Nuclear and postnuclear extracts of NWP cells contain a protein which binds to ERE. Cellular extracts were prepared identically from both the representative NWP B95-8 and OWP MLA cell line. Before extraction, cells were ruptured and intact nuclei were separated from cell cytosol in a low-salt buffer in order to separate the ER, associated with the nuclear pellet (17), from the postnuclear supernatant that is known to be enriched in IDBP under these conditions (10); the presence of endogenous ER in the nuclear pellet was confirmed by Western blot and band-shift analysis of nuclear extract using the C-314 anti-ER antibody (Chen, H., and J.S. Adams, manuscript in preparation). The high-salt nuclear extract was adjusted to 0.1 M KCl by dialysis, while the low-salt nuclear extract was adjusted to same salt concentration by dilution. The postnuclear and nuclear extracts were matched for protein concentration and used in EMSA. We first examined the potential for nuclear and postnuclear extracts of steroid-resistant NWP cells and steroid-responsive OWP cells to bind to a panel of synthetically prepared oligonucleotides including the consensus

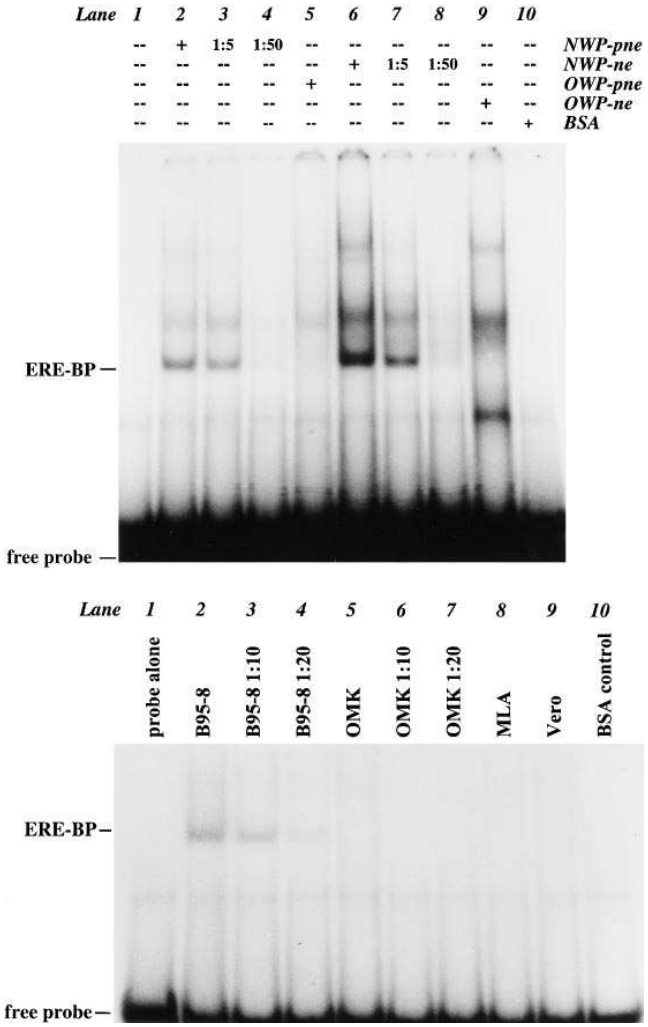

Figure 1. Specificity of the NWP ERE-BP for the ERE. The top shows that both postnuclear (*NWP-pne*, lanes *2–4*) as well as nuclear extracts (*NWP-ne*, lanes *6–8*) of the steroid-resistant B95-8 cell line contain an ERE-BP that binds to a consensus ERE. Serial dilution of the postnuclear and nuclear extracts results in disappearance of the ERE-BP-ERE complex (lanes *3* and *4*, and *7* and *8*, respectively). The labeled ERE-BP-ERE complex was not identified when extracts of a similarly prepared OWP B cell line were used in EMSA (lanes *5* and *9*). Control lanes containing probe alone (lane *1*) and BSA (lane *10*) matched for protein concentration with the cell extracts used demonstrate no specifically retarded bands. The bottom shows the specificity of ERE-BP-ERE complex formation for serially diluted extract obtained from NWP cells with the steroid-resistant phenotype in vivo (lanes *2–4*). ERE-BP-ERE complex was noted only in the B-lymphoblastoid cell line B95-8 but not in renal epithelial cell lines from the owl monkey (*OMK*) and green monkey (*Vero*) or in B-lymphoblasts from a gibbon (*MLA*).

ERE (AGGTCAcagTGACCT) (Fig. 1). The top of Fig. 1 shows that the postnuclear extract, and to lesser extent nuclear extract, of steroid-resistant B95-8 cells contained a protein(s) (labeled ERE-BP) which retarded the mobility of the labeled ERE (lanes *2* and *6*). The presence of this complex was specific for extracts of cells with the steroid-resistant phenotype. The wild-type OWP postnuclear and nuclear extracts, matched for protein concentration with the respective NWP extracts, did not retard the ERE in this fashion (lanes *5* and *9*); the predominant, retarded band observed after incubation of the ERE probe with nuclear extract from either NWP or OWP cells (lanes *6–9*) was not specific for the ERE (data not shown). As anticipated, serial dilution of either the postnuclear (lanes *3* and *4*) or the nuclear NWP extract (lanes *7* and *8*) resulted in gradual disappearance of the retarded band. The specificity of ERE-BP overexpression for NWP with the steroid-resistant phenotype is shown in the bottom half of Fig. 1. Postnuclear extracts of OMK derived from the steroid-responsive NWP *Aotus trivergatus* (lanes *5–7*), transformed B-lymphoblasts (MLA) from the steroid-responsive OWP *Hylobates* (lane *8*), and kidney cells (Vero, lane *9*) from the steroid-responsive OWP *Cercopithecus sabaeus* possessed no ERE-BP detectable by EMSA.

Sterol/steroid ligands do not alter the ERE-BP-ERE interaction. Modification of steroid receptor proteins by their cognate ligands in vivo is often critical in determining the pattern of dimerization of the receptors as well as the response element binding capacity of the receptors. We next examined whether the ERE-BP interaction with the ERE was also modified by preexposure steroid-resistant NWP B95-8 cells to ligand before extraction or by exposure to ERE-BP–containing extracts to potential ligands before incubation with probe in vitro. For these experiments we preincubated cells or extracts of cells known to be enriched in IDBP (10) to an ER-saturating concentration of 17β-estradiol (100 nM) as well as the same concentration of 25-OHD_3 and 1,25-$(OH)_2D_3$, compounds exhibiting the highest affinity for the IDBP (11), be-

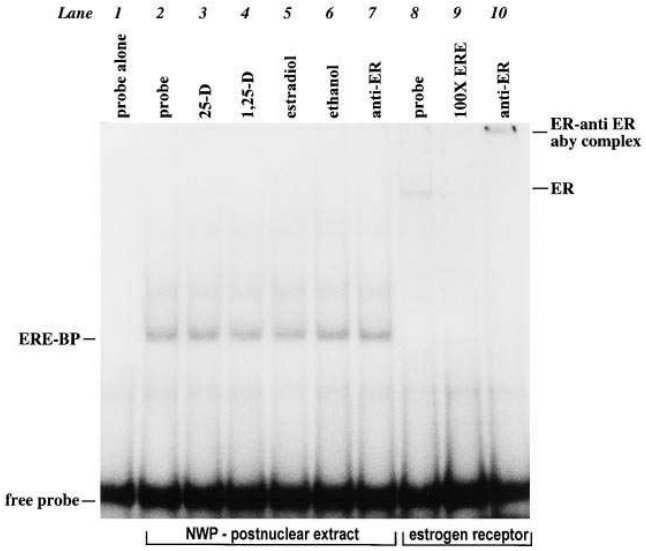

Figure 2. Failure of potential steroid/sterol ligands to modulate ERE-BP interaction with the labeled ERE. Compared with the human ER (lanes *8–10*) which homodimerizes and specifically binds to the ERE upon exposure to 100 nM 17β-estradiol, the ERE-BP in postnuclear extracts of the NWP B cell line B95-8 is not influenced by preincubation with 100 nM 25-hydroxyvitamin D_3 (*25-D*; lane *3*), 1,25-dihydroxyvitamin D_3 (*1,25-D*; lane *4*), or 17β-estradiol in the absence (*estradiol*; lane *5*) or presence of anti-ER antibody (*anti-ER*; lane *7*) which is known to supershift the ER-ERE band (lane *10*). 0.02% ethanol used to solubilize ligands in the reaction mixture is without effect on the mobility of the ERE-BP-ERE complex (*ethanol*; lane *6*).

fore interaction with the probe. Preexposure of cells before harvest as well as incubation with postnuclear extracts and nuclear extracts (data not shown) to these ligands before interaction with the labeled ERE was without effect on the mobility of the ERE-BP-ERE complex (Fig. 2). Furthermore, incubation of NWP postnuclear extract with an anti-ER antibody, which supershifts the ER-ERE complex (lanes *8* and *9*), was without effect on the mobility or intensity of the ERE-BP-ERE complex, either before (not shown) or after (lane *7*) coincubation with 100 nM 17β-estradiol. Identical results were obtained in extracts of cells incubated with 100 nM 25-OHD_3 and 1,25-$(OH)_2D_3$ before EMSA.

The NWP ERE-BP competitively disrupts the ER-ERE interaction. If the ERE-BP we have identified in NWP cell extracts is functionally capable of rendering the cell estrogen-resistant in vivo, then the ERE-BP should be able to interrupt the normal interaction of the ER with the ERE by competing with the ER for binding to the ERE. We investigated this possibility in the experiments depicted in Fig. 3. When the ER was incubated with the ERE (lane *2*), the mobility of the ERE through the gel was slowed considerably. Addition of 100-fold excess unlabeled ERE competed away the ER-ERE band (lanes *3*), and the ER-ERE complex was supershifted by incubation with anti-ER antibody (lane *4*). When the ERE-BP–containing nuclear or postnuclear extract was incubated with the ERE probe (lanes *5* and *8*), the ERE-BP-ERE complex was observed to migrate faster than ER-ERE complex. When the ER and ERE-BP of either nuclear (lane *6*) or postnuclear (lane *9*) extract origin were coincubated with the ERE probe, both the ER-ERE and ERE-BP-ERE complexes were identified in the same lane. Dilution of the extract before coincubation with the activated ER and labeled ERE shifted the balance of retarded complexes in favor of the ER-ERE (lanes *7* and *10*). These data clearly showed that competition for ERE binding was dependent upon the relative amount of ERE-BP present in the extracts of NWP cells with the steroid-resistant phenotype. A second set of experiments was designed to determine the relative affinity of the ER and the ERE-BP for the ERE. Considering the likelihood that the yeast extract overexpressing the human ER was relatively enriched for ER compared with the NWP cell extract containing the ERE-BP, we examined competitive displacement of the ER and ERE-BP from labeled ERE through increasing concentrations of radioinert ERE (2.5×–80×) and increasing dilutions (0–1:15) of the ER-containing yeast extract. Mean densitometric analyses of the EMSA of two such experiments revealed the same result, a sevenfold excess of unlabeled ERE was required to elicit half-maximal displacement of either the ER or ERE-BP from the ERE.

DNA sequence specificity for binding the NWP ERE-BP. The consensus ERE is characterized by an inverted repeat of the 6-bp half-site motif AGGTCA (18). To determine whether binding of the NWP ERE-BP was specific for ERE, we analyzed ability of other receptor response elements containing the AGGTCA half-site motif to compete away labeled ERE-ERE-BP binding (Fig. 4). The list of unlabeled competitive oligonucleotides containing the AGGTCA half-site included the consensus ERE, TRE, TRE palindrome (TREpal), RXRE, and COUP-TF1E. Of these only 100× ERE (lane *3*) completely eliminated labeled ERE-ERE-BP binding. A synthetic oligonucleotide $DR_3$2 (lane *4*), harboring a direct repeat of the AGGTCA half-site separated by the trinucleotide *cag* found in the consensus ERE, was nearly as efficient as the ERE in competing with the labeled ERE probe, and this competitive potential was not related to the sequence flanking the half-sites (data not shown). These results suggested that the AGGTCA half-site as well as the intervening *cag* trinucleotide, not just the AGGTCA half-site motif alone, were important in determining ERE-BP binding to the ERE.

The next question was whether the ERE-BP interacted

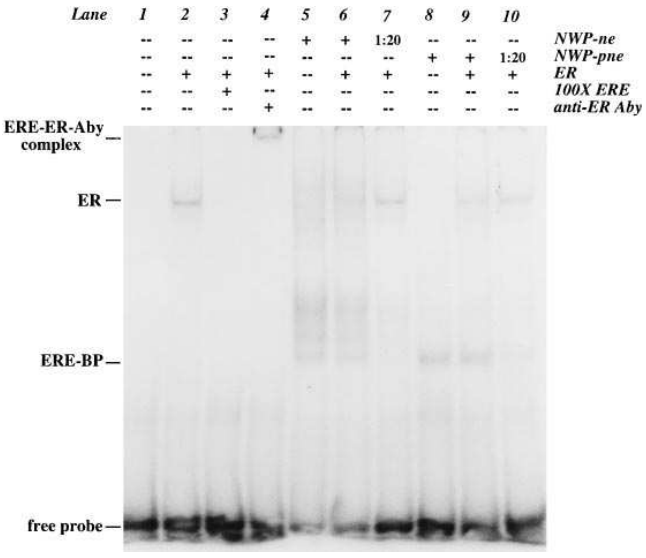

Figure 3. The ERE-BP competitively disrupts the interaction of the ER-ERE interaction. The human ER (lanes 2–4) and undiluted ERE-BP, in either nuclear or postnuclear extracts of NWP B95-8 cells (lanes 5 and 6, and 8 and 9, respectively), bind the ERE probe. In the presence of ER, dilution of either the nuclear (*1:20 NWP-ne*; lane 7) or postnuclear extract (*1:20 NWP-pne*; lane 10) enhances ER-ERE complex formation and diminishes ER-BP-ERE complex formation.

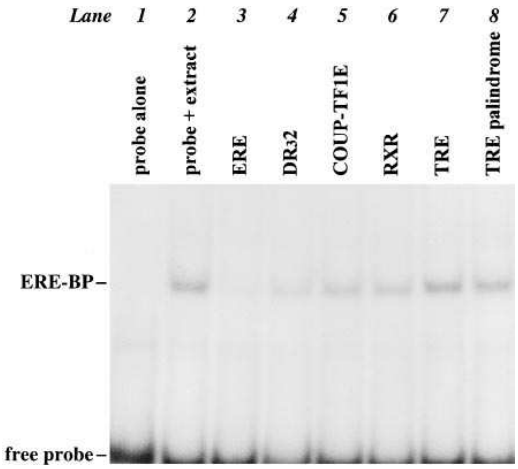

Figure 4. Interaction of the ERE-BP with hormone response elements containing the AGGTCA half-site motif. Shown is the ability of 100-fold molar excess of naturally occurring, consensus hormone response elements (lanes *3* and *5–8*) and of a synthetic oligonucleotide bearing a direct repeat of the AGGTCA motif separated by the trinucleotide *cag* ($DR_3$2, lane *4*) to compete away labeled probe from the ERE-BP contained in the postnuclear extract of NWP cells.

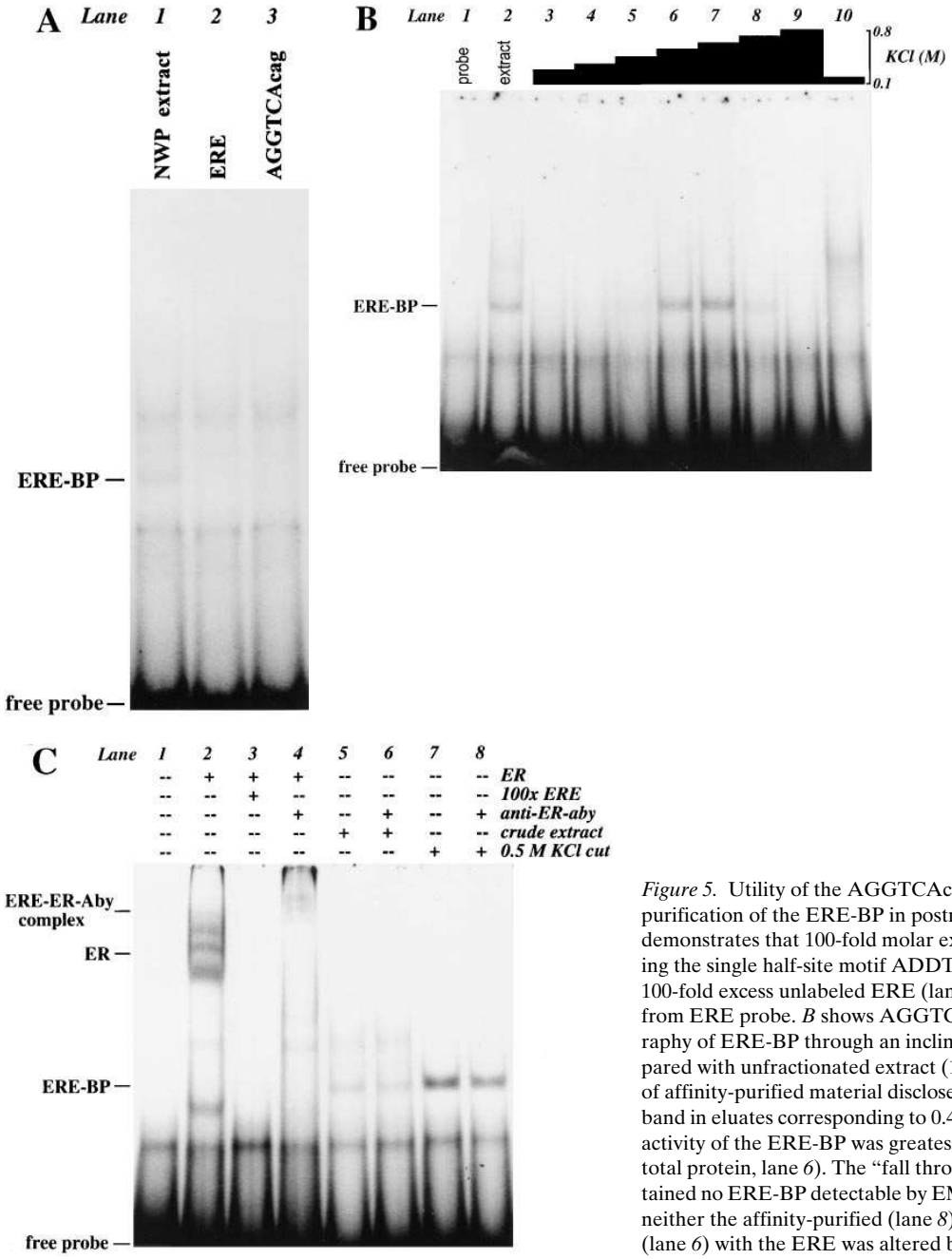

Figure 5. Utility of the AGGTCAcag half-site motif in DNA affinity purification of the ERE-BP in postnuclear extracts of NWP cells. *A* demonstrates that 100-fold molar excess of an oligonucleotide containing the single half-site motif ADDTCAcag (lane *3*) was as effective as 100-fold excess unlabeled ERE (lane *2*) in competing away ERE-BP from ERE probe. *B* shows AGGTCAcag half-site affinity chromatography of ERE-BP through an inclining, stepwise KCl gradient. Compared with unfractionated extract (10 μg total protein; lane *2*), EMSA of affinity-purified material disclosed the presence of a single retarded band in eluates corresponding to 0.4–0.7 M salt (lanes *4–7*); the specific activity of the ERE-BP was greatest in the 0.5 M KCl fraction (120 pg total protein, lane *6*). The "fall through" of the column (lane *10*) contained no ERE-BP detectable by EMSA. *C* confirms that interaction of neither the affinity-purified (lane *8*) nor the unfractionated ERE-BP (lane *6*) with the ERE was altered by the C-314 anti-ER antibody.

with AGGTCAcag in the absence of a second half-site. To answer this question we prepared a synthetic oligonucleotide lacking the 3′ AGGTCA of the ERE but retaining the 5′ AGGTCA and intervening trinucleotide *cag* found in the consensus ERE (Fig. 5 *A*). This oligonucleotide was as effective as the consensus ERE in competing with the labeled probe, indicating that the ERE-BP may bind to a single core element as a monomer.

Affinity purification of the NWP ERE-BP. Because the AGGTCAcag motif was as effective as the consensus ERE in competing for ERE-BP binding (Fig. 5 *A*), we used a Sepharose-linked concatamers of the AGGTCAcag motif for affinity purification of ERE-BP from postnuclear extracts of B95-8 cells; this strategy optimized our opportunity to recover DNA-binding proteins that interacted with the half-site AGGTCA as a monomer and diminished chances of recovering ER that may have been inadvertently retained in our postnuclear extracts. Chromatographic separation of proteins was achieved through a stepwise salt gradient (Fig. 5 *B*). Fractions from the mid portion of the gradient (0.4–0.7 M KCl) were enriched in a protein(s) which interacted with the ERE in EMSA. The specific activity of this ERE binding activity was greatest in fractions containing 0.5 and 0.6 M salt, being 20–30-fold greater than that observed in any of the flanking fractions and 20,000-fold greater than that of the starting material. SDS-PAGE of these two fractions from DNA affinity chromatography disclosed the presence of two distinct silver-stained proteins in the 45-kD range. The protein(s) in the 0.5 M KCl fraction from

affinity chromatography could also be distinguished from endogenous ER (Fig. 5 *C*); the purified ERE-BP did not interact in EMSA with any of the anti-ER antibodies tested and did not specifically bind 17β-estradiol (data not shown).

Discussion

With the exception of animals in the genus *Aotus*, NWP exhibit high circulating levels of the vitamin D hormone 1,25-$(OH)_2$D (7, 9). Using either cultured dermal fibroblasts (19) or established cell lines from vitamin D–resistant NWP species (9, 11), our earlier work suggested that the biochemical phenotype at the level of the vitamin D receptor (VDR) was one of a reduction in the number of receptors per cell, without any change in the affinity of VDR for 1,25-$(OH)_2$D, and a consequent reduction in specific hormone-induced bioactivity. Most intriguing was the observation that the apparent reduction in functional VDR in NWP cells was not associated with a reduction in the amount of immunodetectable VDR (9). These data indicated that NWP cells were somehow limiting the amount of ligand that was accessible to endogenous receptor. Based on the results of heterologous mixing experiments showing that NWP cell extracts contained a factor(s) which inhibited the OWP VDR-1,25-$(OH)_2$D binding interaction (9, 11), our initial hypothesis was that a single factor present in much higher concentrations in NWP than in OWP cells was responsible for both limiting ligand and decreasing VDR-1,25-$(OH)_2$D-specific transactivation. This factor, which we named IDBP, is a 65-kD protein recoverable from both the cytoplasmic and nuclear compartments of steroid-resistant NWP cells and capable of binding 25-hydroxylated vitamin D sterols as well as gonadal steroid hormones including 17β-estradiol (9–11). However, there were two pieces of relevant information that suggested that IDBP or related proteins were not the sole explanation for the steroid-resistant state among NWP. First, IDBP does not bind glucocorticoids or mineralocorticoids (11), so it alone cannot explain the resistance of NWP to steroids of adrenal origin. Second, the quantitative presence of IDBP, an intracellular protein with the potential to compete with steroid/sterol hormone receptor for ligand, in NWP cells could not in all cases explain the severity of biological hormone resistance in all situations (20). Therefore, we undertook the current studies to investigate the possible existence of a second factor in NWP cells that does not influence the receptor-ligand interaction but which may alter hormone-receptor transactivation by binding to the hormone response element.

Using the ER-ERE interaction in an EMSA as a screen for the presence of such a factor, we discovered that both nuclear extracts containing endogenous ER and postnuclear extracts of NWP cells devoid of ER contained a protein(s) unrelated to ER that interacted directly with a consensus ERE (Fig. 1, *top*). With the current experiments we have been able to gain some insight into the potential of this ERE-BP to interact with the ERE. As predicted, detection of the ERE-BP by EMSA technology was specific for cellular extracts from NWP with the steroid-resistant phenotype in vivo (Fig. 1, *bottom*). Although the ERE-BP effectively competed with wild-type human ER for binding to the ERE (Fig. 3), there are a number of pieces of experimental data presented here that indicate that the ER and ERE are structurally distinct. First is the observation that the ER-ERE and ERE-BP-ERE complexes can be observed as distinct bands on EMSA (Figs. 2 and 3) with the apparent molecular mass and/or charge of the ERE-BP-ERE complex distinguishing it from the ER-ERE complex. Second, and not withstanding the fact that there is a distinct species difference between human and nonhuman primates, is the finding that the ERE-BP, in either crude (Fig. 2) or purified form (Fig. 5), was not supershifted by a panel of three antibodies each recognizing a different epitope on the ER. Third is the probability that the ERE-BP interaction with the ERE is as a monomer, whereas the preferred mode of interaction of the ER with the ERE is as a homodimer (18). This is supported by the facts that labeled ER-ERE complex migrates slower in EMSA than the ERE-BP-ERE complex (see Figs. 2, 3, and 5), and that the labeled ERE-BP-ERE complex can be effectively dissociated by addition of the hexanucleotide half-site AGGTCA in context with the trinucleotide, *cag*, that separates the indirect repeats of the consensus ERE (Figs. 4 and 5). And fourth is the evidence that the ERE-BP can be affinity-purified over a support composed of concatamers the half-site motif AGGTCA-*cag* that does not bind wild-type ER (Fig. 5).

The facts that the ERE-BP competes with ER for binding to the ERE in vitro and that NWP must maintain high circulating levels of 17β-estradiol in vivo for reproductive competence (4) suggests that the ERE-BP may function in a "silencer" mode analogous to some of the orphan receptor proteins (18, 20, 21). Like the ERE-BP(s) described here, these orphan receptors can be (*a*) distributed in both nuclear and cytoplasmic compartment of the cell; (*b*) capable of binding to DNA in the absence of a known ligand as a monomer, dimer or sometimes both; and (*c*) also capable of limiting hormone-directed basal or stimulated promoter activity by competitively binding to the HRE (21). An example of an orphan receptor that can apparently bind to the ERE in either a monomeric or dimeric state and can disrupt estrogen-directed gene transcription is the chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) (22). Although initial investigation indicates that the NWP ERE-BP is not recognized by anti–human COUP-TF antibody or dissociated from the ERE with a 100-fold molar excess of an oligonucleotide containing the COUP-TF1 response element, it is possible that the ERE-BP is an orphan receptor protein or the platyrrhine homologue of a COUP-TF1–like receptor.

There is also evidence that the ERE-BP(s) described here is distinct from a VDRE-binding protein (VDRE-BP) that we discovered recently in the same steroid- and vitamin D–resistant B95-8 NWP cell line (23). The ERE-BP appears to be distributed in both nuclear and postnuclear extracts, while the NWP VDRE-BP is concentrated in the nucleus under the same extraction conditions (23). Further, the intensity of the ERE-BP-ERE complex is only minimally reduced in an EMSA by addition of a 100-fold molar excess of the human osteopontin or osteocalcin gene VDRE (data not shown) to which the nuclear VDRE-BP is most avidly bound (23). We are actively investigating the possibility that the ERE-BP and VDRE-BP, while functionally different, may be structurally related and part of a larger family of DNA binding proteins.

Our most recent work with VDRE-BP (23) and that presented here with the ERE-BP clearly suggest that vitamin D sterol and gonadal steroid resistance in NWP genera is probably due to more than just simple overexpression of an IDBP which can trap sterol/steroid ligands en route to their cognate receptor protein. Furthermore, there is little doubt that IDBP, a largely cytoplasmic protein, now known to be a member of

the hsp-70 family of proteins (24) is distinct from the ERE-BP. The latter appears to be a ubiquitously distributed, nucleic acid binding protein without ligand (steroid) binding potential as determined by the evidence to date. Therefore, our current working hypothesis has been revised to presuppose that the more proximal etiology of estrogen resistance in NWP is overexpression of ERE-BP, and that IDBP expression in the same cell occurs either in response to or to further bolster the actions of ERE-BP to silence receptor transactivation of estrogen-responsive genes. In this hypothesis IDBP serves as a high capacity concentrator for intracellular estrogen either acting to prevent estrogen catabolism and bring the ligand into close approximation with the ER or further silence estrogen action on the cell. The first scenario would promote effective ER homodimerization and, in turn, provide the cell with enough competent transactivator to compete effectively with the ERE-BP for the ERE. Given the fact that the ERE-BP possesses ERE binding potential in the NWP cell comparable with that of the ER homodimer, at least in vitro, one would expect to find a new steady state in ER-directed transcription of estrogen-regulated genes that is dependent upon a quantitative balance among ligand, ER, IDBP, ERE-BP, as well as other transcription factors. In the second scenario, overexpression of ERE-BP and IDBP may be designed for cooperative "braking" of steroid/sterol hormone action by inducing a state of hormone resistance. Regardless, confirmation of this hypothesis will be dependent upon the eventual isolation and expression of the NWP ERE-BP cDNA in a wild-type OWP cell to determine whether the ERE-BP characterized here will alter ER-directed transactivation.

Acknowledgments

We dedicate this manuscript to the memory of Bayard "Skip" Catherwood whose suggestions were crucial to the advancement of this work. We are also grateful to Dr. Gregory Brent for his helpful comments.

This work was support by National Institutes of Health grants AR37399 (J.S. Adams) and DK07682 (H. Chen).

References

1. Chrousos, G.P., D. Renquist, D. Brandon, C. Eil, M. Pugeat, R. Vigersky, G.B. Cutler, Jr., D.L. Loriaux, and M.B. Lipsett. 1982. Glucocorticoid hormone resistance during primate evolution: receptor-mediated mechanisms. *Proc. Natl. Acad. Sci. USA.* 79:2036–2040.

2. Chrousos, G.P., D.L. Loriaux, D. Brandon, J. Schull, D. Renquist, W. Hogan, M. Tomita, and M.B. Lipsett. 1984. Adaptation of the mineralocorticoid target tissues to the high circulating cortisol and progesterone plasma levels in the squirrel monkey. *Endocrinology.* 115:25–32.

3. Lipsett, M.B., G.P. Chrousos, M. Tomita, D.D. Brandon, and D.L. Loriaux. 1985. The defective glucocorticoid receptor in man and non-human primates. *Rec. Prog. Horm. Res.* 42:199–246.

4. Brandon, D.D., A.J. Markwick, G.P. Chrousos, and D.L. Loriaux. 1989. Glucocorticoid resistance in humans and nonhuman primates. *Cancer Res.* 49: 2203–2213.

5. Chrousos, G.P., D. Brandon, D.M. Renquist, M. Tomita, E. Johnson, D.L. Loriaux, and M.B. Lipsett. 1984. Uterine estrogen and progesterone receptors in an estrogen and progesterone-resistant primates. *J. Clin. Endocrinol. & Metab.* 58:516–520.

6. Takahashi, N., S. Suda, T. Shinki, N. Horiuchi, Y. Shiina, Y. Tanioka, H. Koizumi, and T. Suda. 1985. The mechanism of end-organ resistance to 1,25-dihydroxycholecalciferol in the common marmoset. *Biochem. J.* 227:555–563.

7. Adams, J.S., M.A. Gacad, A.J. Baker, B. Gonzales, and R.K. Rude. 1985. Serum concentrations of 1,25-dihydroxyvitamin D_3 in platyrrhini and catarrhini: a phylogenetic appraisal. *Am. J. Primatol.* 9:219–224.

8. Gacad, M.A., M.W. Deseran, and J.S. Adams. 1992. Influence of ultraviolet B radiation on vitamin D_3 metabolism in vitamin D_3-resistant New World primates. *Am. J. Primatol.* 28:263–270.

9. Gacad, M.A., and J.S. Adams. 1991. Endogenous blockade of 1,25-dihydroxyvitamin D-receptor binding in New World primate cells. *J. Clin. Invest.* 87:996–1001.

10. Gacad, M.A., and J.S. Adams. 1993. Identification of a competitive binding component in vitamin D-resistant New World primate cells with a low affinity but high capacity for 1,25-dihydroxyvitamin D3. *J. Bone Miner. Res.* 8: 27–35.

11. Gacad, M.A., and J.S. Adams. 1992. Specificity of steroid binding in New World primate B95-8 cells with a vitamin D-resistant phenotype. *Endocrinology.* 131:2581–2587.

12. Moore, C.C.D., S.H. Mellon, J. Murai, P.K. Siiteri, and W.L. Miller. 1993. Structure and function of the hepatic form of 11β-hydroxysteroid dehydrogenase in the squirrel monkey: an animal model of glucocorticoid resistance. *Endocrinology.* 133:368–375.

13. Zerivitz, K., and G. Akusjarvi. 1989. An improved nuclear extract preparation method. *Gene Anal. Tech.* 6:101–109.

14. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

15. Pham, T.A., Y.P. Hwung, D. Santiso-Mere, D.P. McDonnell, and B.W. O'Malley. 1992. Ligand-dependent and independent function of the transactivation region of the human estrogen receptor in yeast. *Mol. Endocrinol.* 6: 1043–1050.

16. Kadondaga, J.T., and R. Tijan. 1986. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA.* 83:5889–5893.

17. Tzukerman, M.T., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell. 1994. Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol. Endocrinol.* 8:21–30.

18. Tsai, M.J., and B.W.O. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* 63:451–486.

19. Adams, J.S., and M.A. Gacad. 1988. Phenotypic diversity of the cellular 1,25-dihydroxyvitamin D_3-receptor interaction among different genera of New World primates. *J. Clin. Endocrinol. & Metab.* 66:224–229.

20. Mangelsdorf, D.J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R.M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell.* 83:835–839.

21. Mangelsdorf, D.J., and R.M. Evans. 1995. The RXR heterodimers and orphan receptors. *Cell.* 83:841–851.

22. Peter, J., H. Burbach, S. Lopes da Silva, J.J. Cox, R.A.H. Adan, A.J. Cooney, M.J. Tsai, and S.Y. Tsai. 1994. Repression of estrogen-dependent stimulation of the oxytocin gene by chicken ovalbumin upstream promoter transcription factor I. *J. Biol. Chem.* 269:15046–15053.

23. Arbelle, J.E., H. Chen, M.A. Gacad, E.A. Allegretto, J.W. Pike, and J.S. Adams. 1996. Inhibition of vitamin D receptor-retinoid X receptor-vitamin D response element complex formation by nuclear extracts of vitamin D-resistant New World primate cells. *Endocrinology.* 137:786–789.

24. Gacad, M.A., H. Chen, T.R. LeBon, J.E. Arbelle, and J.S. Adams. 1997. Functional characterization and purification of an intracellular vitamin D binding protein in vitamin D resistant New World primate cells: amino acid sequence homology with proteins in the hsp-70 family. *J. Biol. Chem.* In press.