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The Leucine Zippers of c-*fos* and c-*jun* for Progesterone Receptor Dimerization: A-Dominance in the A/B Heterodimer

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Human progesterone receptors (hPR) exist as two isoforms: 120 kDa B-receptors (hPR_B) and N-terminally truncated 94 kDa A-receptors (hPR_A). When transfected separately, each isoform exhibits different transcriptional properties that are ligand- and promoter-specific. In human target tissues, both receptor isoforms are present, so that a mixture of three dimeric species, A/A, A/B, and B/B, bind to DNA at progesterone response elements (PRE), and regulate transcription. To study the transcriptional phenotype of pure A/B heterodimers uncontaminated by A/A or B/B homodimers, we exploited the property of the leucine zipper (zip) domains of *fos* and *jun*, to form pure heterodimers. Chimeric constructs were made linking the zip of either c-*fos* or c-*jun* to the C-terminus of hPR_B or hPR_A (hPR-zip) to produce A-*fos*, B-*fos*, A-*jun* or B-*jun*. To determine whether the A- or B-isoform is functionally dominant in the A/B heterodimer, cells expressing hPR-zip chimeras were treated with the progestin antagonist RU486, which produces opposite transcriptional effects with the two isoforms. Gel mobility shift and immune co-precipitation assays show that in the presence of RU486 only pure heterodimers form between A-*fos*/B-*jun* or *A-jun*/B-*fos*, and bind DNA at PREs. Thus, in these pairs, interactions between the extrinsic *fos*/*jun* zipper domains override interactions between the intrinsic hPR dimerization domains. We find that under these conditions, antagonist-occupied B-zip homodimers stimulate transcription, while antagonist-occupied A-zip homodimers are inhibitory, and that pure A/B zip heterodimers have the inhibitory transcriptional phenotype of the A-zip homodimers. We conclude that, in pure heterodimers, A-receptors are dominant negative inhibitors of B-receptors. Additionally, the pure PR-zip heterodimers, unlike wild-type receptors, bind a PRE in the absence of hormone but do not activate transcription. Thus, PR dimerization and PRE binding are necessary but, without hormone, not sufficient to activate transcription.

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INTRODUCTION

There are two isoforms of human progesterone receptors (hPR): 94 kDa A-receptors (hPR_A), and the 120 kDa B-receptors (hPR_B), which have a 164 amino acid extension at the N-terminus [1,2]. Nine messages encoding these isoforms are transcribed from two promoters, which are similarly regulated by estradiol when they are transiently transfected into human cervical carcinoma (HeLa) cells [3]. As a result, in human endometrial and breast cancer cell lines in which they have been measured, the levels of the A- and B-isoforms are approximately equivalent [4, 5]. However, important exceptions may exist to this rule. In one subline of T47D breast cancer cells, the levels of the B-isoform are preferentially increased after estradiol treatment [6]. A recent quantitative analysis of human breast tumors shows that A-receptor levels exceed those of B-receptors in 76% of PR-positive tumors [6]. There are also reports that the A- to B-receptor ratio is developmentally and hormonally regulated [7, 8]. These findings are all consistent with the possibility that A-and B-receptor levels are independently regulated.

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The ratio of B- to A-receptors in target tissues is of interest, since there are important functional differences between the two isoforms in ligand-induced transcription [9–12]. With agonist occupancy, we [12] and others [10, 11] have shown that transactivation induced by hPR_A is often much lower than that induced by hPR_B in transient transfection assays. When occupied by the progesterone antagonist, RU486, hPR_A are transcriptionally inactive in settings in which hPR_B elicit agonist-like responses. The functional deficiencies of ligand-occupied hPR_A compared to hPR_B, in the identical cell and promoter context, suggest that the A-isoform acts as a negative regulator of its B counterpart. In fact, dominant negative effects of A-receptors are seen following agonist or antagonist treatment, when equimolar amounts of hPR_A and hPR_B are expressed [11, 12]. The dominant, progesterone A-receptor mediated repression extends even to other members of the steroid receptor subfamily that are bound to DNA at hormone response elements [11].

When both A- and B-isoforms of hPR are simultaneously synthesized in wild-type PR-positive cells, or transiently coexpressed in PR-negative cells, they dimerize in solution [13] and bind to DNA as three dimeric species: A/A and B/B homodimers, and A/B heterodimers [14]. By random assortment, A/B heterodimers represent 50% of the DNA-bound dimeric species, but there are always mixtures of homo- and heterodimers. Due to this complex dimeric mixture, it is not known whether the dominant negative effects of hPR_A are mediated only by A/A homodimers, or by both A/A homo- and A/B heterodimeric species.

The goal of the present study was to determine whether the inhibitory phenotype of the A-isoform is dominant in the A/B heterodimer. Our strategy was to create a pure population of A/B heterodimers by constructing fusion proteins of hPR_A or hPR_B with the leucine zipper (zip) of either c-*jun* or c-*fos* [15]. This approach exploits the strong and quantitative heterodimeric association established between the *fos* and *jun* zipper domains, to force the assembly of pure A-*fos*/B-*jun* or A-*jun*/B-*fos* heterodimers [16]. It is based on the observation that when c-*fos* and c-*jun* are coexpressed at equivalent levels, they preferentially form heterodimers over homodimers by at least 1000-fold [16]. We have used these hPR-zip chimeras to study the DNA binding and transactivation properties of pure A/B heterodimers in the presence of RU486, which phenotypically distinguishes A- from B-effects. We find here, and as we reported previously in preliminary form [12], that A-receptors are indeed dominant over B-receptors in the A/B heterodimer, and demonstrate that in the absence of hormone, receptor dimerization and progesterone response element (PRE) binding are insufficient to promote transcription.

MATERIALS AND METHODS

Recombinant plasmids

pSG5-hPR2 and pSG5-hPR1, the hPR_A and hPR_B expression vectors cloned into the eukaryotic expression vector, pSG5 [17], were gifts from P. Chambon. Rat c-*fos* and c-*jun* cDNAs, cloned in pTZ*fos* and pTZ*jun* [18], were gifts from D. Cohen and T. Curran. Construction of the chimeric cDNAs encoding the hPR-leucine zipper fusion proteins has been described previously [12]. PRE_2-tk-CAT was constructed as described previously [12] from the tk-CAT reporter [19], which was a gift from P. Chambon. Sense and antisense oligonucleotides containing two tandem repeats of the PRE from the tyrosine aminotransferase gene promoter [20] and *Xba*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, were cloned upstream of the tk promoter.

Immune coprecipitation and immunoblot assays

Whole cell (WCE) 0.4 M KCl extracts containing wild-type or transiently expressed chimeric A- or B-receptors were prepared from transiently transfected COS-1 cells, T47D (V22) or MDA breast cancer cells, and immune precipitated with the B-receptor specific B-30 monoclonal antibody (MAb) as described previously [13, 21]. The eluted complexes containing hPR_B or hPR_B-zip chimeras and any coprecipitated A-receptors, were then resolved on a denaturing 7.5% polyacrylamide gel, transferred to nitrocellulose, and probed with MAb AB-52 which binds to both hPR isoforms and their respective chimeras. Antibody-bound proteins were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified by densitometry.

Ligand binding assay

Binding of the ^3H-labeled synthetic progestin, R5020, to WCE containing hPR or hPR-zip chimeras was measured by a modified charcoal-dextran method described previously [22]. The affinity of RU486 was also quantitated using a competition assay in which cell extracts containing wild-type hPR or hPR_B-*jun* were incubated with 20 nM [^3H]R5020 in the presence or absence of 20 nM to 2 μM of unlabeled RU486 or R5020.

Gel mobility shift assay

WCE 0.4 M KCl extracts prepared from COS-1 cells transiently transfected with receptor constructs, were incubated with a ^{32}P-end-labeled, 27-bp perfect palindromic PRE (5′-AAA GTC AGA ACA CAG TGT TCT GAT CAA-3′), and analyzed by non-denaturing gel electrophoresis as described previously [23].

CAT assay

Transiently transfected HeLa cells expressing receptor constructs, were lysed by freeze–thawing; the

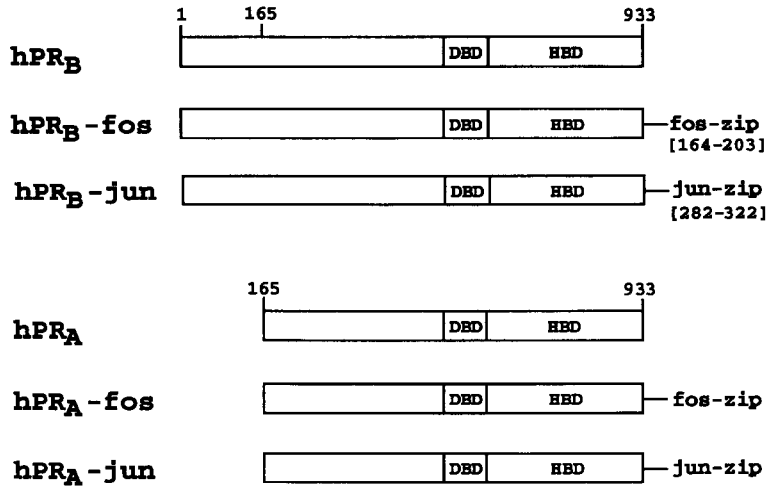

Fig. 1. The chimeric hPR used in these studies. The 40 amino acid (aa) leucine zipper of *fos* (*fos*-zip, spanning aa 164–203 of the fos protein), or 41 aa leucine zipper of *jun* (*jun*-zip, spanning aa 282–322 of the *jun* protein) [18] were fused to the C-terminus of wild-type hPR$_B$ (aa 1–933) or N-terminally truncated hPR$_A$ (aa 165–933), through a two amino-acid linker. DBD, DNA binding domain; HBD, hormone binding domain.

amount of lysate to be used per sample was normalized to β-galactosidase activity; and chloramphenicol acetylation was quantified by phosphorimaging analysis (Molecular Dynamics) of the thin-layer chromatographic plates as described previously [24].

RESULTS

Construction of hPR-zip chimeras

To independently study the transcriptional properties of pure A/B heterodimers, the leucine zipper dimerization domains of rat c-*fos* and c-*jun* were fused to the C-terminal end of full-length hPR$_B$ or hPR$_A$ as shown in Fig. 1. Two new amino acids (*ala-ser*) were inserted at the hPR–leucine zipper junction in constructing the chimeric cDNAs.

Wild-type and chimeric cDNAs were transiently transfected into COS-1 cells and WCE containing the expressed proteins were subjected to SDS–polyacrylamide gel electrophoresis and immunoblot analysis with MAb AB-52 as shown in Fig. 2. Wild-type hPR, and hPR-zip chimeras were of appropriate size; approx. 94 kDa for the A-receptors (lanes 1–3) and 120 kDa for the B-receptors (lanes 3–6). The hPR$_B$-zip chimeras had the triplet band structure characteristic of wild type hPR$_B$, indicating that they had been appropriately phosphorylated. The chimeras ran with slightly slower mobility than the corresponding wild-type hPR, presumably reflecting the additional 42–43 amino acids contributed by the leucine zipper domains.

As shown in Fig. 3, specific binding of the ^{3}H-labeled PR agonist, R5020, to extracts prepared from COS-1 cells transiently transfected with the hPR-zip chimeras was similar to the binding obtained with an extract from T47D-V22 cells, that contain high levels of both A- and B-receptor isoforms. Competition assays, in which binding of [^{3}H]R5020 was measured in the presence of excess unlabeled competitor R5020 or RU486 indicated that RU486 binds to the hPR-zip chimeras with an affinity similar to that of R5020 (data not shown). Thus, the hPR-zip chimeras are expressed at levels equivalent to wild-type receptors, are the correct size, appear to be appropriately modified post-translationally, and retain ligand binding capacity for agonist and antagonist equivalent to wild type hPR.

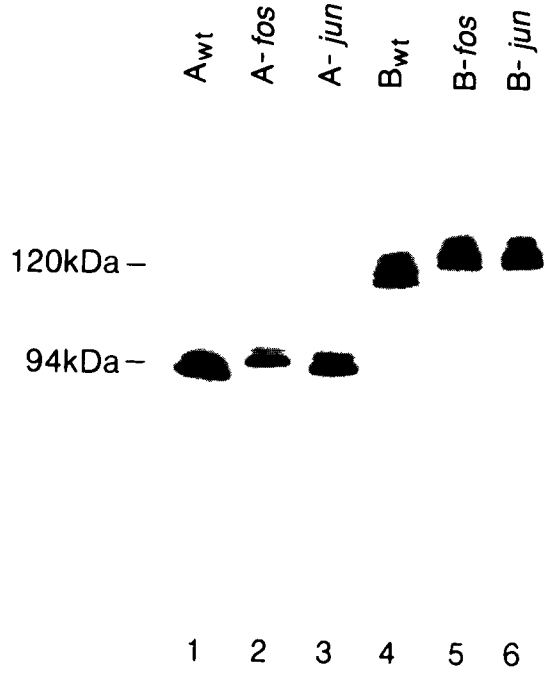

Fig. 2. Expression levels and protein structure of wild-type and chimeric hPR. Expression vectors encoding wild-type hPR (A$_{wt}$, B$_{wt}$) or the four chimeric species (A-*fos*, A-*jun*, B-*fos*, B-*jun*) were transiently transfected into COS-1 cells. Total cellular receptors were extracted with 0.4 M KCl, analyzed by denaturing gel electrophoresis, immunoblotted with MAb AB-52 and autoradiographed by enhanced chemiluminescence.

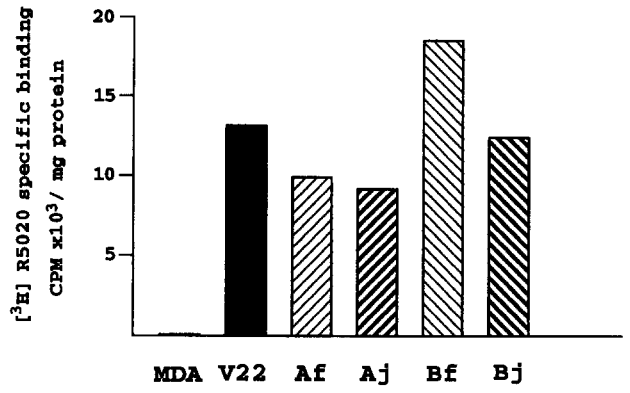

Fig. 3. Binding of [^{3}H]R5020 to wild-type and chimeric hPR. Total cellular receptors were extracted with 0.4 M KCl from MDA and T47D-V22 breast cancer cell lines, or from COS-1 cells transiently expressing the chimeric receptors (Af, A-*fos*; Aj, A-*jun*; Bf, B-*fos*; Bj, B-*jun*). Extracts were diluted and incubated 16 h at 0°C with 20 nM [^{3}H]R5020 with or without a 100-fold molar excess of unlabeled R5020. Protein-bound radioactivity was counted and normalized to total protein levels present in the extract.

Production of pure heterodimers using the hPR-zip chimeras

In T47D cells containing equivalent amounts of hPR_A and hPR_B isoforms, the ligand-activated receptors bind to a PRE as mixtures of A/A, A/B, and B/B dimers in a 1:2:1 ratio [13, 14]. In order to determine the proportion of these dimeric forms obtained after transient cotransfection with equivalent amounts of A and B hPR-zip chimeras, gel mobility supershift and direct protein–protein interaction assays were employed. The molecular size differences between the A- and B-receptor isoforms, and the stoichiometry and specificity of antibody binding, can be used to separate and quantitate each dimeric form when bound to DNA [14]. Figure 4(A) shows a gel mobility shift assay using extracts prepared from COS-1 cells cotransfected with wild type or hPR-zip chimeras and treated with RU486. Extracts were incubated with a ^{32}P-labeled PRE oligonucleotide in the absence or presence of MAb B-30, a monoclonal antibody that binds specifically to the B isoform, and incubation mixtures were then subjected to non-denaturing gel electrophoresis. Since the B/B, A/B and A/A dimers bind, respectively, 2, 1 and 0 molecules of MAb B-30, the different dimer–antibody complexes are easily resolved based on their molecular weight [14]. Thus, in Fig. 4(A) the 3 dimeric species formed when wild-type hPR_A and hPR_B are cotransfected (lane 1) are resolved by MAb B-30 (lane 2). When A-*fos*/B-*fos* (lanes 3 and 4) or A-*jun*/B-*jun* (lanes 5 and 6) were coexpressed and treated with the antagonist RU486, all three dimeric species were observed after MAb B-30 supershift (lanes 4 and 6). Since the *fos*-zipper cannot form an homodimeric interface [16, 25], A-*fos*/B-*fos* probably dimerize only through the intrinsic PR dimerization domains, analogous to wild type receptor dimerization, thus yielding the 3 dimers (lane 4). With A-*jun*/B-*jun*, homodimers can potentially form either through the extrinsic *jun* zipper or the intrinsic PR dimerization domains, and again, 3 dimeric species are observed (lane 6). By contrast, when A-*fos*/B-*jun* (lanes 7 and 8) or A-*jun*/B-*fos* (lanes 9 and 10) were coexpressed and treated with RU486, only A/B heterodimers were observed after MAb B-30 supershift (lanes 8 and 10). Clearly, in these chimeras, heterodimerization is controlled primarily through the extrinsic zipper domains. Of interest is the fact that, unlike RU486, when cells coexpressing A-*fos*/B-*jun* or A-*jun*/B-*fos* chimeras are treated with the agonist, R5020, some homodimeric species are observed (not shown). This suggests that the extrinsic *fos*/*jun* zipper dimerization domains are more likely to control dimerization when antagonists rather than agonists are bound in the ligand binding domain.

To further analyze dimer composition in a set that appeared to contain pure heterodimers an immune coprecipitation assay was employed [13], again using MAb B-30. Since only hPR_B-containing dimers (A/B and B/B) bind antibody, a 1:2:1 starting ratio of A/A, A/B, and B/B dimers would yield a 1:2 mixture of A- to B-receptors in the precipitate and then on immunoblots, while a starting extract containing pure A/B heterodimers would yield a 1:1 ratio of A- to B-receptors on immunoblots. Figure 4(B) shows such a study. Extracts were prepared from RU486-treated wild-type T47D-V22 cells, and COS-1 cells coexpressing A-*jun* and B-*fos* chimeras. Receptor complexes were immunoprecipitated with MAb B-30. Quantitation of wild-type A and B receptor levels by immunoblotting with MAb AB-52 yielded an A- to B-receptor ratio of 1:3 as compared to the expected 1:2 ratio [Fig. 4(B), lane 2]. This discrepancy, which has been observed previously [13], would result if dimers were unstable in solution, or if not all monomers associate into dimers, allowing both B monomers and B-containing dimers to be immunoprecipitated. On the other hand, extracts prepared from mixtures of A-*jun* and B-*fos* chimeras yielded the expected 1:1 ratio (lane 3), suggesting not only that virtually all the receptors present were associated into dimers, but also that they remained stably associated during the immunoprecipitation. Thus, extrinsic dimerization through *fos*/*jun* appears to produce more stable dimers than dimerization through the intrinsic PR dimerization domains. These data are also consistent with the gel mobility supershift studies [Fig. 4(A)] which show that, in the presence of RU486, the *jun* and *fos* zipper domains can override the intrinsic PR dimerization domains, and force the formation of pure A/B heterodimers.

Transcriptional activation by heterodimeric forms of hPR-zip chimeras: A-receptor dominance

To study the transcriptional activity of pure A/B heterodimers, HeLa cells were transiently

cotransfected with wild-type receptors or the PR-zip chimeras, and with a PR-regulated reporter, PRE_2-tk-CAT (Fig. 5). We have previously shown that on PRE-tk-CAT both wild-type PR isoforms strongly stimulate transcription when occupied by R5020, but that when occupied by RU486, hPR_A are inactive, while hPR_B stimulate transcription [12]. The isoform-specific difference in transactivation obtained with RU486 allowed us to use this experimental model to determine which isoform predominates functionally in the heterodimer, as reported previously in preliminary form [12].

Each homodimer was assessed first. RU486-occupied pure B/B homodimers (Fig. 5, set A) produced from wild-type B-receptors, or from B-*fos* or B-*jun* chimeras, all stimulated transcription. B-*jun*/B-*jun* homodimers had lower activity than the other two dimeric species, due perhaps to steric restriction of the PR C-terminus by this strong dimerization domain (see Discussion). None of the three classes of pure A/A homodimers (set B) were transcriptionally active in the presence of the antiprogestin. In Fig. 5, analysis of heterodimer function has been segregated into two groups (sets C and D) based on the gel mobility shift data [Fig. 4(A)]. In set C, cotransfection of B_{wt}/A_{wt} produces all 3 dimeric species through the intrinsic PR dimerization domains, as does cotransfection with B-*fos*/A-*fos*, since homodimerization does not occur through *fos* [16]. Similarly, all 3 dimeric species are produced by random assortment and strong *jun* homodimerization between B-*jun*/A-*jun*. Thus, the receptor combinations in set C are not informative, since all produce mixtures of homo- and heterodimers. By contrast, cotransfection of B-*fos*/A-*jun* or B-*jun*/A-*fos* is informative (set D). Both the gel mobility shift data (Fig. 4, lanes 8 and 10), and other studies of *jun*/*fos* dimerization [16, 25, 26], show that only heterodimers assemble in this setting. When occupied by RU486, these A/B heterodimers have very low transcriptional activity (Fig. 5), analogous to the levels seen with A/A homodimers (set B). The B-*fos*/A-*jun* pair is of particular interest, since B-*fos* alone (set A) is strongly

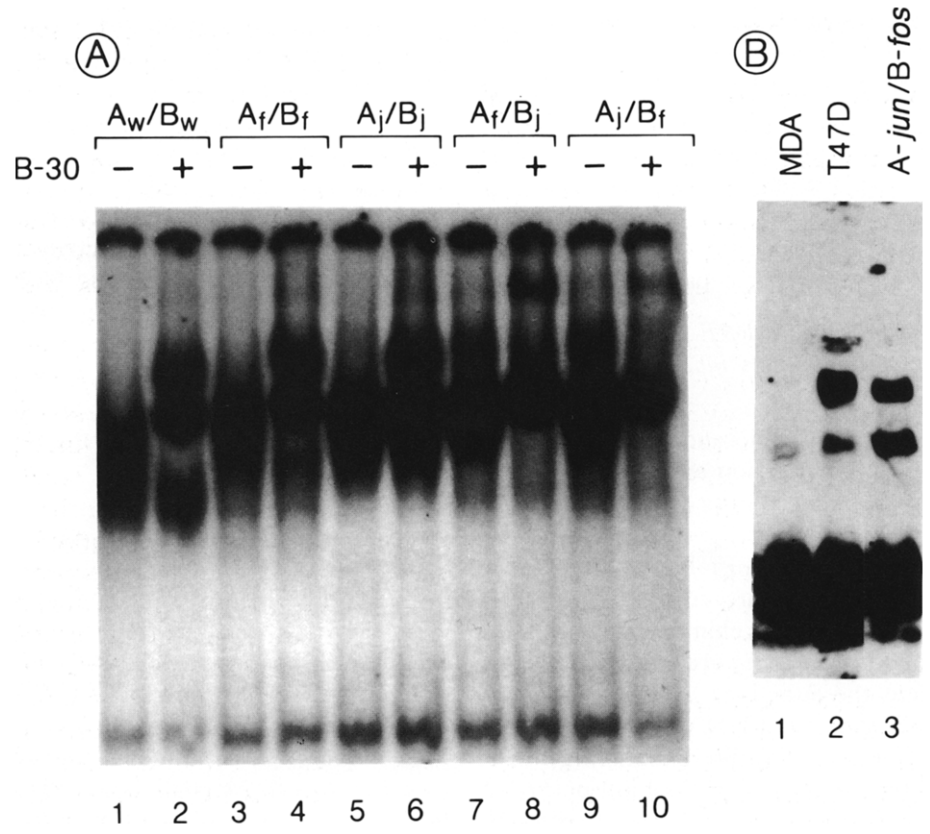

Fig. 4. The dimeric structure of RU486-occupied wild-type and chimeric hPR. (A) Dimeric species analyzed by gel mobility shift assay. COS-1 cells were cotransfected with equimolar concentrations of expression vectors for wild-type A-receptors (A_w) and B-receptors (B_w) or the A- and B-zip receptors shown, treated with 100 nM RU486, and the extracted receptor mixtures were incubated with a ^{32}P-labeled oligomer containing a PRE, in the absence (−) or presence (+) of the B-receptor specific MAb B-30. The reactants were separated on a non-denaturing gel, and the dried gel was autoradiographed. (B) Immune coprecipitation assay of A- and B-receptor mixtures. Whole cell extracts were prepared from 100 nM RU486-treated PR-negative MDA breast cancer cells, PR-positive T47D breast cancer cells, and COS-1 cells cotransfected with equimolar concentrations of the expression vectors for A-*jun* and B-*fos*. Extracts were desalted and receptors were immunoprecipitated with MAb B-30 and cyanogen bromide-activated Sepharose 4B coupled to goat anti-mouse immunoglobulin. The precipitated proteins were eluted with gel loading buffer, separated by denaturing gel electrophoresis, blotted to nitrocellulose, and detected with MAb AB-52 and enhanced chemiluminescence.

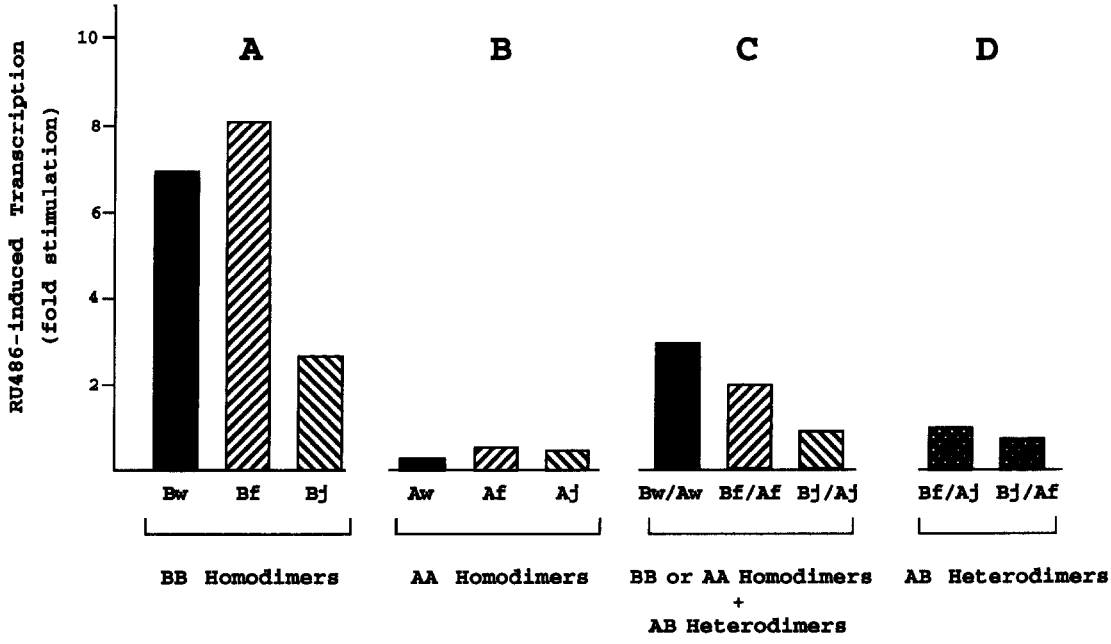

Fig. 5. Transcriptional activity of RU486-occupied mixtures of A/B homo- or heterodimers. HeLa cells were transiently transfected with expression vectors encoding the receptor isoforms shown, together with a β-galactosidase expression vector, and the PRE_2-tk-CAT reporter, and treated with RU486. Cell extracts were normalized to β-galactosidase activity and measured for CAT expression by TLC and phosphorimaging. Transcription values represent fold stimulation over the corresponding values obtained in the absence of RU486.

activated by RU486. We conclude that in the RU486-occupied pure A/B heterodimer, A-receptors are functionally dominant over B-receptors.

hPR-zip heterodimers bind a PRE in the absence of hormone but are transcriptionally inactive

In the absence of hormone, wild type hPR are inactivated by heat shock proteins and assume a conformation that prevents dimerization and DNA binding, while maintaining the high affinity hormone binding site [13, 27, 28]. Occupancy by hormone leads to the dissociation of heat shock proteins followed by receptor dimerization, both of which are required for DNA binding [13]. Since the hPR-zip chimeras dimerize and bind to DNA constitutively, they are a novel tool with which to dissociate the functions of ligand binding from those of dimerization and DNA binding. The gel mobility shift assay in Fig. 6(A) shows that unliganded, coexpressed wild type hPR_A and hPR_B bind poorly to DNA in the absence (lane 1), or presence of antibody (lane 2). In contrast, the hPR-zip chimeras, A-*fos*/B-*jun* (lane 3) or A-*jun*/B-*fos* (lane 5), produce heterodimers that bind strongly to DNA in the absence of hormone, and are quantitatively supershifted with MAb B-30 (lanes 4 and 6). Tight DNA binding apparently occurs *in vivo* as well, judging from the salt requirement for extraction (not shown).

Figure 6(B) shows the ability of these receptors to transactivate a PRE_2-tk-CAT reporter in the presence of the agonist R5020. Although the unliganded hPR-zip heterodimers bind strongly to DNA [Fig. 6(A)], they are transcriptionally inactive in the absence of hormone [Fig. 6(B), lanes 3 and 5], but like wild-type receptors (lanes 1 and 2) they retain the ability to induce transcription in the presence of R5020 (lanes 4 and 6). We conclude that constitutive dimerization and DNA binding are necessary but not sufficient for transactivation, without concomitant ligand binding.

DISCUSSION

Use of fos/jun zippers to form pure hPR heterodimers

We have previously demonstrated that antagonist-occupied B/B, but not A/A, homodimers can activate gene transcription [12]. Our goal in the present study was to analyze the functional properties of pure A/B heterodimers; the major activated dimeric species present in tissues that contain both hPR isoforms. For this purpose, we constructed fusion proteins linking full-length B- or A-receptors to the leucine zipper domains of the nuclear protooncogenes, c-*jun* and c-*fos*. Our strategy was to take advantage of the unique dimerization properties of the *fos/jun* zippers, which, as shown by O'Shea *et al.* [16] form highly stable heterodimers in a 1000:1 ratio over homodimers, due to the instability of the *fos* homodimers [16, 25]. The strong interaction between *fos/jun* zippers has also been used by Spanjaard and Chin [29] to restore function when the ligand binding domain of glucocorticoid receptors on

one molecule was cotransfected with the N-terminal half of the receptors on another molecule. In doing so, they were able to reconstitute hormone-regulated transcription, *in trans*.

In the studies described here, the *fos* or *jun* zipper domains were fused to the C-terminus of the hPR ligand binding domain, the region thought to contain the intrinsic hPR dimerization function [30]. We reasoned that dimerization at the C-terminus would be least likely to disrupt receptor function. By gel mobility supershift and immune coprecipitation analyses, we confirmed that, in the absence of hormone, or after treatment with RU486, a pure population of A/B-zip heterodimers was formed in cells coexpressing A-*fos* and B-*jun* or A-*jun* and B-*fos* chimeras. Since RU486 produces opposite transcriptional effects on a PRE-tk-CAT reporter when bound to hPR_B as compared to hPR_A, treatment with this progesterone antagonist provided a suitable assay system to study dominance of one monomer over the other in the pure heterodimer.

A-receptor transdominance in receptor mixtures

Previous studies have measured the transcriptional activity of hPR-regulated promoters under conditions in which one or both hPR isoforms were present, so that the homodimers alone, or mixtures of homodimers plus heterodimers were formed. When hPR_B is a strong transactivator and hPR_A is weak, the dominant negative transcriptional properties of the A-isoform in the mixed homodimeric/heterodimeric populations have been documented by our group [12] and others [11]. A-receptors are not only dominant when cotransfected with B-receptors, but can also dominantly inhibit ligand-dependent transactivation by other members of the GRE/PRE binding subfamily, and, as recently shown, can inhibit transcription by estrogen receptors bound to estrogen response elements [31]. Moreover, this unusual property of A-receptors does not require that they be bound to DNA, and occurs in the presence of agonists or antagonists [11, 12]. It is not clear whether in all these experimental models, the presence of at least

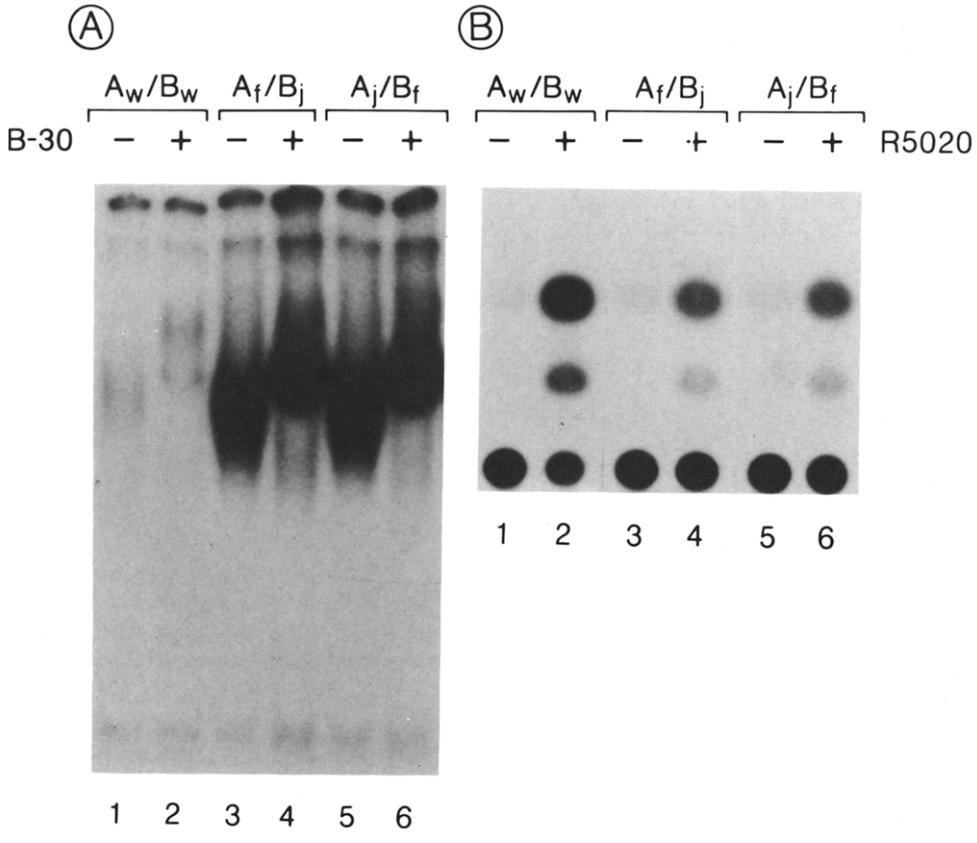

Fig. 6. The dimeric structure and transcriptional activity of unliganded wild-type and chimeric hPR. (A) Dimeric species analyzed by gel mobility shift assay. COS-1 cells were cotransfected with equimolar concentrations of expression vectors for wild-type A-receptors (A_w) and B-receptors (B_w) or the A- and B-zip receptors shown, and the extracted receptor mixtures from hormone-untreated cells were incubated with a ^{32}P-labeled oligomer containing a PRE, in the absence (−) or presence (+) of the B-receptor specific MAb B-30. The reactants were separated on a non-denaturing gel, and the dried gel was autoradiographed. (B) Transcriptional activity of dimeric species in the absence and presence of hormone. HeLa cells were transiently cotransfected with expression vectors encoding the receptor isoforms shown, together with a β-galactosidase expression vector, and the PRE_2-tk-CAT reporter, without (−) or with (+) 100 nM R5020 treatment. Cell extracts were normalized to β-galactosidase activity and measured for CAT expression by TLC.

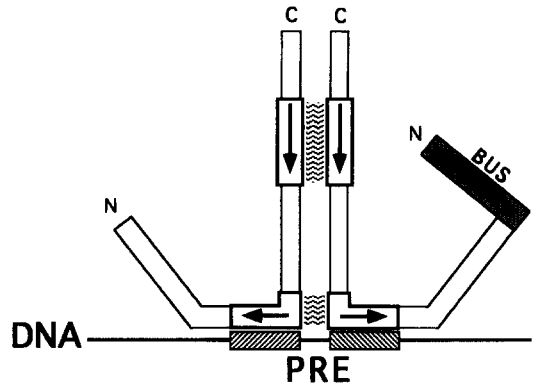

Fig. 7. A model for the structure of the A/B heterodimer showing parallel dimerization at the hormone binding domain (HBD), with antiparallel dimerization and DNA binding at the DNA binding domain (DBD). The HBD at the C-terminus is shown with its dimerization interface (≈); the DBD binds a palindromic PRE and also has a dimerization interface [44]; and the B-upstream segment (BUS) at the N-terminus accounts for the unique functional properties of B-receptors as proposed by Sartorius *et al.* [38].

25% A/A homodimers subverts the activity of the other two dimeric species. It has been proposed that a transcriptionally unproductive complex is formed when hormone-occupied A-receptors, as dimers, bind a common coactivator(s) required for steroid receptor-mediated transactivation from GRE/PRE regulated promoters [32]. Because A-receptors are effective at stoichiometric levels, it is unlikely that they act solely by a DNA-binding independent squelching mechanism [33] which generally requires a large excess of the interfering factor. Rather, they may also form an unproductive complex, either as A/A homodimers or as A/B heterodimers, which can directly compete for DNA binding, analogous to the inhibitory effects of the thyroid hormone receptor (TR) splice variant, TRα2, on transactivation by the active form, TRα1 [34]. Alternatively, the non-DNA-bound A/A homodimers or A/B heterodimers may directly interfere with interactions between upstream GRE/PRE-bound B/B homodimers, or other members of the GR subfamily, and the general transcription complex. Finally, since it is not known whether hPR_A can heterodimerize with other members of the steroid receptor subfamily, A-receptor dominance through such a mechanism cannot be ruled out.

Intermolecular A-receptor dominance in the A/B heterodimer

In the present study we specifically analyzed the transcriptional properties of pure A/B heterodimers by using hPR-zip chimeras treated with the progestin antagonist, RU486. This experimental model creates cellular conditions in which only A/B heterodimers are present, and in which hPR_B are strong transactivators while hPR_A are inactive, allowing the phenotype of the dominant monomer to be determined. The resulting transcription resembled that obtained with pure A/A homodimers, suggesting that A-receptors are dominant over B-receptors in the heterodimer. How do A-receptors inhibit B-receptors when the two are dimerized? Two mechanisms could account for negative dominance by hPR_A in the A/B heterodimer. First, A-receptors could destabilize A/B dimers, preventing their DNA binding [35,36]. This mechanism is unlikely since RU486-treated hPR have been shown by gel mobility assays to bind strongly to a GRE/PRE as A/B heterodimers [10, 37]. Recent studies from our laboratory [38] and that of Meyer *et al.* [39] suggest an alternative mechanism. We have identified a third transactivation function (AF3) located in the far N-terminus unique to the B-isoform. AF3 appears to be responsible for promoter-specific transactivation differences between the A- and B-isoforms by binding a nuclear factor(s), raising the possibility that in the A/B heterodimer, the A-isoform, either directly, or after binding an inhibitor [40], blocks binding of the coactivators at AF3 on the B-isoform.

DNA binding and transactivation by unliganded hPR-zip chimeras

We have shown here that, in the absence of ligand, A-*fos*/B-*jun* or A-*jun*/B-*fos* form pure heterodimers and bind strongly to DNA. However, these unliganded receptors do not activate transcription (Fig. 6). A similar transactivation failure was observed when bivalent antibodies were used to facilitate the DNA binding of unliganded receptors [41]. It is not known whether heat shock proteins are dissociated when receptor dimerization and DNA binding are artificially imposed either by leucine zippers, or by antibodies. However, it has also been previously reported that in unliganded receptors, removal of heat shock proteins does not, by itself, promote receptor binding to DNA [42]. Taken together, these data suggest that neither heat shock protein dissociation, nor dimerization nor DNA binding either alone, or in combination, can substitute for ligand binding to fully activate receptors, and suggest that the hormone subserves some additional function, perhaps following DNA binding.

Wild-type hPR dimerization

We found that with RU486-occupied A-*fos*/B-*jun* or A-*jun*/B-*fos*, only heterodimers are present (Fig. 4), but that with R5020-occupancy some homodimers are formed (not shown). This suggests that in the presence of the agonist, but not the antagonist, the intrinsic hPR dimerization domains can override the strong extrinsic zip dimerization domains. These results support the model of Allan *et al.* [41, 43], who recently demonstrated that R5020 allosterically induces a conformational change in hPR resulting in a coiled structure at the C-terminus, making this region inaccessible to digestion by proteases. By contrast, RU486 induces a more protease accessible or uncoiled configuration at the C-terminus. We believe that the allosteric

structural differences conferred on the C-terminus by RU486 versus R5020 occupancy affects the stability of the *fos/jun* zipper, and in the case of R5020, they are sufficiently unstable so that homodimerization can occur through the intrinsic PR dimerization domains.

With respect to the overall dimeric structure of full-length hPR, O'Shea *et al.* [16] demonstrated that the structure of the *fos/jun* leucine zipper complex is that of a parallel, heterodimeric coiled coil. This suggests that the hPR-zip chimeras also form parallel dimers, and that in this orientation, the dimers are completely functional as shown by the transcriptional activity of agonist- or antagonist-occupied A-*fos*/B-*jun* or A-*jun*/B-*fos* heterodimers (Figs 5 and 6). Juxtaposition of the two hormone binding domains as parallel dimers, is consistent with the binding of dimeric hPR to PRE half-sites oriented as inverted repeats, analogous to the binding of the homeodomains in Athb-1 and -2 to dyad-symmetric DNA sequences, while they are dimerized through leucine zippers [4]. In this configuration (Fig. 7) the dimeric receptor molecules could be aligned in a parallel orientation through the hormone binding domain at the C-terminus, yet as the crystallographic data show, still be aligned in an antiparallel orientation [45] to the inverted dyads of the GRE/PRE.

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