Pergamon

0960-0760(95)00035-6

J. Steroid Biochem. Molec. Biol. Vol. 53, No. 1–6, pp. 9–17, 1995

0960-0760/95 $9.50 + 0.00

Novel Mechanisms of Antiprogestin Action

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When hormone antagonists have unexpected agonist-like effects, the clinical consequences are grave. We describe novel molecular mechanisms by which antiprogestin-occupied progesterone receptors behave like agonists. These mechanisms include agonist-like transcriptional effects that do not require receptor binding to DNA at progesterone response elements, or that result from cross-talk between progesterone receptor and other signalling pathways. We discuss the complex structural organization of progesterone receptors and demonstrate that the B-receptor isoform has a unique third activation domain that may confer agonist-like properties in the presence of antiprogestins. By contrast, the A-receptor isoform is a dominant-negative inhibitor. We argue that these novel mechanisms play a role in the apparent hormone resistance of breast cancers and the variable tissue-specific responses to progestins.

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INTRODUCTION

The mechanisms by which steroid hormone antagonists produce unexpected agonist-like effects, or different tissue-specific effects, are unknown, but have important clinical implications. For example, tamoxifen, the estrogen antagonist used widely to treat breast cancers, is an agonist in bone and uterus and has estrogenic effects on lipid and lipoprotein levels. Tamoxifen can even be an agonist in breast cancers, producing undesirable side effects that exacerbate the disease. Thus, at the start of tamoxifen therapy patients often experience an estrogenic tumor flare, and after long-term tamoxifen therapy inappropriate proliferative effects camouflage as "resistance" [1].

Antiprogestins may also prove to be useful hormonal agents for the treatment of breast cancer [2]. However, like tamoxifen, agonist-like proliferative effects have been reported with the progesterone antagonist RU486 in cultured breast cancer cell lines and in postmenopausal women, under conditions in which inhibition would be expected [3, 4]. Recent studies in our laboratory have addressed molecular mechanisms by which this occurs. They focus on the two natural isoforms of human progesterone receptors (hPR): B-receptors (hPR_B), which are 933 amino acids in length, and A-receptors (hPR_A), which lack 164 amino acids from the N-terminus. When A- and B-isoforms are present in equimolar amounts in wild-type PR-positive cells, or are transiently co-expressed in PR-negative cells, they dimerize and bind DNA as three species: A/A and B/B homodimers, and A/B heterodimers ([2] and references therein). As shown here, this heterogeneity has complicated the study of antiprogestins.

This chapter reviews our recent work with antiprogestins and hPR [5–9]. It demonstrates the extraordinary complexity of antiprogestin action and underscores the fact that steroid receptors regulate transcription through multiple mechanisms, some of which may not require direct interaction of the receptors at canonical DNA hormone response elements (HRE), as previously thought. We show: (1) that on certain promoters, transactivation by antagonist-occupied B-receptors can occur without receptor binding to progesterone response elements (PRE); and (2) that the extent of transcription by antagonist-occupied hPR can be synergistically enhanced by raising intracellular cAMP levels. This requires that hPR be bound to PREs. (3) We have developed new breast cancer cell lines that express only one or the other hPR isoform and show that only antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP. (4) We demonstrate that antagonist-occupied A-receptors are transdominant repressors of antagonist-occupied B-receptors. Thus, under conditions in which both isoforms are present, the B/A ratio may dictate the direction of transcription by antagonists. (5) We show that repression by A-receptors can be achieved without their binding to a canonical PRE. (6) We demonstrate

Proceedings of the IX International Congress on Hormonal Steroids, Dallas, Texas, U.S.A., 24–29 September 1994.
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that the unique 164 amino acid N-terminal segment of hPR_B—the B-upstream segment, or BUS—contains a third transactivation domain (AF3) which, depending on the cell or promoter context, can either function autonomously or can synergize with downstream activation domains of B-receptors to enhance their activity. Thus, the presence or absence of BUS may explain the functional differences between B- and A-receptors. We argue that antagonist-occupied hPR use novel mechanisms to control transcription, and believe that these data begin to explain the unexpected agonist-like effects of progesterone antagonists—an issue of importance in hormone resistance and tissue-specificity.

CONVENTIONAL ACTIONS OF ANTIPROGESTINS

Two fundamentally different mechanisms underly the actions of antagonist–hPR complexes. First is the classical effect of antagonists; namely, their ability to directly inhibit agonist actions. In this scenario, agonist-occupied hPRs regulate transcription by binding as dimers to PREs present on the regulated gene. Antagonist-occupied hPR complexes also bind to PREs but are non-productive. Thus, by this mechanism, antagonist inhibition involves competition between the two ligands, agonist vs antagonist, for hPR occupancy, followed by competition between the two ligand–hPR classes for binding to PREs. With agonists, DNA binding leads to a specific transcriptional response, while with antagonists, the DNA binding is nonproductive. It follows that the nonproductive or inhibitory potency of an antagonist is controlled by numerous factors which include its affinity for the receptors, the affinity of antagonist-occupied hPR complexes for PREs, the number and occupancy of PREs on a promoter, and probably other factors ([2] and references therein).

NOVEL TRANSACTIVATION BY ANTAGONIST-OCCUPIED hPR_B WITHOUT BINDING A PRE [5]

Additionally, there are now data suggesting that alternative mechanisms exist by which antagonist effects are mediated. By one of these, antagonist-occupied hPR complexes have inadvertent transcriptional stimulatory actions through DNA-binding sites or DNA-binding proteins that do not involve the canonical PREs. These novel mechanisms could, in theory, affect not only genes that contain PREs, but even genes that were never meant to be regulated by PR and upon which agonists have no effects. Such mechanisms could explain how an antiprogestin can have effects on genes that are not targets of progesterone. There are several experimental models which demonstrate these unusual mechanisms.

We have studied the transient transcriptional activity of antagonist-occupied hPR using a chloramphenicol acetyltransferase (CAT) reporter driven by a PRE cloned upstream of the thymidine kinase (*tk*) gene promoter [5]. Treatment of HeLa cells expressing hPR_A with the agonist R5020 leads to a 20-fold increase in transcription compared to basal levels, while none of three antiprogestins, RU486, ZK112993 or ZK98299, stimulate transcription. Instead, the antiprogestins typically suppress basal levels of transcription. However, in cells expressing hPR_B, not only the agonist, but all three antagonists, strongly stimulate transcription. We were surprised that ZK98299 was a transcriptional activator, because in our hands, receptors occupied by this antagonist do not bind to DNA *in vitro* or *in vivo*. This suggested that transcriptional activation by antagonist-occupied hPR_B was independent of PRE binding. We therefore removed the PRE from the promoter-reporter constructs. As expected, agonist-dependent transcription was eliminated when the PRE was removed, but to our surprise, the anomalous antagonist-dependent transactivation was retained. Similar results were observed with a DNA-binding domain (DBD) hPR mutant whose specificity was altered so that it would no longer recognize a PRE but would instead bind an estrogen response element. When occupied by antiprogestins, this mutant still activated transcription of the PRE-containing reporter.

Recent data show that other members of the steroid receptor superfamily can have effects independent of the canonical HREs. Potential mechanisms fall into two broad categories. Either the receptors bind to novel DNA sites that differ substantially from the consensus HREs, or the receptors do not bind DNA at all, but interact with other DNA-binding proteins instead [10–17]. By the latter mechanism, termed factor tethering, two factors establish protein–protein contacts on the DNA, but only one of the two actually binds DNA. However, both the DNA-bound protein and its tethered partner contain a DBD. This model is of particular significance for antagonist-occupied hPR_B-mediated transcription, because we also find a requirement for an intact DBD. Thus, an hPR_B mutant lacking an ordered first zinc finger fails to stimulate transcription when occupied by RU486. In addition to its DNA-binding function, the DBD of steroid receptors is implicated in mediating protein–protein interactions [12, 13, 15], perhaps through conserved surfaces that face away from the DNA. Indeed, several recent studies show that glucocorticoid receptors (GR) and c-Jun repress one another's activity by protein–protein binding mechanisms that are independent of DNA binding. Nevertheless, to produce repression, an intact GR DBD is required. Additionally a dimerization function has been assigned to the second zinc finger [18], providing further evidence that the DBD mediates protein interactions. We speculate that induction of transcription by antagonist-occupied hPR_B can proceed through a mechanism in which the receptors are tethered to a DNA-bound protein partner, but do not bind DNA themselves. Alternatively, hPR_B

could function by linking an activator protein bound to the *tk* promoter, to the basal transcriptional machinery.

ANTAGONIST-OCCUPIED HUMAN hPR_B BOUND TO DNA ARE FUNCTIONALLY SWITCHED TO TRANSCRIPTIONAL AGONISTS BY cAMP [6]

By contrast, we have described an entirely different antagonist-mediated activation mechanism, in which hPR do have to be bound to DNA. Because we have a specific interest in the actions of steroid antagonists in breast cancer, we studied antiprogestins in a derivative of T47D human breast cancer cells which express high endogenous levels of hPR_B and hPR_A and which stably express the mouse mammary tumor virus (MMTV) promoter cloned upstream of the CAT gene [6]. Treatment of these cells with the agonist R5020 produces high levels of CAT. When tested alone, the three antiprogestins, RU486, ZK98299 and ZK112993, are unable to stimulate transcription, and all three inhibit R5020-mediated transcription. Thus, in this model, all three antiprogestins are good antagonists.

However, when cellular cAMP levels are raised, two of the antagonists demonstrate a surprisingly strong agonist activity: when present alone, RU486 and ZK112993 are transcriptionally inactive, but in the presence of 8-Br-cAMP, their transcription is agonist-like. Of interest is the fact that ZK98299 is entirely different, and despite elevated cAMP levels, this antagonist does not function as an agonist. Recall that ZK98299-occupied hPR either do not bind to DNA at PREs or have anomalous DNA-binding properties. From this and other controls we deduce that in order for antagonist-occupied hPR to become transcriptional activators under cAMP control, the receptors have to be bound to DNA.

The amplification of steroid-mediated responses in the presence of cAMP is not limited to hPR. While PR levels are overexpressed in T47D cells, the levels of GR, androgen (AR), and estrogen receptors (ER) are extremely low. In addition to hPR, the PREs of the MMTV promoter can be regulated by AR and GR [19, 20]. However, the MMTV promoter lacks an estrogen response element, and is not regulated by ER. In T47D cells expressing the MMTV-CAT reporter, neither dexamethasone nor dihydrotestosterone stimulate CAT transcription, suggesting that GR and AR levels are too low in these cells to activate this promoter in the absence of other influences. However, when cAMP levels are raised, the cells acquire sensitivity to the steroid hormones, resulting in strong transcription. Thus, 8-Br-cAMP sensitizes the MMTV promoter to the actions of glucocorticoids and androgens. In contrast, no transcriptional amplification is seen with estradiol, consistent with the inability of ER to bind the MMTV promoter. This again suggests that the cooperative effects of 8-Br-cAMP require that the receptors be bound to DNA.

Signal transduction pathways ultimately converge at the level of transcription to produce patterns of gene regulation that are specific to the gene and cell in question. Composite promoters may be regulated by multiple independent and interacting factors. In extreme cases, a transcription factor can yield opposite regulatory effects from one DNA-binding site due to modulation by a second factor. A case in point are GRs, which regulate proliferin gene transcription either positively or negatively. The direction of transcription by glucocorticoids is selected by DNA-bound Jun and Fos, which are postulated to interact with GR at PREs. cAMP-responsive signal transduction pathways are often involved in such cooperative interactions. These models suggest that on complex promoters, non-receptor factors, among which are cAMP-regulated proteins, can interact with steroid receptors to select the direction of transcription [12, 21].

Our studies demonstrate the cAMP can both amplify the transcriptional signals of agonist-occupied steroid receptors, and can switch the transcriptional direction of some antiprogestins to render them potent agonists; an effect that can have unintended clinical consequences. We believe that this functional reversal requires that hPR bind to DNA, and that it is not due to ligand-independent receptor phosphorylation, or direct activation of the receptors by protein kinase A-dependent pathways. We find that elevated cAMP levels do not enhance phosphorylation of hPR in breast cancer cells, and do not modulate the hormone-dependent phosphorylation induced by progestins [6], and therefore conclude that cAMP does not directly influence hPR activity by phosphorylating the receptors. Instead, our data are consistent with a model in which the direction of transcription by DNA-bound hPR is indirectly regulated by coactivator proteins whose activity is perhaps controlled by cAMP-dependent phosphorylation. This cooperativity between two signal transduction pathways, one involving steroid receptors, the other involving cAMP-regulated proteins, requires that the steroid receptors bind to DNA. It therefore does not occur on the MMTV promoter with ER, or when hPR are occupied by ZK98299.

NEW T47D BREAST CANCER CELL LINES FOR THE INDEPENDENT STUDY OF PROGESTERONE B- AND A-RECEPTORS: ONLY ANTIPROGESTIN-OCCUPIED B-RECEPTORS ARE SWITCHED TO TRANSCRIPTIONAL AGONISTS WITH cAMP [7]

The studies with wild-type T47D cells described above do not permit analysis of the relative contributions of hPR_B and hPR_A to the synergism observed with cAMP since these cells contain mixtures of the two receptors. However, their constitutive high level

production of PR have made T47D cells the major model in which to study the actions of progesterone in breast cancer, unencumbered by the need for estradiol priming. Because of several special phenotypic properties of T47D cells and because factors other than receptors may be missing in persistently receptor-negative cells, we thought it prudent to retain the T47D cellular milieu in developing new models to study the independent actions of the two PR isoforms [7]. First we needed a PR-negative T47D subline. We developed a monoclonal PR-negative cell line, called T47D-Y, by selecting a PR-negative subpopulation from a parental T47D line that contained mixed PR-positive/PR-negative cells identified by flow cytometry. T47D-Y cells are PR-negative immunologically and by ligand binding assays, by growth resistance to progestins, by failure to bind a PRE *in vitro*, and by failure to transactivate PRE-regulated promoters.

T47D-Y cells were then stably transfected with expression vectors encoding one or other PR isoform, and two monoclonal cell lines were selected that express only B-receptors (called T47D-YB), or only A-receptors (called T47D-YA). The ectopically expressed receptors are properly phosphorylated, and like endogenously expressed receptors, they undergo ligand-dependent down-regulation. The expected B/B or A/A homodimers are present in cell extracts from each cell line, but A/B heterodimers are missing in both [7].

An immunoblot (Fig. 1) demonstrates the equimolar mixture of B- and A-receptors that are present in wild-type T47D cells (Fig. 1, lanes 1 and 2), the absence of either receptor isoform in T47D-Y cells (Fig. 1, lanes 3 and 4), and the unique presence of one or the other receptor isoform in T47D-YB and T47D-YA cells (Fig. 1, lanes 5–8). The levels of each isoform in YB and YA cells are approximately the same as the levels of that isoform in wild-type T47D cells. The structure of the receptors in YB and YA cells is also analogous to that of the wild-type receptors. The triplet banding pattern of wild-type B-receptors is retained in the ectopically expressed B-receptors of hormone untreated (−R) YB cells, and the characteristic molecular weight upshifts of B- and A-receptors produced by R5020 treatment (+R) are also retained in the new cell lines. Both structural features are due to receptor phosphorylation.

The new cell lines were used to study isoform-specific effects of agonists and antagonists when cAMP levels are raised. In the study shown in Fig. 2, YA or YB cells were transiently transfected with the MMTV-CAT reporter and treated with R5020 or the three antiprogestins in the presence or absence of 8-Br-cAMP. 8-Br-cAMP alone does not stimulate CAT synthesis in either cell line. In YA cells, R5020 alone moderately stimulates CAT transcription from MMTV-CAT, and the agonist effect is synergistically enhanced by raising cAMP levels. Thus, agonist-occupied A-receptors are relatively weak transactivators, the activity of which is strongly enhanced by cAMP. When only A-receptors are available as they are in YA cells, the three antiprogestins RU486, ZK98299, or ZK112993 (lanes 7–15) have no intrinsic agonist-like activity and 8-Br-cAMP does not alter this.

However, the agonist and antagonists have quite

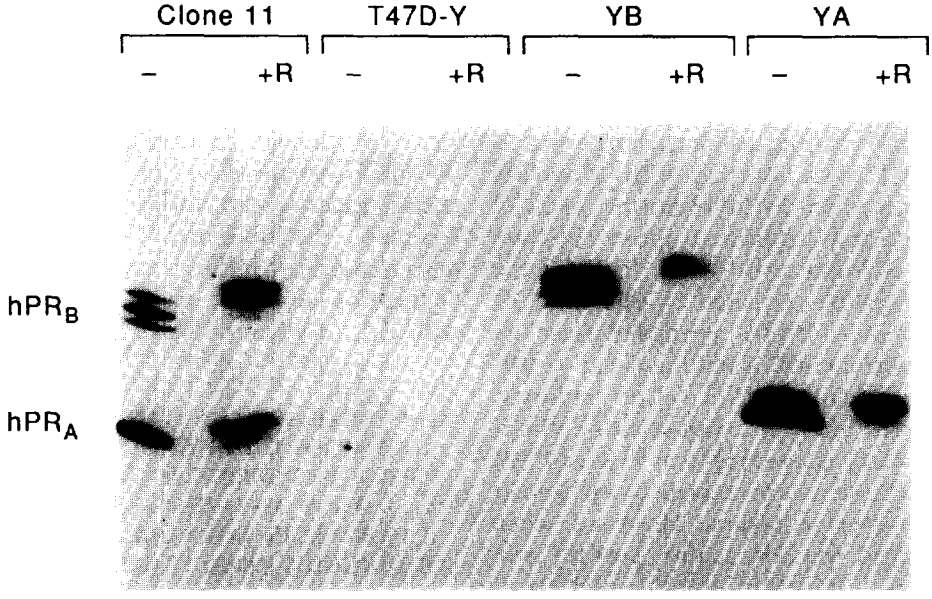

Fig. 1. PR content and structural analysis of wild-type T47D cells (clone 11) and three new T47D cells lines by immunoblotting. The cell lines indicated were treated (+R) or not treated (−) with 100 nM of the agonist R5020 for 1 h and then harvested. Total cellular receptors were extracted by freeze–thawing with 0.4 m KCl, desalted, resolved by SDS–PAGE, and electroblotted to nitrocellulose. The nitrocellulose sheet was probed with the anti-PR antibody AB-52, and protein bands were detected by enhanced chemiluminescence and autoradiography. Reproduced with permission from Sartorius *et al.* [7].

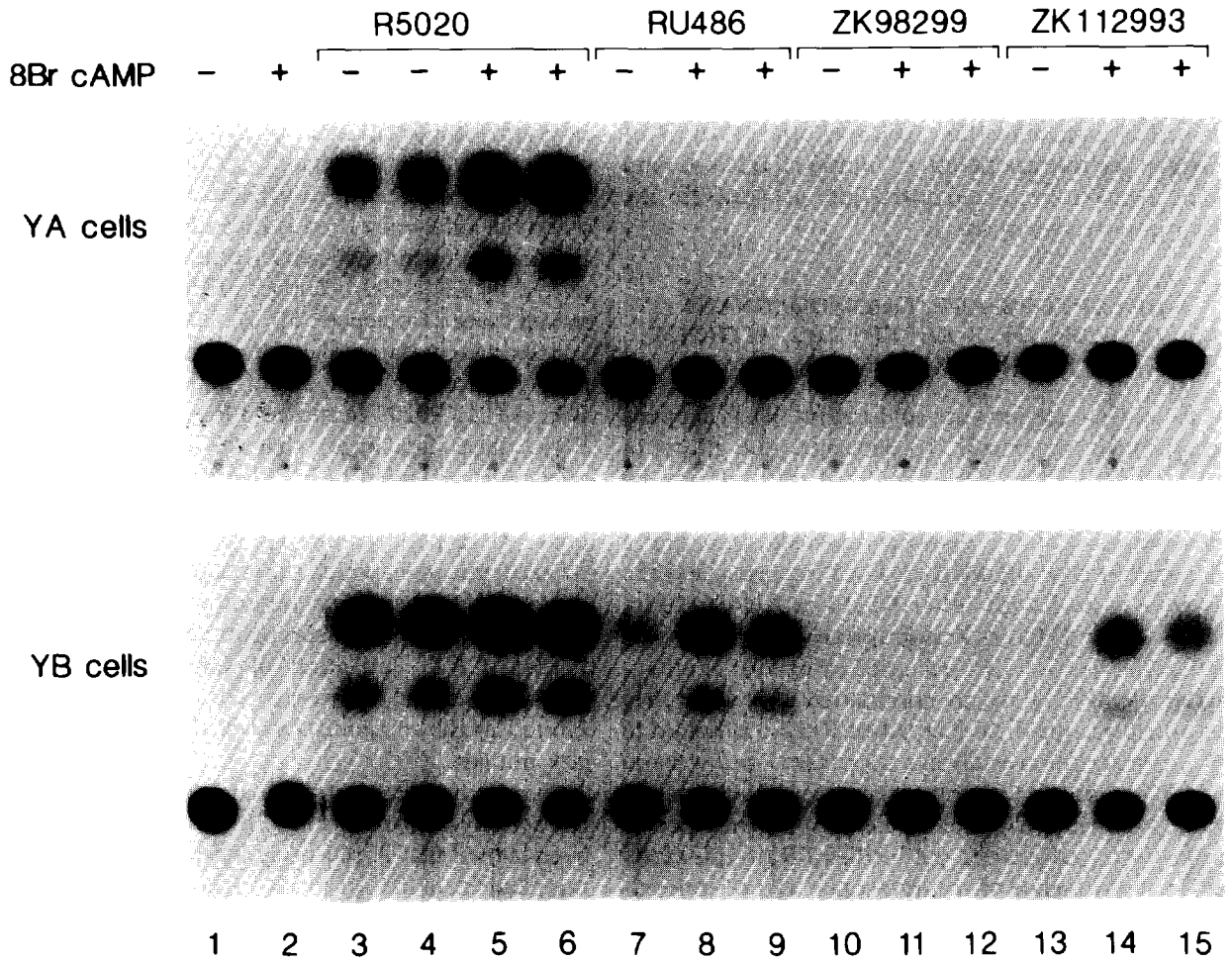

Fig. 2. When cAMP levels are elevated, RU486 and ZK112993 but not ZK98299 are strong transactivators in T47D-YB cells and not in T47D-YA cells. T47D-YA (top) and T47D-YB (bottom) cells were transiently transfected with the MMTV-CAT reporter and treated with the steroid hormone indicated in the presence (+) or absence (−) of 1 mM 8-Br-cAMP; CAT activity levels normalized to β-galactosidase activity were measured by thin layer chromatography. Hormone concentrations were 50 nM for R5020, RU486, and ZK112993 and 100 nM for ZK98299. YA cells contain only A-receptors; YB cells only B-receptors. Reproduced with permission from Sartorius *et al.* [7].

different effects in the B-receptor-containing YB cells. R5020-regulated transcription from the MMTV-CAT reporter is very strong in these cells, making the cAMP-mediated synergism more difficult to observe than in YA cells. Also, in YB cells, 8-Br-cAMP strongly enhances the transcriptional phenotype of the antagonists RU486 and ZK112993. Both of these antiprogestins are weak agonists on the MMTV-CAT promoter, but become strong agonists when 8-Br-cAMP is added (lanes 8, 9 and 14, 15). The antagonist ZK98299 is entirely different since it has no intrinsic agonist activity alone and no enhancement is produced by 8-Br-cAMP (lanes 12, 13). This resistance of ZK98299 in YB cells to the activating effects of cAMP is similar to the one we described in wild-type T47D cells that contain the natural mixture of both receptors.

These studies using our new stable cell lines show that the two PR isoforms behave dissimilarly in their cooperativity with cAMP. With regard to R5020, the synergism between cAMP and agonist-occupied receptors is most pronounced in YA cells. We speculate that in YA cells, cAMP sensitizes the MMTV promoter to the weak signal transmitted by R5020-occupied A-receptors; this is similar to the manner in which cAMP amplifies the weak signals transmitted by hormone-occupied GR and AR in wild-type T47D cells. Since in YB cells agonist-occupied B-receptors are already strong transactivators, cAMP has only modest further effects on this isoform.

With regard to progesterone antagonists, the isoform specificity of the cAMP effect is even more interesting. We find absolutely no effect of cAMP in YA cells, perhaps due to the fact that the antagonists (specifically RU486 and ZK112993) exhibit no agonist-like activity on A-receptors. Is there no minimal signal for cAMP to amplify? In contrast, the two antagonists appear to have some weak agonist-like activity in YB cells; hence, cAMP strongly amplifies this signal, converting the antagonist-occupied B-receptors to potent transactivators. Therefore, it is significant that B-receptors occu-

pied by the antiprogestin ZK98299 are not subject to this functional modulation by cAMP. We speculate that ZK98299-occupied PR are physically removed from cAMP control by their failure to bind DNA. This again implies that cooperativity between DNA-bound PR and a cAMP-regulated coactivator accounts for the transcriptional synergism.

A-RECEPTORS ARE TRANSDOMINANT REPRESSORS OF B-RECEPTORS WITHOUT BINDING A PRE [5, 9]

If B- and A-receptors are so different, what happens when the two are mixed? To determine the effects of A-receptors on antagonist-stimulated transcription by B-receptors, expression vectors encoding hPR_B and increasing levels of hPR_A were cotransfected into HeLa cells together with the PRE-*tk*-CAT reporter, and the cells were treated with either R5020 or RU486. hPR_B alone stimulate CAT transcription in this model whether the receptors are occupied by agonist or antagonist, while hPR_A alone are stimulatory only when they are agonist-occupied. In fact, when RU486 is bound to hPR_A, transcription is always suppressed below basal levels. When the two receptor isotypes are coexpressed, strong transcription is maintained in the presence of the agonist, regardless of the hPR_B–hPR_A ratio. However, in the presence of the antagonist and at approximately equimolar amounts of the two receptors, the transcriptional phenotype of hPR_A predominates, so that hPR_B-stimulated transcription is almost entirely extinguished [5].

When hPR_A and hPR_B are equimolar, a 1:2:1 ratio of A/A, A/B, and B/B dimers is expected. The extensive inhibition by A-receptors suggested that A/B heterodimers have the same inhibitory transcriptional activity as A/A homodimers, and that only B/B homodimers are stimulatory. However, presence of the two competing homodimeric species complicates functional analysis of the heterodimers and B/B homodimers probably account for the incomplete suppression of transcription seen when A- and B-receptors are coexpressed. We, therefore, decided to construct receptors in which the heterodimeric species was the only class present.

When they are mixed, c-Jun and c-Fos preferentially form heterodimers over homodimers by at least 1000-fold [22]. Therefore, to force heterodimerization of hPR, the leucine zippers of c-Fos or c-Jun were fused to the C-terminus of hPR_A or hPR_B [9]. These chimeric hPR retain agonist and antagonist binding capacity, and agonist or antagonist-occupied hPR_A-Jun and hPR_B-Fos, when each is expressed alone, have the same transcriptional phenotype as the wild-type receptors. However, when the two are cotransfected, the weak residual transcription seen with wild-type RU486-occupied B/A receptor mixtures, is entirely eliminated. Thus, CAT levels are reproducibly below control values with B-Fos/A-Jun. These data confirm the A-dominance hypothesis and show that antagonist-occupied pure A/B heterodimers exhibit exclusively the inhibitory transcriptional phenotype of antagonist-occupied A/A homodimers.

The dominance of A-receptors is observed even when the antagonist used is ZK98299. The strong PRE binding-independent transcriptional stimulation imparted by ZK98299-occupied hPR_B in the *tk* promoter model [see reference 5] is 80% suppressed by approximately equimolar concentrations of hPR_A and fully suppressed by a 2-fold molar excess of hPR_A. Because ZK98299-occupied hPR_A do not bind to a PRE, these data imply that the inhibitory effects of antagonist-occupied hPR_A, like the stimulatory effects of antagonist-occupied hPR_B, are mediated by novel non-PRE-dependent mechanisms. This was confirmed by experiments in which the antagonist-occupied A-receptor DBD specificity mutant, which cannot bind a PRE, was used as the competing receptor species. On PRE-*tk*-CAT, activation of CAT transcription by RU486-occupied wild-type hPR_B was completely inhibited by the antagonist-occupied hPR_A-DBD specificity mutant.

Our studies demonstrate that A-receptors can inhibit the activity of B-receptors [5]. In related studies it has been shown that A-receptors inhibit not only B-receptors, but also the activities of other members of the steroid receptor family, including ER [23, 24]. Thus, the dominant inhibitory effects of A-receptors are extensive, and may explain some of the "antiestrogenic" actions reported for antiprogestins. The mechanisms underlying these "trans" inhibitory effects are unknown. Meyer *et al.* [25] demonstrated several years ago that transcription by PR is inhibited by coexpressed ER, and that both PR and GR expression inhibits activation by ER. They suggested that steroid hormone receptors compete for limiting transcription factors that they all use in common. Since ER and PR bind to different DNA response elements, their mutual inhibition appears to occur without the direct DNA binding of the interfering receptor. Thus, when PR interferes with ER action, the gene being suppressed need not contain a PRE or be otherwise progestin-regulated.

A THIRD TRANSACTIVATION FUNCTION (AF3) OF HUMAN PROGESTERONE RECEPTORS LOCATED IN THE UNIQUE N-TERMINAL SEGMENT OF THE B-ISOFORM—THE B-UPSTREAM SEGMENT (BUS) [8]

Why do B-receptors differ from A-receptors? We postulated that the unique 164 amino acid B-upstream segment (BUS) is in part responsible for the functional differences between the two isoforms, and constructed a series of hPR expression vectors encoding BUS fused

to individual downstream functional domains of the receptors (Fig. 3, top) [8]. These include the two transactivation domains, AF1 located in a 90 amino acid segment just upstream of the DBD and nuclear localization signal (NLS); and AF2 located in the hormone binding domain. BUS is a highly phosphorylated domain and contains the serine residues responsible for the hPR_B triplet protein structure. The construct containing BUS-DBD-NLS binds tightly to DNA when aided by accessory nuclear factors. In HeLa cells, BUS-DBD-NLS strongly and autonomously activates CAT transcription from a promoter containing two progesterone response elements (PRE_2-$TATA_{tk}$-CAT) (Fig. 3, bottom). This study shows that the empty expression vector is inactive (lane 1), and that hPR_B are inactive in the absence of hormone (lane 4), but are strongly active in the presence of R5020 (lanes 2–6). Cells transfected with BUS-NLS lacking the DBD show no CAT activity over basal levels (lanes 7 and 8), but with BUS-DBD-NLS a dose-dependent increase in transcriptional activity is observed (lanes 9–18), and at the highest plasmid concentrations (lanes 15–18), BUS-DBD-NLS constitutively activates transcription to levels comparable to those of hormone-activated, full-length B-receptors. Thus, we conclude that this construct contains an autonomous third transactivation function, AF3.

In HeLa cells, transcription levels with BUS-DBD-NLS are equivalent to those seen with full-length hPR_B, and are higher than those seen with hPR_A. Additional studies show that BUS specificially requires an intact hPR DBD in order to be transcriptionally

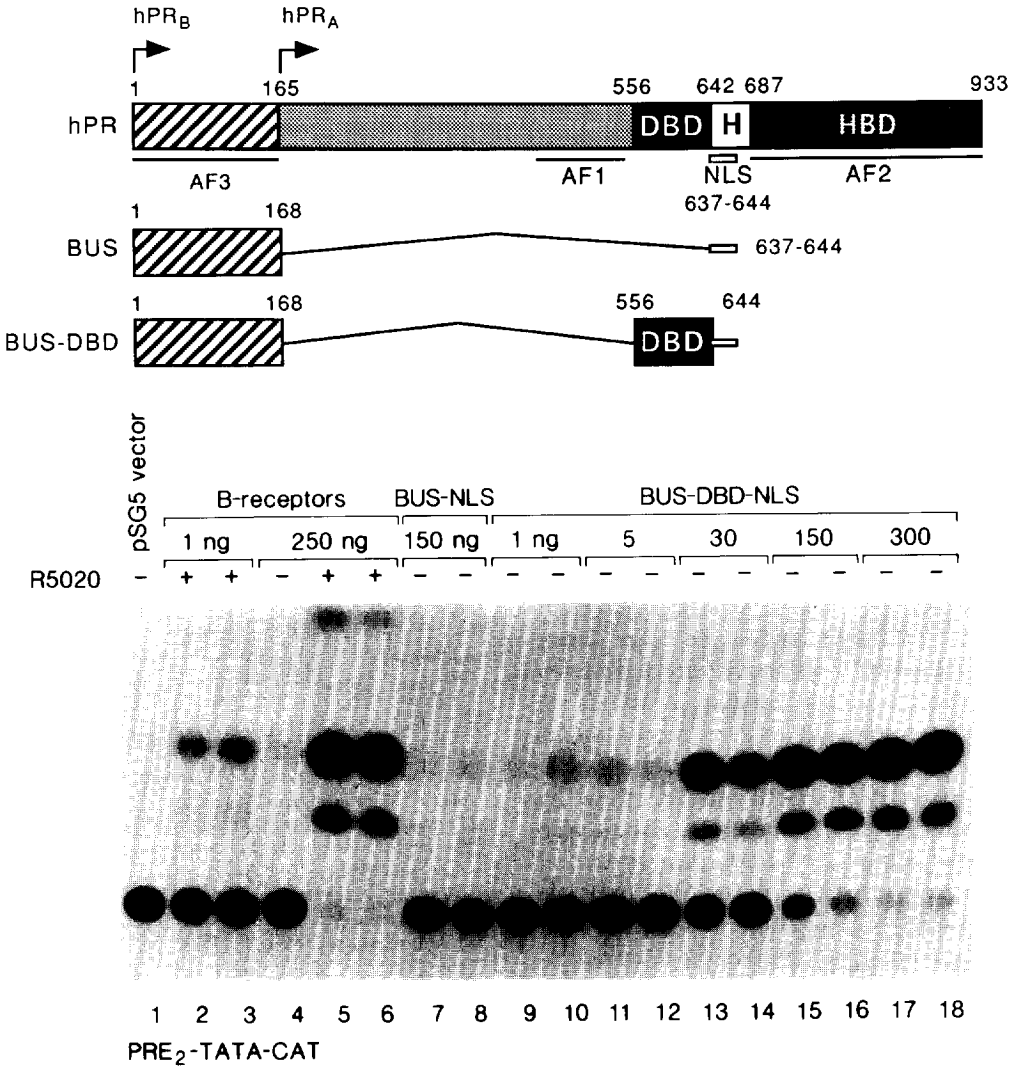

Fig. 3. BUS-DBD-NLS contains a third hPR transcription activation function, AF3. (Top) Structure of the fusion proteins tested below. BUS, B-upstream segment; AF, activation domain; DBD, DNA-binding domain; NLS, nuclear localization signal; HBD, hormone binding domain. (Bottom) HeLa cells were cotransfected with 2 μg of PRE_2-$TATA_{tk}$-CAT reporter and the empty expression vector pSG5 (lane 1), or the indicated concentrations of expression vectors encoding hPR_B (lanes 1–6), BUS-NLS (lanes 7 and 8), or BUS-DBD-NLS (lanes 9–18). 24 h after transfection, the medium was replenished and cells were either untreated (−) or treated with 50 nM of the agonist R5020 (+). Cell lysates were normalized to β-galactosidase activity and CAT assays were performed by thin layer chromatography. Reproduced with permission from Sartorius *et al.* [8].

active. DBD mutants that cannot bind DNA, or whose DNA binding specificity have been altered, cannot cooperate in BUS transcriptional activity. This suggests that the autonomous AF3 activity resides in a discontinuous domain formed from BUS and the hPR DBD. We also find that the autonomous function of BUS-DBD-NLS is promoter and cell-specific. BUS-DBD-NLS does not transactivate MMTV-CAT in HeLa cells, and poorly transactivates PRE_2-$TATA_{tk}$-CAT in the PR-negative T47D-Y breast cancer cells. In the latter case, however, transcription can be restored either by elevating cellular levels of cAMP, or by linking BUS to AF1 or AF2, each of which alone is also inactive in T47D-Y cells. Thus, while in T47D-Y cells each AF alone is inactive, when AF3 is linked to either of the other two AFs (AF3 + AF1 or AF3 + AF2), strong transcriptional activation is regenerated, which is approximately equal to that obtained with B-receptors. These data suggest that in the appropriate cell or promoter context, BUS can supply an important transactivation function in two different ways: either by autonomously activating transcription in the absence of the other two AFs, as it does in HeLa cells on PRE_2-$TATA_{tk}$-CAT; or by synergizing with the other AFs on the hPR molecule, as it does in T47D-Y cells on PRE_2-$TATA_{tk}$-CAT [8]. Is it the autonomous function that produces agonist-like effects from antagonist-occupied B-receptors?

CONCLUSION

We are beginning to accept that a single model cannot describe the actions of steroid receptors. The conventional model, which depicts receptors as ligand-activated proteins that bind to specific DNA sequences at "consensus" hormone response elements and activate transcription, is not incorrect. It is, however, oversimplified and we now appreciate that other models are also applicable. This should not have been surprising given the complex regulatory demands on these receptors. These demands include requirements for both positive and negative transcriptional regulation; for tissue specificity of action; and for regulation of composite and simple gene promoters. It should also not have been surprising given the complex structural organization of these proteins. This includes multiple covalent modifications by phosphorylation; and multiple functional domains that control intramolecular contacts, intermolecular protein–protein interactions, and DNA binding. Finally, given the fact that steroid antagonists are synthetic rather than natural hormones it is perhaps not surprising that their binding produces structural alterations in the receptors that unveil additional novel interactive capabilities. Thus, while antiprogestins can indeed competitively inhibit agonists by forming non-productive receptor-DNA complexes, this is not their sole mechanism of action. Depending on the promoter and cell regulated, antiprogestin effects may also be mediated by receptor interactions with coactivators whose function is in turn controlled by non-steroidal signals. Therefore, when two different signalling pathways are simultaneously activated they can cooperate to produce unintended effects. Additionally, it seems clear from several studies that antagonist-occupied receptors can act without binding to canonical PREs, or without binding to DNA at all, relying perhaps on tethering proteins. This may be a consequence of the unusual allosteric structure imparted on the receptors by synthetic ligands. Because of these, and undoubtedly other mechanisms yet to be discovered, the most serious mistake that investigators in this field can make when studying antiprogestins is to assume that a specific mechanism is operating.

With respect to protein structure, we are only beginning to appreciate receptor complexity. For example, it appeared at first blush that the structural independence of functional domains permitted the analysis of receptor fragments by fusing them to heterologous proteins. However, we now know that important functional domains can overlap; that other functional domains may be discontinuous; and that one domain can modulate the activity of another. This means that analysis of receptor fragments in chimeras is an incomplete test of domain function, and that we need innovative experimental strategies to understand this intramolecular cross-talk. Finally, what could be more unexpected than finding that one receptor isoform can inhibit not just its mate, but even distantly related cousins! Stay tuned for more surprises from this fascinating protein family.

Acknowledgements—The studies described herein were generously supported by the NIH through CA26869, CA55595 and DK48238, and by the National Foundation for Cancer Research. They were originally published in *J. Biol. Chem.* 5 (1993) 9262; *Molec. Endocr.* 7 (1993) 1256; *Cancer Res.* 54 (1994) 3868; and *Molec. Endocr.* 8 (1994) 1347. We thank the publishers for permission to reproduce data and text.

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