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RU486 Exerts Antiestrogenic Activities through a Novel Progesterone Receptor A Form-mediated Mechanism*

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The human progesterone receptor (hPR) exists in two distinct forms in most cells, hPR-A and hPR-B. Both receptor isoforms exhibit distinct biological functions and demonstrate a cell- and promoter-specific ability to regulate gene transcription. Interestingly, in cell contexts where PR-A is transcriptionally inactive, it acts as a progesterone-dependent inhibitor of estrogen receptor function. Coexpression of the human estrogen receptor with the A form (but not the B form) of the human progesterone receptor resulted in a ligand-dependent inhibition of estrogen receptor-mediated gene transcription. The antiprogestins RU486 (Mifepristone) and ZK98299 (Onapristone) and related antiprogestins were all effective "noncompetitive" inhibitors of the estrogen receptor in this assay as none of these compounds interacted directly with the estrogen receptor. This observation may explain in part the observed tissue-specific antiestrogenic effects of RU486 and further indicates that the antiestrogenic activities of antiprogestins may be intrinsic to their biological function. This important new information defines novel activities of progesterone receptor ligands and may alter the way in which we define progesterone receptor modulators for future clinical applications. In addition, these data reveal that the A form of the progesterone receptor plays a key role in modulating estrogen receptor function in cells where both receptors are expressed.

The human progesterone receptor (hPR)[1] can exist in two distinct molecular forms in cells, hPR-A and hPR-B (1, 2). These forms differ at the amino terminus, where hPR-B is 164 amino acids longer than hPR-A. We have shown previously that the biological activities of these two isoforms are distinct in that they function as cell- and promoter-specific modulators of transcription (3). Interestingly, in cell and promoter contexts where PR-A does not activate transcription, the protein functions as a specific ligand-dependent inhibitor of glucocorticoid, androgen, and mineralocorticoid receptor transcriptional activity. Both progestin agonists and antagonists inhibit glucocorticoid receptor function when PR-A is coexpressed in the cell. These results define a central role for PR-A in regulating the activity of a number of the steroid receptors.

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[1] The abbreviations used are: hPR, human progesterone receptor; hER, human estrogen receptor; MMTV, mouse mammary tumor virus; ERE, estrogen response element.

We hypothesized that PR-A might function analogously as a modulator of human estrogen receptor (hER)-mediated transactivation. The antiprogestin RU486 ((11β,17β)-11-[4-(dimethylamino)phenyl]-17-hydroxy-17-(1-propynyl)estra-4,9-diene-3-one) inhibits estrogen action in the endometrium, but not in the oviducts of non-human primates (4–6). Tissue-specific modulation of hER action by hPR-A might explain such effects of antiprogestins. In addition, we considered that PR-A modulation of hER function could explain the observed antiestrogenic activities of some progesterone receptor agonists used in hormone replacement therapy (7) and the antiestrogenic activity of progestin agonists in the human endometrium (8). Because of the possibility that antiestrogenic activity is an intrinsic property of progesterone receptor ligands and may in fact be required for clinical efficacy, we sought to elucidate the biological mechanism of these antiestrogenic effects using a reconstituted estrogen-responsive transcription system *in vitro*.

MATERIALS AND METHODS

Chemicals—Restriction and modification enzymes were obtained from Promega (Madison, WI), Boehringer Mannheim, or New England Biolabs Inc. (Beverly, MA). Polymerase chain reaction reagents were obtained from Perkin-Elmer. Chemicals were purchased from Sigma. The antiprogestins ZK112993 (11β-(4-acetylphenyl)-17β-hydroxyl-17α-(1-propinyl)-4,9-estradiene-3-one) and ZK98299 [(11β-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13α methyl-4,9-gonadien-3-one] were a gift from Dr. David Henderson (Schering AG, Berlin). The antiestrogen ICI 164384 (*N*-(*n*-butyl)-11-[3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl]-*N*-methylundecanamide) was generously supplied by Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, England).

Construction of MMTV-ERE Reporter—The plasmid ΔMTV-LUC containing a deletion of the sequences from positions 190 to −88 was obtained from Dr. Ron Evans (Salk Institute, San Diego, CA). This plasmid was digested with *Hin*dIII, and five copies of a 33-base pair vitellogenin ERE were inserted. The sequence of the positive strand of this oligonucleotide was 5′-AATTCAAAGTCAGGTCACAGTGACCTGATCAAA.

Cell Culture—Monkey kidney CV-1 fibroblasts were routinely maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT).

Transient Transfection Assays—Cells were seeded in 12-well, 96-well, or 10-cm tissue culture plates. DNA was introduced into cells using calcium phosphate coprecipitation (9). 20 μg of DNA/ml of transfection buffer was used in each transfection reaction. In this mixture, the concentration of the luciferase plasmids and that of the internal control plasmid pCH110 (which contains the gene for the β-galactosidase enzyme) remained constant (5 μg of each plasmid DNA), while the receptor plasmid concentration varied as indicated for each experiment. Different amounts of the receptor parental plasmid pSV2-neo were included to keep constant the total amount of the SV40 enhancer-containing vectors. pGEM4 plasmid DNA was added to balance the total DNA concentration to 20 μg/reaction. For the 96-well plate experiments, transfections were performed on a Biomek 1000 automated laboratory workstation (Beckman Instruments), and cells were incubated with the precipitate for 6 h. Cells were washed with phosphate-buffered saline and incubated for 40 h with or without hormones as indicated in the figure legends. Cell extracts were prepared as previously described (9) and assayed for luciferase and β-galactosidase activities.

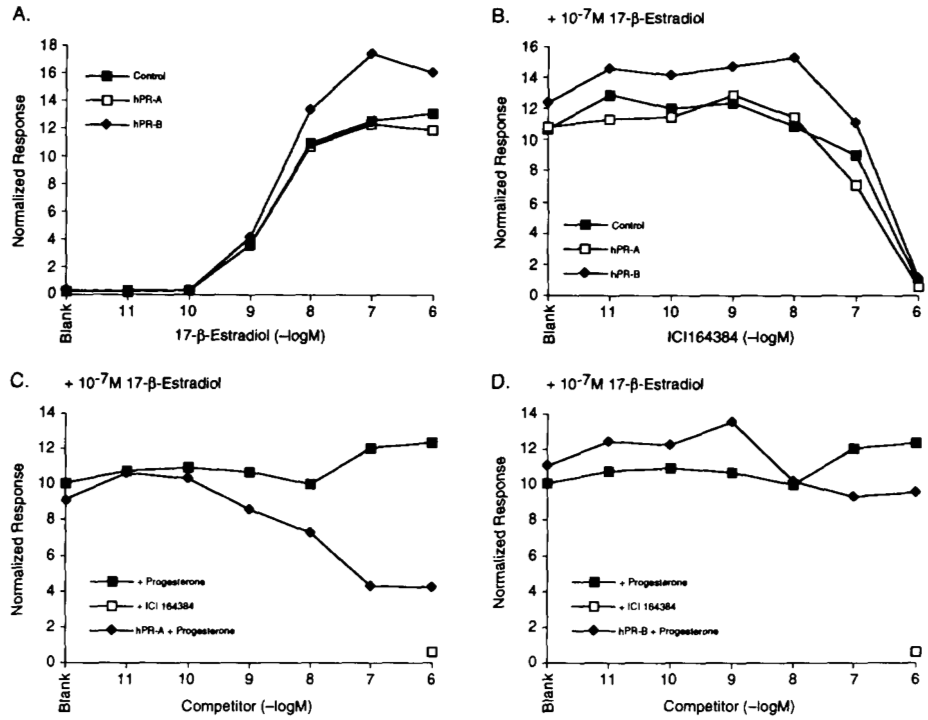

FIG. 1. **Noncompetitive inhibition of estrogen receptor transcriptional activity by progesterone.** Monkey kidney CV-1 cells were transiently transfected with vectors expressing the human estrogen receptor alone or in combination with a vector expressing either the human progesterone receptor A or B form as indicated. The transcriptional activity in these setups was measured following the addition of increasing concentrations of 17β-estradiol (*A*), increasing concentrations of the pure antiestrogen ICI 164384 in the presence of a saturating concentration of 17β-estradiol (10^{-7} M) (*B*), and increasing concentrations of progesterone in the presence of 10^{-7} M 17β-estradiol (*C* and *D*). In these experiments, CV-1 cells were transiently transfected with a MMTV-ERE-LUC (where LUC represents luciferase) reporter and vectors producing the human estrogen receptor (pRST7hER) or the human progesterone receptor B form (pSVhPR-B) or A form (pSVhPR-A) as indicated. The construction of these vectors has been described elsewhere (3, 13). The concentration of the hPR-A vector chosen was determined by performing the experiment at several different concentrations of vector and then using that concentration optimal for transcriptional read-out. The concentration of the hPR-B vector was chosen based upon the amount required to give an identical amount of immunoreactive hPR-A and hPR-B. The normalized luciferase activity was calculated by dividing the raw luciferase ($\times 10^4$ units) for each point by the β-galactosidase activity (($A_{415\ nm} \times 10^5$)/time in minutes) at that point. A representative experiment is shown. Each data point shown represents the average of triplicate determinations of the transcriptional activity under a given experimental condition. The average coefficient of variation at each hormone concentration was <15% in this experiment.

RESULTS AND DISCUSSION

To examine the interplay between hER- and hPR-mediated signal transduction pathways, we reconstituted an estrogen-responsive transcription unit in an ER/PR-negative CV-1 cell line. These cells were transfected with an estrogen-responsive MMTV-ERE promoter (mouse mammary tumor virus in which glucocorticoid response elements have been replaced with EREs) and expression vectors for hER alone or hER in combination with either hPR-A or hPR-B. The use of heterologous promoters allowed the examination of direct effects of agents on hPR and hER function without the complications associated with cross-regulation of their respective natural promoters. The results of these analyses are shown in Fig. 1. In this system, hER responded appropriately to 17β-estradiol, demonstrating an EC_{50} of 3 nM (Fig. 1*A*). The activity of 17β-estradiol on hER was unaffected by coexpression of either hPR-A or hPR-B (Fig. 1*A*). The ability of 17β-estradiol to induce transcriptional activation by hER could be inhibited by the addition of the pure antiestrogen ICI 164384 (10) (Fig. 1*B*). As expected, coexpression of hPR-A or hPR-B did not affect the activity of ICI 164384. Interestingly, activation of hER by 17β-estradiol could be inhibited by progesterone when hER and hPR-A were coexpressed (Fig. 1*C*). Maximal inhibition (55%) was achieved at 100 nM progesterone. This inhibition of hER function mediated by hPR-A was specific as hPR-B would not inhibit under the same experimental conditions (Fig. 1*D*). Hormone binding analysis supported equivalent levels of expression of hPR-A and hPR-B in this system. These data indicate that progesterone can function as a partial hER antagonist in cells where hPR-A is coexpressed. This inhibition is mediated by a noncompetitive mechanism as no direct effects on hER were observed. This new information indicates that a considerable degree of cross-talk exists between the ER and PR signal transduction pathways. These experiments indicate that PR-A should be considered a key modulator of steroid receptor function in certain cells and that the biological activity of progesterone extends beyond its ability to modulate PR function directly.

To evaluate whether antiprogestins could modulate hER transcriptional activity through its interaction with PR-A, we examined the ability of antiprogestins to modulate hER function in the presence or absence of coexpressed hPR-A. In the absence of hPR-A, none of the compounds tested exhibited any significant effects on hER activity (Fig. 2, *A–C*). However, when PR-A was coexpressed in the cell, all three antiprogestins tested exhibited a potent antiestrogenic activity. When compared to the activity of the pure antiestrogen ICI 164384, RU486 and ZK112993 each demonstrated >80% efficacy (Fig. 2, *A* and *B*). The compound ZK98299 was slightly less effective (70%) (Fig. 2*C*). The ability of these compounds to inhibit the transcriptional activity of hPR-B directly was also examined; RU486, ZK112993, and ZK98299 displayed IC_{50} values of 0.5, 0.6, and 2.7 nM, respectively (Table I). In this experiment, hPR-B was used to evaluate progestin activity as our previous studies suggested that hPR-A is not an effective activator of transcription in CV-1 cells (3). The concentrations of antiprogestins (RU486, ZK112993, and ZK98299) required for half-

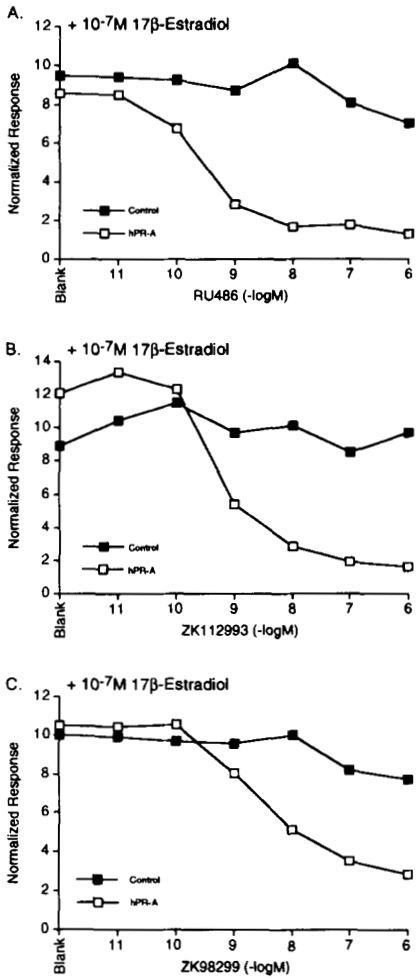

FIG. 2. **Noncompetitive inhibition of estrogen receptor transcriptional activity by progesterone antagonists.** In this experiment, monkey kidney CV-1 cells were transiently transfected with a MMTV-ERE-LUC reporter and vectors producing the human estrogen receptor (pRST7hER) alone or in combination with a vector producing the human progesterone receptor A form (pSVhPR-A). The transcriptional activity of the estrogen receptor in this experiment was measured following the addition of 10^{-7} M 17β-estradiol alone or in combination with the antiprogestins RU486 (*A*), ZK112993 (*B*), and ZK98299 (*C*). Experimental protocols and data calculation are as described for Fig. 1. The experiment detailed above is representative of independent assays ($n > 3$). The average coefficient of variation at each hormone concentration was <15% in this experiment.

maximal inhibition of hER in the presence of hPR-A were 0.3, 0.5, and 3 nM, respectively. These results suggest that both processes, direct inhibition of PR and indirect inhibition of hER, can occur simultaneously in certain cells upon administration of antiprogestins. In the presence of coexpressed hPR-B and hER, the antiprogestins examined exhibited no measurable antiestrogenic activity (>5%), confirming the specificity of this inhibitory process. In previous studies, we observed distinct differences in the functional roles of hPR-A and hPR-B (3). In the presence of hPR-A (but not hPR-B), antiprogestins were shown to inhibit the transcriptional activity of the androgen, mineralocorticoid, and glucocorticoid receptors, but not that of the vitamin D receptor or unrelated transcription factors (3). These results are in agreement with the hypothesis that antihormone-activated hPR-A sequesters a transcription factor required for the function of the ER/PR class of receptors. Unfortunately, because all the known antiprogestins are of the same chemical derivation (11β-substituted steroids), it is not possible to determine whether anti-ER activity is a function of all antiprogestins or just this particular chemical series. These data indicate that the development of a pure antiprogestin to modify the transcriptional activity in cells expressing hPR-A together with other steroid receptors will be difficult using classical approaches.

TABLE I

Ligand-dependent activation and inhibition of progesterone receptor transcriptional activity

For Part A, CV-1 cells were transfected with an expression vector encoding the human progesterone receptor B form (pSVhPR-B) and a progesterone-responsive MMTV-LUC promoter. The transcriptional activity of the progesterone receptor was measured following the addition of increasing concentrations of progesterone receptor agonist. The efficacies of the selected agonists were compared to that of progesterone (100%). The potency (EC_{50}) was calculated as the concentration of hormone required to give half-maximal activation. For Part B, using an identical set of transfections, we measured the transcriptional activity of hPR-B in the presence of 10^{-8} M progesterone (EC_{50} concentration) and increasing concentrations of the indicated receptor antagonists. The value representing 100% inhibition is the transcriptional activity of hPR-B in the absence of added progesterone. In this experiment, CV-1 cells were transiently transfected with a MMTV-LUC reporter and a vector producing the human progesterone receptor B form (pSVhPR-B) as described under "Materials and Methods," with the exception that each transfection mixture included 0.5 μg of pSVhPR-B, 10 μg of MMTV-LUC, 5 μg of pCH110, 5 μg of pGEM4 carrier DNA, and 4.5 μg of pSV2-neo. The EC_{50}, IC_{50}, and efficacy values were calculated from multiple experiments using the same experimental protocol.

	Efficacy	Potency (EC_{50})
	%	nM
A. Activation of progesterone receptor transcriptional activity		
Progesterone	100	7.8 ± 2.2
17α-Hydroxyprogesterone	34 ± 2	2250 ± 150
Medroxyprogesterone acetate	94.5 ± 10.5	0.12 ± 0.04
Norethynodrel	111 ± 13	1.6 ± .4
Norethindrone	117 ± 6	7.8 ± 2.2
	Efficacy	Potency (IC_{50})
	%	nM
B. Inhibition of progesterone receptor transcriptional activity		
RU486	94 ± 5	0.5 ± 1
ZK112993	97 ± 1	0.6 ± 0.1
ZK98299	95 ± 2	2.7 ± 0.8

The intriguing possibility that some of the biological actions of progestins and antiprogestins may be related to their antiestrogenic activities prompted us to examine several additional hPR agonists. The results of these experiments are shown in Fig. 3. Interestingly, norethynodrel and norethindrone (which are derived from 19-nortestosterone) and medroxyprogesterone acetate (which is derived from hydroxyprogesterone) were effective antiestrogens in our assay (Fig. 3, *A*, *C*, and *D*). The compound 17α-hydroxyprogesterone was considerably less effective (Fig. 3*B*). The amount of agonist required for half-maximal activation of hPR-B (EC_{50}) (Table I) was consistently greater than that required for half-maximal inhibition of hER (IC_{50}) (Fig. 3). This may indicate that inhibition of hER activity may require less hormone-activated hPR than it takes to activate progesterone-responsive promoters. These results prompt a careful examination of the antiestrogenic effects of progestin agonists *in vivo* and a determination of the tissue specificity of these responses. Interestingly, in humans, it has been shown that compounds of the 19-nortestosterone series reverse the beneficial effects of estrogen on high density lipoprotein cholesterol when used in hormone replacement therapy, whereas those compounds derived from hydroxyprogesterone do so to a lesser degree (7). Since elevated levels of high density lipoprotein cholesterol are negatively correlated with heart disease, it appears from our data that use of progestins such as the hydroxyprogesterone derivatives would be desirable in hormone replacement therapy (11). It is unclear what effect antiprogestins have on serum low and high density lipoprotein choles-

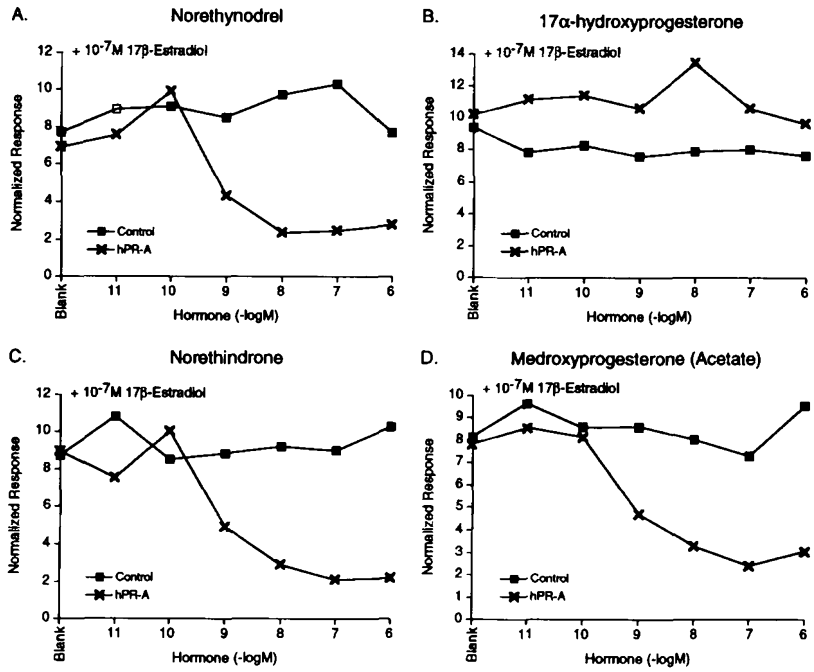

FIG. 3. **Progesterone receptor agonists differ in their ability to inhibit noncompetitively estrogen receptor transcriptional activity.** Monkey kidney CV-1 cells were transiently transfected with a MMTV-ERE-LUC reporter and vectors producing the human estrogen receptor (pRST7hER) alone or in combination with a vector producing the human progesterone receptor A form (pSVhPR-A). The transcriptional activity of the estrogen receptor was determined following the addition of 10^{-7} M 17β-estradiol alone or in combination with increasing concentrations of norethynodrel (*A*), 17α-hydroxyprogesterone (*B*), norethindrone (*C*), and medroxyprogesterone acetate (*D*). Experimental protocols and data calculation are as described for Fig. 1. The experiment detailed above is representative of independent assays ($n > 3$). Each data point is assayed in triplicate.

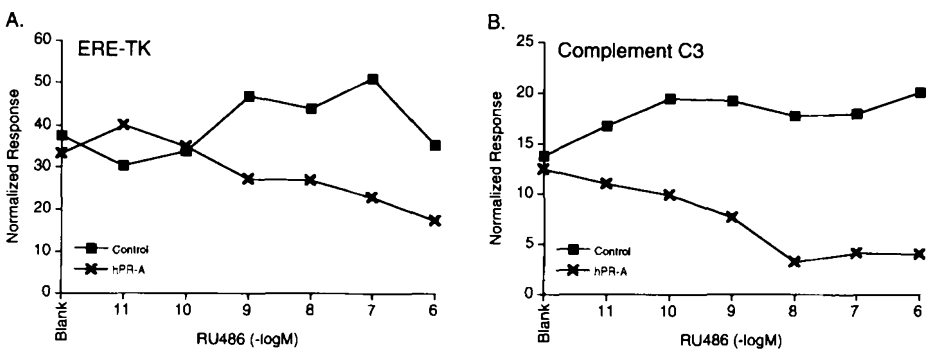

FIG. 4. **Noncompetitive antiestrogenic activity of RU486 is not promoter-restricted.** Monkey kidney CV-1 cells were transiently transfected with vectors expressing the human estrogen receptor (pRST7hER) alone or in combination with a vector expressing the human progesterone receptor A form (pSVhPR-A). The transcriptional activity of estradiol-induced estrogen receptor transcriptional activity was measured on using a synthetic ERE-TK-LUC (*A*), or a natural complement C3 promoter in the presence or absence of increasing concentrations of RU486 (*B*). The construction of these reporter plasmids has been described elsewhere (13). Experimental protocols and data calculation are as described for Fig. 1, with the exception that the MMTV-ERE-LUC plasmid was replaced with either ERE-TK-LUC or C3-LUC. The experiment detailed above is representative of several independent assays. Each measurement was performed in triplicate.

terol. Our new insights may also explain the effectiveness of high doses of medroxyprogesterone acetate as a chemotherapeutic agent in the treatment of breast cancer and suggest that the 19-nortestosterone-derived progesterone agonists or PR antagonists may function analogously.

Analysis of the activity of RU486 *in vivo* indicates that this compound does not oppose all estrogenic responses. In the primate uterus, for instance, in addition to functioning as an antiprogestin, it inhibits estrogen-stimulated uterine proliferation (4–6). However, RU486 acts primarily as an antiprogestin in the oviducts of the same animal and does not block estrogen-dependent oviductal differentiation (4, 5). Interestingly, it has been shown that hPR-A is well expressed in the endometrium throughout the human menstrual cycle, suggesting that the contraceptive efficacy of RU486 may relate to its ability to function both as an antiprogestin in late cycle and as an antiestrogen throughout the cycle (12). In our previous analysis of hPR-A function, we concluded that it operates as a cell- and promoter-specific inhibitor of the PR-related intracellular receptors (3). To examine whether the antiestrogenic effects of hPR-A were similarly restricted, we examined the transcriptional activity of MMTV-ERE in HepG2 (human hepatocellular carcinoma) and HS578T (human breast cancer) cells. In HepG2 cells, the antiprogestins did not exhibit any antiestrogenic activity, whereas in HS578T cells, they functioned as antiestrogens (data not shown). The observation that the identical promoter is differentially regulated in different cell lines strongly suggests that estrogen receptor-mediated activation of transcription is not identical in all cells. Since PR-A is not ubiquitously expressed in all tissues, our data suggest a novel mechanism to generate cell-specific estrogen antagonists.

The promoter specificity of the antiestrogenic activity of RU486 was also examined. The results shown in Fig. 4 indicate that RU486 effectively suppressed estrogen receptor-mediated activation of a synthetic ERE-TK (where TK represents thymidine kinase) promoter (Fig. 4*A*) in CV-1 cells in the presence of coexpressed hPR-A as well as the natural complement C3 promoter, which contains an endogenous ERE (Fig. 4*B*) (13). Thus, hPR-A can function as either a transcriptional activator or a repressor depending on the promoter and cell line examined (3). It is reasonable to postulate that differences in cellular transcription factor pools dictate hPR-A biological function.

Therefore, it is likely that the ability of RU486 to function as an antagonist of some, but not all, estrogen-regulated responses is a consequence of the cellular context of the target gene.

Our findings indicate a molecular basis for the observed noncompetitive antiestrogenic effects of antiprogestins. They highlight the need to understand clearly how antiprogestins mediate their biological effects to permit an optimization of their clinical utility. A considerable amount of effort has been expended to develop compounds that display absolute hPR specificity in order to develop a "clean" antiprogestin (14). This may be a futile exercise if the ability of PR to inhibit steroid receptor function noncompetitively is an intrinsic property of all antiprogestins. These data suggest that alternate pharmacological approaches to inhibit PR selectively must be developed.

We propose that the mechanism of noncompetitive inhibition of hER involves the sequestration by hPR-A of a transcription factor required for hER transcriptional activity. We feel that this transcription factor or cofactor is required at the distal stages of the signal transduction pathway where the receptor directly interfaces with the transcription apparatus. If antiprogestins allow us to interfere pharmacologically with this vital step in activation, they may have clinical utility in the treatment of breast cancer(s) wherein estrogen receptor activity may be constitutively manifested in a hormone-independent manner (15) or wherein a hormone-binding defective ER mutant constitutively activates transcription and, in so doing, escapes standard endocrine intervention protocols (16).

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