MOLECULAR AND CELLULAR BIOLOGY, Dec. 1994, p. 8356–8364
0270-7306/94/$04.00+0

Vol. 14, No. 12

The A and B Isoforms of the Human Progesterone Receptor Operate through Distinct Signaling Pathways within Target Cells

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Received 27 July 1994/Returned for modification 25 August 1994/Accepted 8 September 1994

The biological response to progesterone is mediated by two distinct forms of the human progesterone receptor (hPR-A and hPR-B). In most cell contexts, hPR-B functions as a transcriptional activator of progesterone-responsive genes, whereas hPR-A functions as a transcriptional inhibitor of all steroid hormone receptors. We have created mutations within the carboxyl terminus of hPR which differentially effect the transcriptional activity of hPR-B in a cell- and promoter-specific manner. Analogous mutations, when introduced into hPR-A, have no effect on its ability to inhibit the transcriptional activity of other steroid hormone receptors. The observed differences in the structural requirements for hPR-B and hPR-A function suggest that transcriptional activation and repression by PR are mediated by two separate pathways within the cell. In support of this hypothesis, we have shown that hPR-A mediated repression of human estrogen receptor (hER) transcriptional activity is not dependent on hER expression level but depends largely on the absolute expression level of hPR-A. Thus, it appears that hPR-A inhibits hER transcriptional activity as a consequence of a noncompetitive interaction of hPR-A with either distinct cellular targets or different contact sites on the same target. We propose that hPR-A expression facilitates a ligand-dependent cross-talk among sex steroid receptor signaling pathways within the cell. It is likely, therefore, that alterations in the expression level of hPR-A or its cellular target can have profound effects on the physiological or pharmacological responses to sex steroid hormone receptor ligands.

The growth and development of the human mammary gland are regulated in part by the actions of estrogen and progesterone. In this tissue, estrogen appears to have a proliferative effect on mammary epithelial cells, whereas progesterone functions as a modulator of estrogen action (8). The mechanism by which progesterone affects estrogen-stimulated responses is unclear, though its ability to modulate estrogen receptor (ER) expression is likely to be important (8). In addition, accumulating evidence indicates that progestins can exhibit both proliferative and antiproliferative activities on breast cancer cells in vitro independent of estrogen stimulation, suggesting that progesterone may act directly on target gene transcription in mammary cells (6).

Progesterone mediates its biological activity following its interaction with a specific, high-affinity progesterone receptor (PR) located within target cell nuclei (29). In the T47D human breast cancer cell line, PR occurs as two distinct molecular forms (18), termed hPR-B (116 kDa) and hPR-A (94 kDa). These two isoforms, different only at the N terminus, are derived from transcripts initiated from two distinct estrogen-inducible promoters within a single-copy PR gene (21).

The mechanism by which the PR modulates target gene transcription remains to be determined. However, it has been shown in vitro that the receptor directly contacts the basal transcription machinery and stabilizes the formation of the transcription preinitiation complex (19, 23). Detailed molecular dissection of PR structure has permitted the definition of two distinct regions (activation functions [AFs]) within hPR which likely permit its interaction with the general transcription machinery. One of these AFs is located in the N-terminal region (AF-1) of PR, and the other is within the C-terminal ligand-binding domain (AF-2) of PR (27). By assessing the transactivational capacity of chimeric fusions containing the PR N terminus and the GAL4 DNA-binding domain, Meyer et al. were able to localize AF-1 to a 91-amino-acid region which was capable of functioning as an independent activator in the context of a GAL4 chimera (28). The exact boundaries of the C-terminal AF-2 region are less well defined. In the ER, the AF-2 function has been shown to occur within a region of the carboxyl terminus which is conserved among all nuclear hormone receptors (9). Mutations of three invariant charged residues in this region of mouse ER (9) and human ER (hER) (35) inactivated AF-2 activity of this receptor without altering its ligand binding specificity. It is likely, therefore, that the corresponding region within PR mediates AF-2 activity, although this hypothesis awaits further investigation.

We are interested in defining the biological role of the hPR-A and hPR-B isoforms in mediating the cellular responses of PR to hormones and antihormones. Our work and that of others has demonstrated that while the DNA and hormone binding properties of the hPR isoforms are similar, they display different transcriptional activities. Using reconstituted progesterone-responsive transcription systems in a series of heterologous cell lines, our laboratory has revealed distinct roles for hPR-B and hPR-A (36). Specifically, it was determined that hPR-B functions predominantly as an activator of progesterone-responsive genes, while hPR-A functions as a modulator or repressor of hPR-B activity. Furthermore,

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hPR-A, but not hPR-B, was capable of repressing glucocorticoid receptor (GR), androgen receptor, mineralocorticoid receptor, and ER-mediated gene transcriptional activation, suggesting that hPR-A is an important modulator of steroid hormone receptor action (25, 26, 36). Similarly, Tung et al. demonstrated that the partial agonist effects manifest by RU486-activated hPR-B can be inhibited by coexpression of hPR-A, supporting the premise that the functional activities of hPR-A and hPR-B are distinct (34).

The experiments described in this report address the questions of whether the structural sequences within hPR-A and hPR-B required for transcriptional activation and repression are the same and whether these receptor isoforms utilize similar or distinct signaling pathways in target cells.

MATERIALS AND METHODS

Materials. DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Indianapolis, Ind.), New England Biolabs (Beverly, Mass.), or Stratagene (San Diego, Calif.). PCR reagents were obtained from Perkin-Elmer Cetus (Norwalk, Conn.). Progesterone, dexamethasone, and 17-β-estradiol were purchased from Sigma (St. Louis, Mo.). [1,2-^{3}H]progesterone was purchased from Amersham (Arlington Heights, Ill.).

Plasmids. Construction of the mammalian expression plasmids phPR-B, phPR-A, and pRShGR has been described elsewhere (36), as has the construction of PRE_2-TK-LUC, TAT-LUC, and ERE-TK-LUC reporters (3, 35, 36). Plasmid MMTV-ERE-LUC was constructed as follows. Plasmid ΔMTV-LUC containing a deletion of the sequences from +190 to −88 was obtained from Ron Evans (Salk Institute, San Diego, Calif.). This plasmid was digested with *Hin*dIII to remove the glucocorticoid response elements, and five copies of a 33-bp oligonucleotide containing the consensus vitellogenin A2 estrogen response element were inserted. The sequence of the oligonucleotide used was 5′-AATTCAAAGTCAGGT CACAGTGACCTGATCAAA-3′.

Site-directed mutagenesis. The mutations E907A and E911A were introduced into phPR-B and phPR-A by PCR (16). Glutamic acid residues at positions 907 and 911 were substituted with alanines by using PCR primers containing two base changes (underlined) as indicated: 5′-CCAGCAATGAT GTCTGCAGTTATTGC-3′ and 5′-GCAATAACTGCAGA CATCATTGCTGG-3′ (National Biosciences Inc., Plymouth, Minn.). The *Eco*NI-*Kpn*I fragment of DNA containing the mutated receptor sequences was subcloned into plasmids phPR-B and phPR-A and sequenced to confirm the mutations.

Cell culture. The human mammary epithelial cell line MCF-10 was obtained originally from Samuel Brooks (Michigan Cancer Foundation). This cell line was routinely maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (Biofluids, Rockville, Md.) and Ham's F12 medium (Biofluids) with 20 ng of epidermal growth factor (Sigma) per ml, 100 ng of cholera toxin (Sigma) per ml, 0.01 μg of insulin (Biofluids) per ml, 500 ng of hydrocortisone (Sigma) per ml, and 5% horse serum (Biofluids). The human breast adenocarcinoma cell line MCF-7 was obtained from Marc E. Lippman (Vincent T. Lombardi Cancer Center, Georgetown University) and maintained in Iscove's modified Eagle's medium (Biofluids) with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah). Monkey kidney CV-1 fibroblasts were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Cotransfection assays. Cells were plated in 12- or 96-well tissue culture plates the day before transfection. DNA was introduced into cells by the calcium phosphate coprecipitation method (3). For each transfection reaction, 20 μg of DNA per ml of transfection buffer was used. For the 96-well plate experiments, transfections were performed with a Biomek 1000 automated laboratory workstation (Beckman, Fullerton, Calif.). Cells were incubated with the precipitate for 6 h, then washed with phosphate-buffered saline, and incubated for 40 h with or without hormones as indicated in the text. Cell extracts were prepared as previously described (3) and assayed for luciferase and β-galactosidase activities.

Hormone binding assays. The wild-type PR and mutant receptor proteins were produced by in vitro translation of mRNA synthesized by using wild-type and mutant PR templates. The binding assay buffer consisted of 10% glycerol, 10 mM Tris, 2 mM dithiothreitol, 2 mM 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, and 1.5 mM EDTA (pH 7.5). The binding assays were performed in a 500-μl volume containing 10 μl of reticulocyte lysate (containing PR) and various concentrations of [^{3}H]progesterone in the absence or presence of 10 μM progesterone. Incubations were carried out at 4°C for 16 h. At the end of the incubation period, bound and unbound progesterone were separated by using dextran-coated charcoal. The supernatants containing bound progesterone were drawn off, and the radioactivity retained was estimated by liquid scintillation counting. Data were analyzed by the method of Rosenthal (31). For competition binding assays, a similar protocol was used except that a fixed concentration of [^{3}H]progesterone was added to extracts in the presence or absence of competing ligands (1 nM to 10 μM). After correcting for nonspecific binding, 50% inhibitory concentration values were determined graphically from a log-logit plot of the data. K_is were determined from the calculated 50% inhibitory concentration values by using the Cheng-Prusoff equation (7).

RESULTS

hPR-B, but not hPR-A, activates transcription in a hormone-dependent manner in human mammary cells. It has been shown previously that hPR-A and hPR-B are cell- and promoter-specific regulators of target gene transcription (27, 34, 36). In addition, we have demonstrated that in cellular contexts in which hPR-A was unable to activate transcription, it functioned as a potent transdominant inhibitor of hPR-B (36). To further understand the mechanism of hPR-A- and hPR-B-mediated effects on transcription, we examined the function of these proteins in MCF-10 cells. MCF-10 is an ER/PR-negative, nontransformed mammary cell line, allowing specific examination of the transcriptional activity of transfected receptors. Receptor expression plasmids encoding either hPR-A (phPR-A) or hPR-B (phPR-B) together with a luciferase reporter driven by the progesterone-responsive mouse mammary tumor virus (MMTV) promoter were cotransfected into MCF-10 cells. Receptor-mediated transcriptional activation of the transfected MMTV promoter was measured in the absence or presence of increasing concentrations of progesterone. The results shown in Fig. 1A indicate that a 400-fold induction of MMTV promoter activity occurs in the presence of cotransfected phPR-B and progesterone relative to the basal activity observed in the absence of hormone. In contrast, no progesterone-mediated activation of the MMTV promoter by hPR-A was evident under identical conditions.

The transcriptional activity of the nuclear hormone receptors has been shown to be influenced by cell and promoter

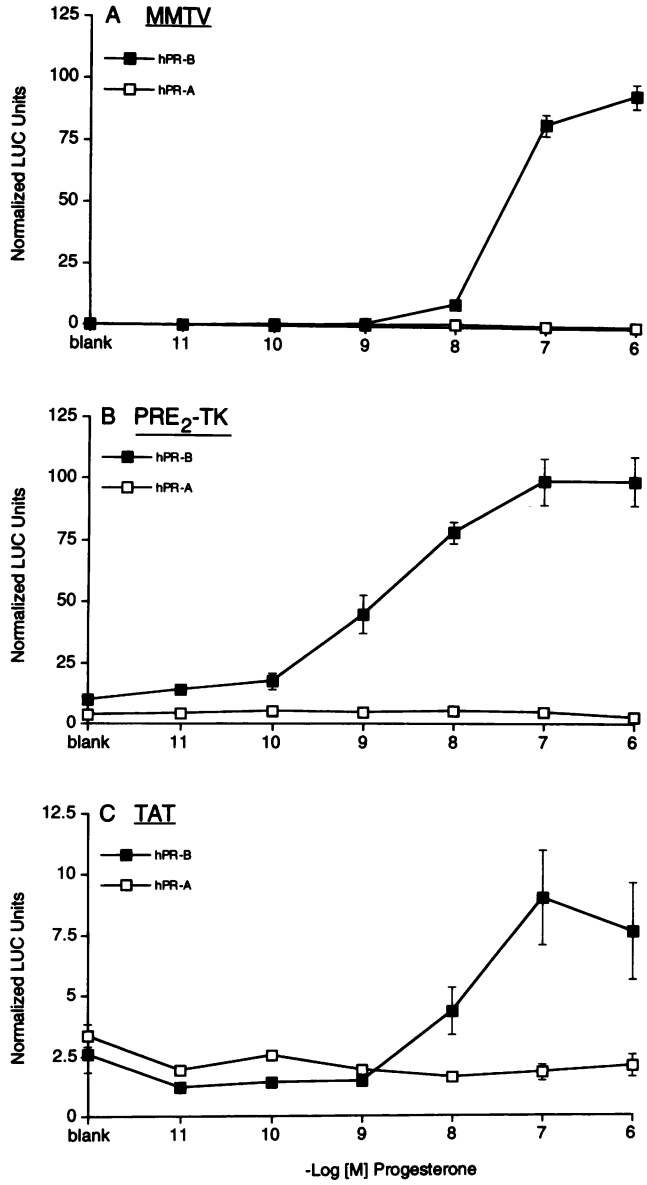

FIG. 1. hPR-B but not hPR-A activates target gene transcription in cultured MCF-10 cells. The receptor expression plasmid phPR-A or phPR-B (5 μg) was transfected into cultured MCF-10 cells together with 10 μg of either MMTV-LUC (A), PRE_2-TK-LUC (B), or TAT-LUC (C) reporter plasmid per ml. In addition, the transfection mixes contained 5 μg of plasmid pCH110 (a simian virus 40–β-galactosidase expression vector) per ml as an internal control. The amount of expression vector chosen in these studies was that which permitted maximal transcriptional responsiveness. The cells were incubated for 40 h in the absence or presence of increasing concentrations of progesterone, as indicated. Following incubation, the cells were harvested and assayed for luciferase and β-galactosidase activities. The data are presented as normalized luciferase (LUC) units. Normalization was calculated by dividing the raw luciferase activity (relative light units × 10^4) for each point by the β-galactosidase activity (A_{415} × 10^5)/time in minutes) at that point. Data shown represent the mean values ± standard errors of the means of 12 replicates.

context (4, 33, 35). Consequently, we examined whether hPR-A could function as a transcriptional activator of other progesterone-responsive promoters when analyzed in MCF-10 cells (Fig. 1B and C). This was accomplished by transfecting either the hPR-A or hPR-B expression plasmid together with the PRE_2-TK reporter, in which two copies of a consensus progesterone response element were cloned upstream of the thymidine kinase promoter (Fig. 1B), or a reporter containing the progesterone-responsive tyrosine aminotransferase promoter (Fig. 1C). These analysis revealed that in MCF-10 cells, hPR-B, but not hPR-A, was capable of activating the PRE_2-TK or tyrosine aminotransferase promoter in a progesterone-dependent manner. From these results and those published previously (34, 36), we conclude that hPR-B is the major transcriptional activator of progesterone-responsive genes and that in most instances, hPR-A functions as a transcriptional inhibitor of this activity.

Specific mutations in the PR AF-2 region affect PR-B transcriptional activity in a cell-dependent manner. We were interested in defining the molecular basis for the differential transcriptional activities manifest by hPR-A and hPR-B. In particular, we wished to determine if the receptor sequences required for transcriptional activation (AFs) were required also for transcriptional repression. Interestingly, PR contains two regions required for maximal transcriptional activity. These AFs, AF-1 in the amino terminus and AF-2 in the carboxyl terminus (27), are thought to permit interaction of PR with the general transcription machinery. It has been shown that both AFs are contained in hPR-A and hPR-B; however, a region specific for hPR-B is required for AF-1 function (28). We reasoned, therefore, that differences in PR AF-1 activity are likely responsible for the observed differences in hPR-A and hPR-B transcriptional activity. Although the precise location of AF-2 within PR has not been defined, Danielian et al. have identified a region contained within all the sex steroid receptors necessary for AF-2 activity (10). Accordingly, we disrupted PR AF-2 function by replacing the glutamate residues at positions 907 and 911 with alanine and examined the effects of these mutations on the ligand binding characteristics and transcriptional activities of both hPR-A and hPR-B.

Analysis of the in vitro binding characteristics of the wild-type and mutant PRs indicated that the two point mutations (E907A and E911A) within the AF-2 region had little effect on progesterone binding. Specifically, the dissociation constants of the mutant receptor $hPR\text{-}A_{(E907A,\ E911A)}$ and $hPR\text{-}B_{(E907A,\ E911A)}$ were 10 and 1.6 nM, respectively, compared with 1.3 and 1.8 nM observed when the wild-type hPR-A and hPR-B were assayed. Similarly, the binding characteristics of RU486 were unaffected by these mutations. These results indicate that these mutants created minimal changes in overall receptor conformation and permit the analysis of AF-1 activity in the context of a minimally perturbed receptor.

We then examined the transcriptional activity of the PR AF-2 mutants in transfected mammalian cells. As expected, wild-type hPR-B activated the MMTV promoter in a hormone-dependent manner in both MCF-10 and CV-1 cells (Fig. 2). Similarly, in MCF-10 cells, $hPR\text{-}B_{(E907A,\ E911A)}$ functioned as a hormone-dependent activator of MMTV gene transcription (Fig. 2A). In contrast, this mutant receptor was unable to activate MMTV gene transcription in CV-1 cells (Fig. 2B). These data indicate that the relative activity contributed by each of the PR AFs is cell dependent. Specifically, we conclude that in MCF-10 cells, the AF-1 domain of hPR-B can function in the absence of AF-2, whereas in CV-1 cells, AF-2 is required for transcriptional activity of hPR-B. These results confirm

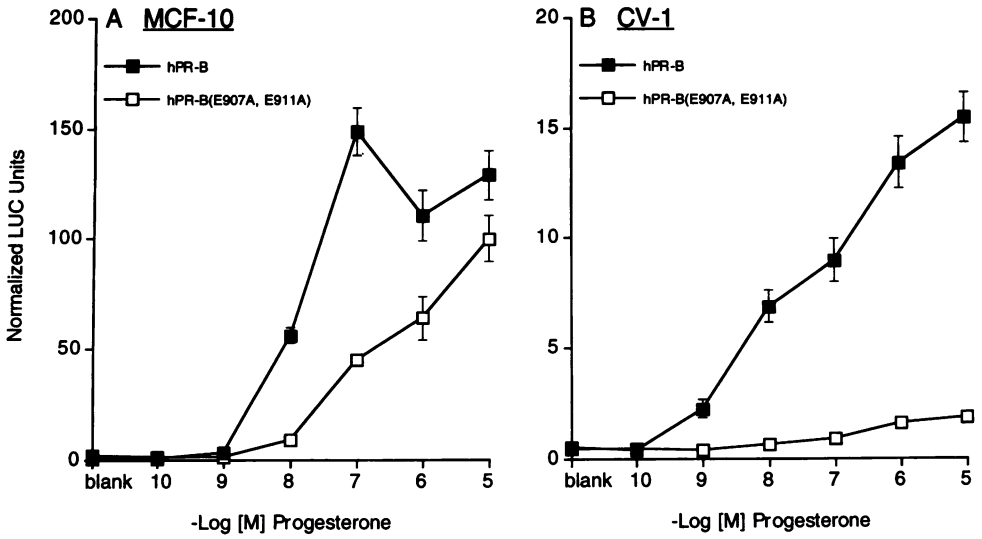

FIG. 2. The AF-2 function of PR-B is required in some cell and promoter contexts for maximal transcriptional activity. The hPR expression plasmid phPR-B or phPR-$B_{(E907A, E911A)}$ was transfected into MCF-10 (A) or CV-1 (B) cells together with an MMTV-LUC reporter plasmid (10 μg/ml) and pCH110 (5 μg/ml) as an internal control. The amount of expression vector chosen in these studies was that which permitted maximal transcriptional activation in each cell line examined. The transfected cells were incubated for 40 h with increasing concentrations of progesterone as indicated and assayed for luciferase and β-galactosidase activities. The data are presented as normalized luciferase (LUC) units and were calculated as for Fig. 1. The data shown represent the mean values ± the standard errors of the means of 12 replicates.

that both AF-1 and AF-2 sequences are critical elements required within hPR-B for maximal stimulation of transcription in response to progesterone.

Transcriptional activation and repression functions are mediated by structurally distinct regions of hPR-B and hPR-A. We have shown previously that hPR-A can inhibit hPR-B- and hGR-mediated transcriptional activation of the MMTV promoter (36). Therefore, we examined the ability of hPR-A and hPR-$A_{(E907A, E911A)}$ to inhibit dexamethasone-induced GR-mediated MMTV gene transcription. This particular assay was chosen since the transcriptional efficiency of GR is about 10 times greater than that achieved by hPR-B, creating a larger window with which to characterize the inhibitory activity of hPR-A and the hPR-A mutants. MCF-10 (Fig. 3A) and CV-1 (Fig. 3B) cells were transfected with the MMTV-LUC reporter and vectors expressing either GR alone or GR in combination with wild-type hPR-A or mutant hPR-$A_{(E907A, E911A)}$ protein. The transcriptional activity of GR was measured in the presence of 5×10^{-8} M dexamethasone and increasing concentrations of progesterone (10^{-11} to 10^{-6} M). Progesterone had no significant effect on dexamethasone-activated GR-mediated transcription activation of the MMTV-LUC reporter in either MCF-10 cells (Fig. 3A) or CV-1 cells (Fig. 3B) when GR was transfected alone. However, when GR and either hPR-A (Fig. 3A) or hPR-$A_{(E907A, E911A)}$ (Fig. 3B) were transfected together, the transcriptional activity of GR was inhibited by progesterone in a dose-dependent manner in both cell lines examined. A 50% reduction in GR activity was observed in the presence of 5×10^{-8} M added progesterone. Thus, hPR-$A_{(E907A, E911A)}$ is functionally similar to wild-type hPR-A as a repressor of GR-mediated transcriptional activity in both MCF-10 and CV-1 cells. Therefore, since transcriptional activation, but not repression by hPR-A, requires an intact AF-2 function in certain contexts, we conclude that these processes are likely to operate through distinct cellular regulatory pathways.

hPR-A represses endogenous hER transcriptional activity in MCF-7 cells. We have used reconstituted transcription systems in heterologous mammalian cell lines to study the transcriptional activity of hPR-A. Our recent observation that hPR-A modulates hER transcriptional activity (25) prompted us to examine hPR-A activity in cell lines which express endogenous hER (15). It has been shown recently, by two independent groups, that the biological activity of ER in cell lines containing endogenous ER was different from the activity in those in which ER had been introduced by transfection (20, 37). Thus, to understand the pharmacology of estrogen and progesterone in normal and malignant breast cells, it was important to analyze hPR-A function in a bona fide ER target cell. For these studies, we chose the human breast adenocarcinoma cell line MCF-7, which expresses both ER and PR (15). Although, the ER expression level in MCF-7 cells is sufficiently high to facilitate estradiol-mediated transcriptional activation of target genes, the endogenous level of hPR did not permit activation of PR target genes (data not shown) or permit transcriptional repression of hER-dependent transcriptional activity (Fig. 4). Therefore, a vector encoding hPR-A or hPR-$A_{(E907A, E911A)}$ was transfected into MCF-7 cells together with an ERE-TK-LUC reporter. The ability of the expressed receptor proteins to modulate the transcriptional activity of endogenous hER was assessed. The results of this analysis are shown in Fig. 4. Addition of the PR antagonist RU486 (or progesterone; data not shown) had no significant effect on 17-β-estradiol-activated, hER-mediated transcriptional activity. However, when either hPR-A or hPR-$A_{(E907A, E911A)}$ was transfected into these cells, hER transcriptional activity was inhibited by RU486 in a dose-dependent manner. This inhibition was not observed in the presence of coexpressed hPR-B (data not shown). Importantly, we determined by radioligand binding assay that RU486 has no effect on hER expression levels in either the absence or presence of transfected hPR-A. Thus, hPR-A can repress the transcriptional activity of endogenously expressed hER in a bona fide target cell, supporting the hypothesis that the in vitro activity exhibited by hPR-A is important in the pharmacological actions of progesterone and estrogen.

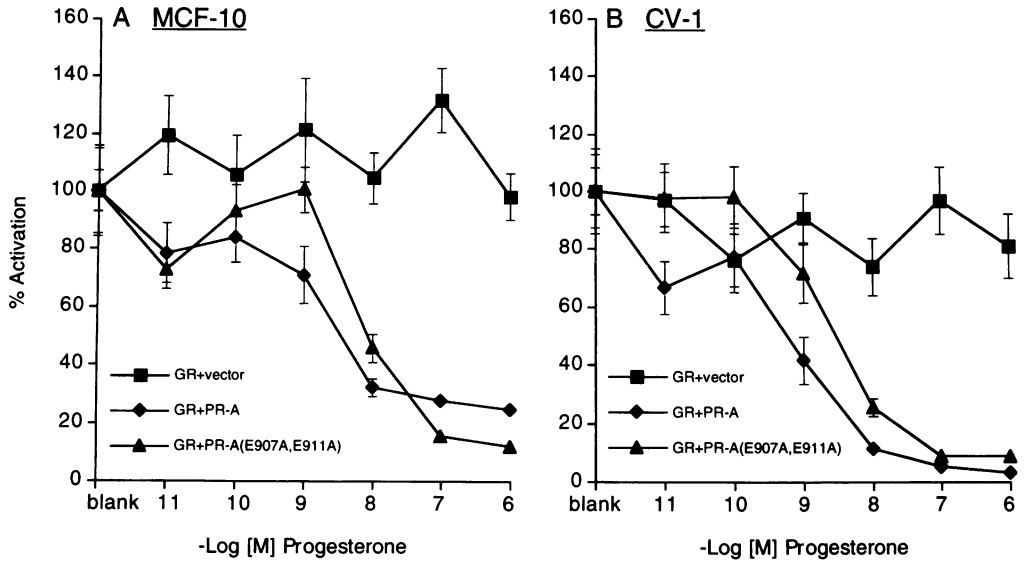

FIG. 3. Transcriptional repression of steroid receptor transcriptional activity by hPR-A does not require an intact AF-2 function. The abilities of hPR-A and hPR-$A_{(E907A,\ E911A)}$ to modulate GR transcriptional activity were assayed in MCF-10 (A) or CV-1 (B) cells. These cells were transfected with the GR expression plasmid pRShGR (3) (0.25 μg/ml) alone or together with an expression vector for either hPR-A or hPR-$A_{(E907A,\ E911A)}$ (0.25 μg/ml). Included in all transfections were the MMTV-LUC reporter plasmid (5 μg/ml) and pCH110 (5 μg/ml) as an internal control. The amount of expression vector chosen in these studies was that which permitted maximal transcriptional activation or repression as indicated. The cells were incubated for 40 h with 50 nM dexamethasone alone or in the presence of increasing concentrations of progesterone as indicated. Following incubation, the cells were harvested and luciferase and β-galactosidase activities were measured. The data are presented as percent activation, the 100% value set as the GR activation in the presence of 50 nM dexamethasone alone. The data shown represent the mean values ± standard errors of the means of 12 replicates.

hPR-A and other sex steroid hormone receptors may interact with distinct targets on sex steroid-regulated promoters. The ER is distinct among steroid hormone receptors in that its DNA-binding-site recognition sequence is distinct from that utilized by GR, PR, the mineralocorticoid receptor, and the androgen receptor. Thus, by focusing on the mechanism by which hPR-A inhibits hER transcriptional activity, we can examine hPR-A activity in the absence of DNA binding. The data described above indicate that repression by hPR-A and activation by sex steroid receptors most likely occur by distinct signaling pathways within the cell. We were therefore interested in determining whether both of these pathways (activation and repression) converge on similar or distinct cellular targets. To specifically address this question, we reconstituted an estrogen-responsive transcription unit in CV-1 cells and examined hER transcriptional activity in the absence and presence of various concentrations of expressed hPR-A. The PR ligands examined, progesterone (Fig. 5A), norethindrone (Fig. 5B), and RU486 (Fig. 5C), had no effect on estradiol-activated hER transcriptional activity in the absence of expressed hPR-A. In the presence of hPR-A, however, we noticed that progesterone, RU486, and norethindrone functioned as noncompetitive hER antagonists. Using a similar strategy, we showed that expression of hPR-B had no effect on hER transcriptional activity (reference 27 and unpublished data). Importantly, the degree of antagonism was related to the expression level of hPR-A. In this assay, we noticed that RU486 displayed some agonist activity; this was not observed when the antiprogestins ZK98299 and ZK112993 were examined. This phenomenon may be related to the observation that some 19-nor testosterone-derived PR ligands (as is RU486) displayed estrogenic activities in vitro (6). Clearly this activity of RU486 needs to be examined more closely. Using this information, we proceeded to determine whether the ex-

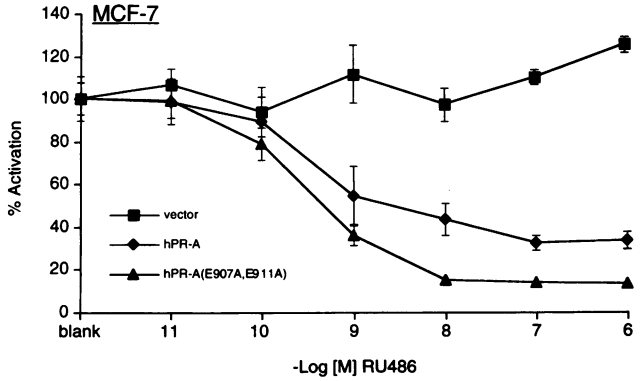

FIG. 4. hPR-A inhibits the transcriptional activity of endogenous hER in MCF-7 cells. The ability of the endogenous hER expressed in human MCF-7 cells to regulate transcription of a transfected ERE-TK-LUC target gene was assayed in the absence or presence of coexpressed hPR-A. For this analysis, MCF-7 cells were transfected with an expression vector encoding hPR-A or hPR-$A_{(E907A,\ E911A)}$ (5 μg/ml) together with an ERE-TK-LUC reporter plasmid (10 μg/ml) and pCH110 (5 μg/ml) as an internal control. The amount of hPR-A and hPR-$A_{(E907A,\ E911A)}$ expression vectors chosen in these studies was that which permitted maximal repression of hER transcriptional activity in MCF-7 cells and all other cell lines examined. The cells were incubated for 40 h in the presence of 100 nM 17-β-estradiol and in the absence or presence of increasing concentrations of the antiprogestin RU486 as indicated. Following incubation, the cells were harvested and assayed for luciferase and β-galactosidase activities. The data are presented as percent activation; the 100% value represents maximally activated hER in the presence of 100 nM 17-β-estradiol alone. The data shown represent the mean values ± standard errors of the means of 12 replicates.

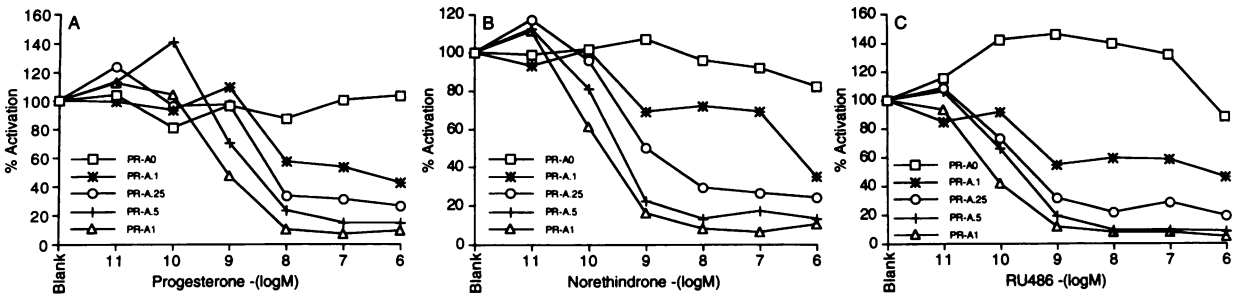

FIG. 5. Inhibition of hER transcriptional activity by PR ligands is influenced by hPR-A expression level. The effects of increasing cellular concentrations of hPR-A on hER-mediated transcriptional activity were measured in CV-1 cells. An expression vector encoding hER (pRST7hER) (5 μg/ml) was transfected into CV-1 cells alone or in the presence of different concentrations of an hPR-A expression plasmid as indicated. All transfection mixes contained an ERE-TK-LUC reporter (10 μg/ml) (25) and pCH110 (5 μg/ml) as an internal control. The transcriptional activity under these conditions was measured following the addition of 10^{-7} M 17-β-estradiol alone or estradiol in the presence of increasing concentrations of progesterone (A), norethindrone (B), or RU486 (C), as indicated. Following incubation, cells were harvested and luciferase and β-galactosidase activities were measured. The data are presented as percent activation, 100% representing the activity of hER in each condition in the absence of any added PR ligand. Each datum point 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.

pressed hPR and hER were competing for the same cellular target.

We considered that if inhibition of hER function by hPR-A was due to competition for a single target protein, then repression should be overcome by increasing the expression level of hER. Conversely, if inhibition were independent of hER expression level, this finding would suggest that inhibition was noncompetitive, possibly representing interactions with distinct target proteins. To address this issue, we assayed the transcriptional activity of different concentrations of expressed hER in the presence of a constant amount of expressed hPR-A (a concentration which we had determined to be subsaturating for hPR-A-mediated inhibition) (Fig. 6). Interestingly, no differences in the ability of hPR-A to inhibit transcription were observed over a 10-fold range of expressed hER. These data suggested that the action of hPR-A was not competitive but rather was noncompetitive. In support of this hypothesis, we observed similar results when the experiment was repeated with a concentration of hPR-A which we had determined to give maximal hPR-A mediated inhibition (data not shown). We conclude from these results that the ability of hPR-A to function as a transcriptional repressor is independent of the concentration of the activator (hER), suggesting that hPR-A and hER interact with distinct cellular targets or contact distinct sites on a common target.

DISCUSSION

hPR exists in two distinct forms within the cell (hPR-A and hPR-B). These proteins are expressed in approximately equimolar amounts in human breast cancer (18, 24) and in human endometrial carcinoma cell lines (12); however, a

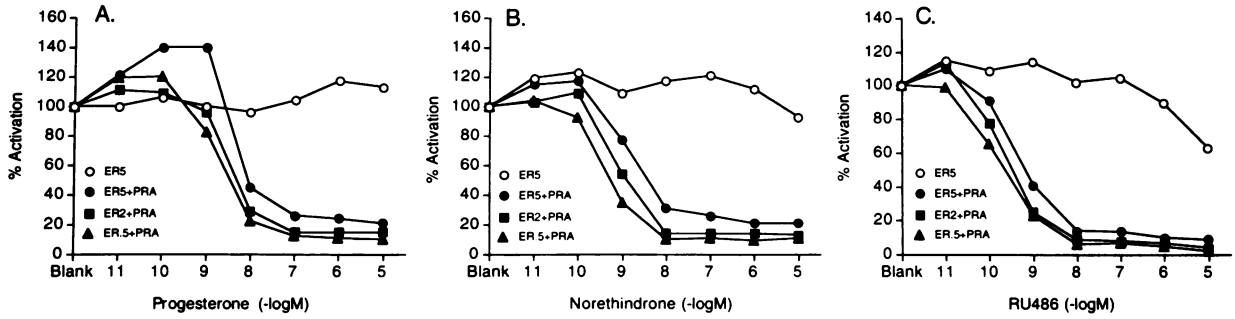

FIG. 6. Inhibition of hER transcriptional activity by subsaturating concentrations of hPR-A is independent of hER expression level. Monkey kidney CV-1 cells were transiently transfected with increasing concentrations of an hER expression plasmid (as indicated) alone or in the presence of a vector expressing hPR-A. The concentration of hPR-A expression vector (0.5 μg/ml) was shown previously to be submaximal for hPR-A-mediated repression of hER activity. Each transfection condition included an MMTV-ERE-LUC reporter plasmid (10 μg/ml) and plasmid pCH110 (5 μg/ml) as an internal control. The transcriptional activity in these setups was measured following the addition of 10^{-7} M 17-β-estradiol alone or estradiol in the presence of increasing concentrations of progesterone (A), norethindrone (B), or RU486 (C), as indicated. Following incubation, the cells were harvested and luciferase and β-galactosidase activities were measured. The data are presented as percent activation; the 100% value represents the activity of hER in each condition in the absence of any added PR ligand. To confirm that the range of ER expression vector chosen allowed an examination of hPR-A activity in the presence of subsaturating and saturating levels of ER, we calculated the fold induction by estradiol in each transfection. The fold inductions in the experiments detailed in panels A to C were as follows: 5 μg of ER expression vector, 31, 26, and 35, respectively; 2 μg of hER expression vector, 28, 24, and 33, respectively; 0.5 μg of hER expression vector, 18, 16, and 24, respectively. Each datum 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. This data presented are representative of several individual experiments.

systematic examination of the expression patterns of these individual isoforms in progesterone target tissues has not yet been accomplished. The recent demonstration that the hPR-A and hPR-B isoforms arise from transcripts initiated from two distinct promoters within the PR gene suggests that independent regulation of these promoters may occur (28). Indeed, in support of this hypothesis, it has been shown that the relative expression of hPR-A and hPR-B receptor proteins in human endometrium changes during the human menstrual cycle, suggesting that the two different PR promoters can be regulated independently in this organ (13). In addition, it has been shown recently that the relative levels of expression of hPR-A and hPR-B are different in biopsies of uterine leiomyomas compared with their expression in adjacent normal myometrium (5). It is not known, however, if the observed alteration in PR expression levels is a cause or consequence of the disease. Nevertheless, this observation lends additional support to the idea that alterations in the expression of hPR-A and hPR-B can exist within PR target cells.

The precise functions of hPR-A and hPR-B have not yet been defined. Previously, it has been demonstrated that the A and B forms of chicken PR exhibit distinct transcriptional activities which are manifest in a cell- and promoter-specific manner (33). Similarly, recent work in our laboratory and that of others has indicated that the transcriptional activities of hPR-A and hPR-B are different and are dependent upon cell and promoter context (28, 36). In most cell and promoter contexts that we have examined, hPR-B functions as a positive regulator of progesterone-responsive genes, whereas hPR-A appears to function as a transdominant inhibitor of hPR-B activity (36). In the current study, we have extended our analysis of hPR-A and hPR-B function to the nontransformed MCF-10 cell line and observe that hPR-B is the dominant transcriptional activator of progesterone-responsive genes and that hPR-A functions as an inhibitor of this activity. Thus, we conclude that in most cells, hPR-B is the dominant activator of progesterone-responsive target genes, whereas hPR-A functions to inhibit this activity.

In addition to its role as a modulator of hPR-B transcriptional activity, hPR-A functions as an inhibitor of the transcriptional activity of other known steroid hormone receptors (25, 26, 34, 36). This activity suggests a central role for hPR-A in regulating steroid hormone action in those cells in which it is expressed. Of particular interest to our group is the ability of hPR-A to inhibit the transcriptional activity of estradiol-activated hER (25). Both progesterone and estrogen are involved in the maintenance and development of female reproductive function and additionally are involved in the progression of hormone-dependent breast tumors (8). The coexpression of hER, hPR-B, and hPR-A in these tissues suggests that the actions of progesterone and estrogen are integrally linked in these target cells through the modulatory activity of hPR-A. Thus, hPR-A may facilitate cross-talk between the progesterone- and estrogen-regulated signaling pathways in these hormone-responsive tissues. Our studies of hER function in MCF-7 breast cancer cells have shown that hPR-A can inhibit the transcriptional activity of endogenous hER expressed in these cells. In addition, the presence of expressed hPR-A allows the PR antagonist RU486 to function as a potent noncompetitive antiestrogen. In view of these data, it is now unclear to what extent the clinical efficacy of RU486 in the treatment of endometriosis, uterine fibroids, brain meningiomas, and hormone-dependent breast cancers results from its ability to function as an antiprogestin or an antiestrogen (1, 11, 22, 30). Resolution of this issue will have important consequences for the use of RU486 and other antiprogestins in these chronic diseases clinically.

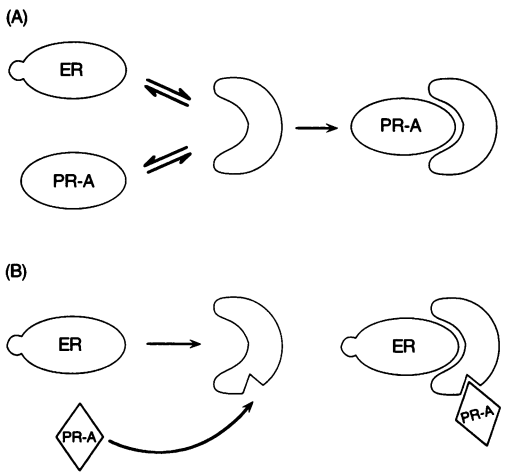

FIG. 7. hPR-A functions as a transdominant inhibitor of hER function. A working model explaining the possible mechanisms by which hPR-A can function as a transdominant repressor of hER function is presented. We propose that hPR-A acts by competing with hER for a common transcription factor (A) or alternatively that hPR-A and hER interact with different cellular targets, but interaction of hPR-A with its targets indirectly blocks hER function (B).

The mechanism of hPR-A-mediated inhibition of steroid hormone action is unknown. Using in vitro DNA and ligand binding assays (data not shown), we have determined that hPR-A (i) does not heterodimerize with hER, (ii) has no effect on the ability of hER to interact with DNA, (iii) has no direct effect on hER biochemistry, and (iv) has no effect on hER cellular expression in transfected mammalian cells. We postulate, therefore, that inhibition may result as a consequence of transcriptional interference by hPR-A of a distal step in the hER signal transduction pathway. Inhibition of the transcriptional activity of one transcription factor by another is a frequently occurring paradigm in modern cell biology (14). In certain contexts, overexpression of a transcriptional activator can squelch the transcriptional activity of another protein by sequestering a required transcription factor. In these cases, the sequences within the protein responsible for activation and repression are the same, and its ability to function as a transcriptional repressor relies heavily on the strength of its activation sequence (2). In previous studies, we have shown that hPR-A functions as a transcriptional repressor of hPR-B function at stoichiometric levels of expression (36). This result would argue against squelching as the mechanism for the inhibitory actions of hPR-A. However, to address this issue directly, we created mutants in the PR-B receptor which prevented hPR-B from activating transcription in CV-1 cells. When these same mutations were introduced into the hPR-A isoform and assayed in CV-1 cells, it was observed that they had little effect on the inhibitory activity of hPR-A. This finding indicated that the receptor sequences required for activation and repression of transcription are distinct; thus, the processes of transcriptional activation and repression are likely also distinct and mediated by separate pathways in the cell.

Accessing currently available information, we have developed two distinct working models to explain how hPR-A can act as a transcriptional repressor of hER (Fig. 7). Although alternative mechanisms are possible, this approach has facilitated our experimental design. The first model proposes that

hER and hPR-A compete for a limiting factor required by hER for maximal transcriptional activity (Fig. 7A). The requirement by hER for this limiting factor would be determined by the cell and promoter context of the hormone-responsive gene, such that hPR-A would not inhibit all hormone-regulated target genes. In the second model, we propose that the targets for hPR-A and hER are distinct (Fig. 7B), existing as either different proteins or different sites on the same protein. In this model, inhibition would be noncompetitive, such that the ratio of hER to hPR-A is less important than the ratio of hPR-A to target. These models were tested experimentally in reconstituted hormone-responsive transcription units in cultured mammalian cells. We observed that increasing the cellular expression of hPR-A leads to an increased repression of hER transcriptional activity. Importantly, however, the ratio of expressed hPR-A to hER has little bearing on the ability of hPR-A to function as a transcriptional repressor. This observation suggests that hPR-A's ability to function as an hER transcriptional repressor does not result from a competitive interaction of hPR-A and hER for a common transcription factor but rather that inhibition results from an interaction of hPR-A with a distinct target within the cell, supporting the model depicted in Fig. 7B.

Conceptually, the target proteins for hPR-A and the steroid receptors could be steroid receptor-specific transcription factors or adapter proteins. Alternatively, they may be members of the general transcription machinery. Interestingly, intracellular hormone receptors have been shown in vitro to interact with the basal transcription factor TFIIB (19), although the functional significance of this interaction is unknown. In addition, several laboratories have shown that enhancer-binding proteins may communicate with the general transcription machinery through interactions with TFIID. Interestingly, it has been shown that TFIID is a multiprotein complex comprising the TATA box-binding protein and TATA box-binding protein-associated factors (TAFs) (32). To date, eight TAFs (TAF250, -150, -110, -80, -60, -40, -30α, and 30β) have been cloned and characterized (32). When assayed in vitro, TAF110 was found to permit SP1 interaction with the general transcription machinery, while TAF40 contacted VP16 (17, 32). In view of the fact that steroid receptors contact the basal transcription apparatus in vitro, we consider that the transcriptional enhancement activities of the steroid hormone receptors and the inhibitory activity of hPR-A could possibly be mediated through interactions with different TAF proteins in the TFIID complex.

The ability of hPR-A to regulate the activities of all of the steroid hormone receptors further substantiates the concept that this family of proteins share a common mechanism of action. Identification of the biochemical targets of hPR-A and hPR-B within the cell and elucidation of their precise mechanism of action will surely facilitate the discovery and development of novel drugs which modulate steroid receptor action and will allow further definition of the specific biological processes which are regulated by hPR-A.

ACKNOWLEDGMENTS

We thank Wes Pike, Marco Gottardis, and Robert Stein for insightful discussions and Shau-Ming Mong for technical assistance. We also thank the Ligand New Leads group for help in performing the cell transfection assays.

This work was supported in part by NIH grant DK43267 to D.P.M.

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