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Modulation of AP-1 activity by the human progesterone receptor in endometrial adenocarcinoma cells

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ABSTRACT **The composite transcription factor activating protein 1 (AP-1) integrates various mitogenic signals in a large number of cell types, and is therefore a major regulator of cell proliferation. In the normal human endometrium, proliferation and differentiation alternate in a cyclic fashion, with progesterone being largely implicated in the latter process. However, the effects of progesterone and the progesterone receptor (hPR) on AP-1 activity in the human endometrium are not known. To address this issue, HEC-1-B endometrial adenocarcinoma cells, which are devoid of hPR, were transfected with luciferase reporter constructs driven by two different AP-1-dependent promoters. Unexpectedly, cotransfection of hPR caused a marked induction of luciferase activity in the absence of ligand on both promoters. The magnitude of this induction was similar to that observed in response to the phorbol ester TPA. Addition of ligand reversed the stimulating effect of the unliganded hPR on AP-1 activity in these cells. These effects were specific for hPR, and were not observed with either human estrogen receptor or human glucocorticoid receptor. Furthermore, they strictly depended on the presence of AP-1-responsive sequences within target promoters. Finally, the described effects of hPR on AP-1 activity were shown to be cell-type specific, because they could not be demonstrated in SKUT-1-B, JEG-3, and COS-7 cells. To our knowledge this is the first report of an unliganded steroid receptor stimulating AP-1 activity. This effect and its reversal in the presence of ligand suggest a novel mechanism, through which hPR can act as a key regulator of both proliferation and differentiation in the human endometrium.**

Cellular proliferation and differentiation processes are regulated by extracellular factors, which, by binding to specific receptors, initiate intracellular signal cascades, and, thus, orchestrate the activity of transcription factors within the cell nucleus (1). Imbalances between proliferation- and differentiation-promoting signals have pathophysiological consequences, such as hyperplasia or even tumor formation (2). The composite transcription factor activating protein 1 (AP-1) is the prototype of a mitogen-activated transactivator, and its transcriptional activity is believed to reflect cell proliferation in many tissues (3–6). In contrast, nuclear receptors, such as the progesterone receptor (PR), represent classic examples of transcription factors that mainly govern cell differentiation (2, 6). Interactions between these two types of transcription factors have only begun to be unraveled at the molecular level.

The human PR (hPR) belongs to the large family of nuclear hormone receptors, which also includes the receptors for other steroid hormones, thyroid hormone, vitamin D, retinoic acid, and for as yet unidentified ligands ("orphan" receptors) (7–10). These receptors are ligand-activated transcription factors that share a characteristic three-domain structure: the highly conserved DNA-binding domain (DBD) consisting of two zinc fingers, the N-terminal transactivation domain (AF-1), and the C-terminal ligand-binding domain. This domain also contains an additional transactivation function (TAF-2) as well as signals for nuclear localization and heat shock protein (HSP) binding (11–13). Although hPR is encoded by a single gene, it occurs in two distinct isoforms, hPR-A and hPR-B, which are generated by alternative promoter usage (14). hPR-B is transcriptionally active on most progesterone-responsive promoters, whereas hPR-A seems to act in a more context-restricted fashion and can even antagonize the effects of hPR-B (15).

In the unliganded state, hPR is anchored to a complex of HSPs, which prevents the receptor from binding to the DNA (16). Ligand binding induces a conformational change in the receptor molecule that initiates a series of events including dissociation from the HSP complex, receptor phosphorylation, dimerization, and binding to specific DNA sequences termed PR response elements (13, 17). This activation cascade was originally considered to be strictly steroid-dependent. However, several laboratories have recently reported an alternative mechanism, by which PR can be activated in the absence of ligand. Dopamine, for instance, has been shown to activate PR ligand independently, both *in vitro* and *in vivo* (18, 19). Similar results were reported for the vitamin D and the retinoic acid receptors (20). These effects probably involved phosphorylation of specific amino acid residues in the receptor molecule, since they were partially mimicked by the phosphatase inhibitor okadaic acid (21). However, activation of PR required the presence of either ligand or extracellular stimuli in all these experiments. Transcriptional activity of PR in the complete absence of any stimulus has not yet been reported.

AP-1 is a homo- or heterodimeric DNA-binding protein composed of either two Jun family proteins or one Jun and one Fos family protein (5, 22). The activity of this transcription factor complex is modulated by growth factors, cytokines, and tumor promoters that activate protein kinase C, such as the phorbol ester 12-*O* tetradecanoylphorbol 13-acetate (TPA) (5, 22). The activated AP-1 dimer binds to specific DNA sequences in the regulatory regions of mitogen-responsive genes, so-called TPA response elements (TREs) (23, 24). Reporter gene constructs driven by a minimal promoter and several TREs provide a simple tool to integrate the various signal transduction pathways and protein subunits contributing to AP-1 activation. The activity of such a reporter gene is, therefore, commonly referred to as AP-1 activity.

Because cell proliferation and differentiation are not regulated independently, it is conceivable that factors modulating these processes interact with each other. In support of this notion, several groups have recently reported extensive cross-talk between AP-1 proteins and nuclear receptors. In most cases, these interactions led to mutual inhibition of transcrip-

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Abbreviations: AP-1, activating protein 1; PR, progesterone receptor; hPR, human PR; hER, human ER; TPA, tetradecanoylphorbol 13-acetate; HSP, heat shock protein; TRE, TPA response element; IL-2, interleukin-2; tk, thymidine kinase; MPA, medroxyprogesterone, acetate.

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tional activity (6). In the context of TRE-dependent promoters, for instance, the ligand-activated glucocorticoid receptor can suppress AP-1 activity (25–28). This effect does not require DNA binding by the receptor, and crosslinking studies indicate that it is due to direct protein/protein interactions between the receptor protein and either c-Jun or c-Fos (28). Similar results have been reported for other nuclear receptors, e.g., the retinoic acid (29, 30) and the thyroid receptors (31).

The human endometrium represents a unique model to study possible interactions between hPR and AP-1, as it proliferates and differentiates in a cyclic fashion under the influence of estradiol and progesterone, respectively (2, 32). To gain insight into the molecular mechanisms controlling these processes, we decided to study the effect of hPR on AP-1 transcriptional activity in human endometrial adenocarcinoma cells. In the course of these studies we found that hPR, when transfected into hPR-devoid HEC-1-B cells, stimulated AP-1 activity in the absence of ligand or any other extracellular stimulus. Addition of ligand reversed this effect. Because estrogens are known to induce hPR expression, our findings suggest a novel mechanism, through which the unliganded hPR could promote cell proliferation in the first half of the menstrual cycle when progesterone levels are low. They also explain the anti-proliferative, differentiation-promoting effect of the hormone-activated hPR in the second, progesterone-dominated phase of the menstrual cycle. Thus, our data define a possible role for hPR as a major regulator of human endometrial proliferation and differentiation.

MATERIALS AND METHODS

Plasmids. AP-1-tk81-luc was constructed as follows: Oligonucleotides 5′-CGTGACTCAGCGCGGTGACTCAGCGCGG3′ and 5′-GATCCCGCGCTGAGTCACCG CGCTGAGTCACGAGCT-3′, containing two TRE consensus sites (underlined), were annealed to generate a double-stranded TRE_2 oligomer. Two TRE_2 oligomers were ligated by means of their compatible *Bam*HI overhangs and inserted into the *Sst*I site of the plasmid tk81-luc via their *SstI* overhangs. The correct insertion of the double-stranded TRE_4 oligonucleotide was confirmed by dideoxynucleotide DNA sequencing. tk81-luc contains the firefly luciferase gene under the control of a truncated herpes simplex virus thymidine kinase (tk) promoter (33) and was a gift from S. K. Nordeen (Denver). ARRE-1-luc was a gift from G. R. Crabtree (Stanford, CA) and contains the luciferase gene under the control of a minimal γ-fibrinogen promoter and four tandem copies of an antigen receptor response element from the human interleukin-2 (IL-2) gene. This element contains a modified TRE, which, in addition to AP-1 proteins, binds the transcription factor Oct-1 (34). The plasmid PRE-tk81-luc was constructed by inserting two copies of the PRE from the tyrosine amino-transferase gene into tk81-luc. For pMSG-luc, the luciferase cDNA from pGEM-luc (Promega) was inserted immediately downstream of the mouse mammary tumor virus long terminal repeat in pMSG (Pharmacia). This promoter construct contains a TRE located 400 bp downstream from the 5′ end of the full-length mouse mammary tumor virus long terminal repeat (35). ERE-tk-luc was a gift from J. H. Segars (36). IL-2-luc was donated by T. M. Williams (37). The hPR expression vectors hPR-0 (hPR-B), hPR-3 (AF-1 deletion mutant of hPR-B), and hPR-5 (ligand-binding domain deletion mutant of hPR-B), all cloned into pSG5, were gifts from P. Chambon (11, 14). pSG5 was used in mock-transfection for a total of 1.4 μg DNA per well. The expression vector encoding the non-DNA-binding hPR-B mutant (hPR-B-DBD_{cys}) was a gift from K. Horwitz (38). The expression vectors for the human glucocorticoid receptor α and the human estrogen receptor (hER) were gifts from R. M. Evans (La Jolla, CA) and R. J. Miksicek (Stony Brook, NY), respectively.

Cell Culture. HEC-1-B endometrial adenocarcinoma cells, COS-7 monkey kidney cells, and JEG-3 chorioacarcinoma cells were obtained from the American Type Culture Collection and maintained in low glucose DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin/streptomycin. SKUT-1-B endometrial leiomyosarcoma cells (American Type Culture Collection) were maintained in a 1:1 mixture of DMEM and Ham's F-12, supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. Cells were passaged twice weekly and plated for transfection when 60–80% confluent.

Transfections and Luciferase Assays. Cells were plated in 12-well plates (Costar) at a density of 2×10^5 cells per well. After 24 h, cells were transfected by the lipofection method as described (39). If not otherwise indicated, the transfection mixture contained 1 μg of reporter plasmid per well and 0.4 μg of expression vector per well. Eight hours after transfection, cells were refed with phenol red-free DMEM containing 10% charcoal-stripped FCS to avoid steroid contamination. After 12 h, cells were treated with steroid hormones and/or antagonists. The steroids tested were medroxyprogesterone acetate (MPA, 2.5×10^{-7} M, Sigma), RU486 (2.5×10^{-7} M, Roussel-UCLAF), estradiol (10^{-9} M, Sigma), and dexamethasone (10^{-7} M, Sigma). When tested, TPA (10^{-7} M, Sigma) was added after an additional 12 h.

Forty-four hours after transfection, cells were trypsinized, washed, pelleted, and lysed with reporter lysis buffer (Promega). After one freeze-thaw cycle, luciferase activity in the lysate was determined in a luminometer (Lumat LB 9501, Berthold, Wildbad, Germany). For some experiments, cell proliferation rates were determined by counting the cells in an automated cell counter (Coulter). All experiments were repeated at least three times in triplicates.

RESULTS

HEC-1-B Human Endometrial Adenocarcinoma Cells Are Devoid of hPR and hER. To study the interactions between hPR and AP-1 in the human endometrium, we decided to use HEC-1-B human endometrial adenocarcinoma cells as a model. These cells have been shown to be devoid of immunoreactive hPR (40). To further characterize this cell line with respect to the presence of functionally active hPR, the activity of the progesterone-responsive reporter plasmids PRE-tk81-luc and pMSG-luc was measured in the presence and absence of cotransfected hPR-B. No stimulation of luciferase activity in response to MPA was observed in untransfected cells. In cells transfected with hPR-B, there was strong induction following progestin treatment: 19 ± 0.9-fold stimulation for the PREtk81-luc construct and 22.6 ± 1.5-fold for pMSG-luc. Similar results were obtained for hER/ERE-tk-luc: no induction following E2-treatment in the absence of receptor, strong induction (34 ± 3-fold) with E2 when hER was transfected. Thus, HEC-1-B cells are devoid of both hPR and hER, yet are able to express functional receptors from transfected expression vectors. Therefore, this cell line represents an excellent model to study the transcriptional effects of these receptors.

The Unliganded hPR Stimulates AP-1 Activity and Progestins Reverse this Effect. HEC-1-B cells were transfected with two different AP-1-responsive luciferase reporter constructs (ARRE-1-luc and AP-1-tk81-luc). Cells were cotransfected with either control vector (pSG5) or the hPR-B expression vector. In mock-transfected cells, TPA stimulated the activity of ARRE-1-luc 6.6 ± 2.7-fold (mean ± SD), whereas MPA had no effect (Fig. 1). When hPR-B was cotransfected with the ARRE-1-luc reporter plasmid, luciferase activity in unstimulated cells was 4.7 ± 0.52-fold higher than in unstimulated mock-transfected cells. TPA treatment further enhanced luciferase activity (21.2 ± 2.0-fold induction as compared with unstimulated mock-tranfected cells). MPA reduced ARRE-1-luc activity both in the absence and in the presence of TPA

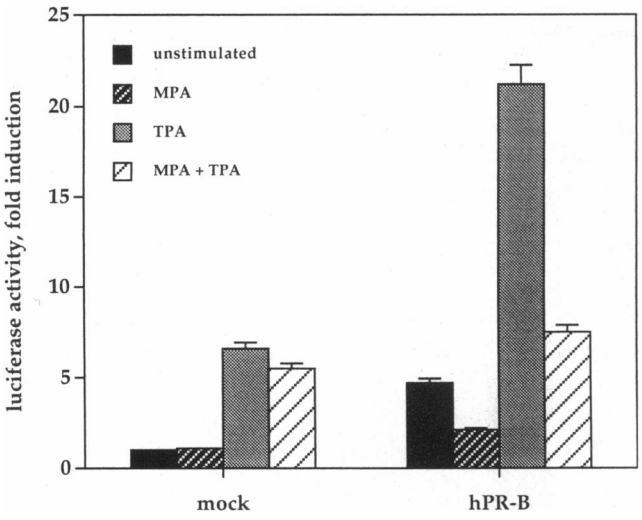

FIG. 1. Stimulation of AP-1 activity by cotransfection of hPR-B and reversal of this effect by MPA. HEC-1-B endometrial adenocarcinoma cells were transfected with the AP-1-responsive reporter plasmid ARRE-1-luc. Cells were cotransfected with either mock or hPR-B vector and stimulated with the substances indicated. All data are expressed as means ± SD.

(55.3 ± 8.9% and 64.6 ± 7.0% reduction, respectively). Similar results were obtained in experiments analyzing AP-1-tk81-luc activity. This reporter plasmid was induced 2.8 ± 0.1-fold by TPA, 4.3 ± 0.09-fold by cotransfected hPR-B, and 7.0 ± 0.38-fold by the combination of both. MPA also reversed these effects (57.1 ± 1.1% reduction in the absence of TPA and 31.5 ± 7.2% reduction in the presence of TPA).

All experiments were performed in phenol red-free DMEM containing charcoal-treated serum to exclude the possibility that residual steroids in the medium could account for any of the observed effects. To also exclude activation of hPR by nonsteroidal substances present in serum, the experiments were repeated in serum-free DMEM. These experiments gave the same results as those performed with serum (data not shown).

In transfection experiments similar to those described above, we also determined the cell number within each well. These experiments indicated that there were no significant differences in cell numbers between the different treatment groups (data not shown). The observed effects are, therefore, not caused by different cell proliferation rates.

The Observed Effects Are hPR-Specific. To determine whether the enhancing effect of the unliganded hPR on AP-1 activity was specific for this receptor, HEC-1-B cells were cotransfected with ARRE-1-luc and the hER expression vector. Cells were treated with physiological concentrations of estradiol (10^{-9} M) and/or with TPA. As shown in Fig. 2, cotransfection of hER *per se* had no effect on AP-1 activity. Furthermore, there was no difference in TPA inducibility between mock- and hER-transfected cells. Similar results were obtained with the human glucocorticoid receptor α expression vector (data not shown).

The Effects of hPR Are Promoter-Specific. To analyze the requirement for the presence of TREs to elicit the above effects, the hPR-B expression vector was cotransfected with different TRE-containing and TRE-devoid promoter-luciferase constructs. Fig. 3 compares the ability of unliganded hPR-B to activate these promoters. In addition to AP-1-tk81-luc and ARRE-1-luc, the more complex mouse mammary tumor virus long terminal repeat (pMSG-luc) and the human IL-2 promoter exhibited this type of inducibility. Both promoters contain functional TREs (34, 35, 37). In contrast, the activity of tk81-luc and PRE-tk81-luc, which do not contain TREs, was not significantly altered by cotransfected hPR-B.

Stimulation of AP-1 Activity by hPR Is Cell Type-Specific. We were further interested to determine whether the observed effects of cotransfected hPR could also be elicited in other cell lines. The results of these experiments are shown in Fig. 4. In SKUT-1-B and COS-7 cells, AP-1 activity was not induced by cotransfection of hPR-B. A minimal, yet not significant induction was seen in JEG-3 cells. Except for HEC-1-B cells, MPA had no effect on AP-1 activity in any of these cell lines. The same results were obtained when TPA was added to the cell culture medium (data not shown).

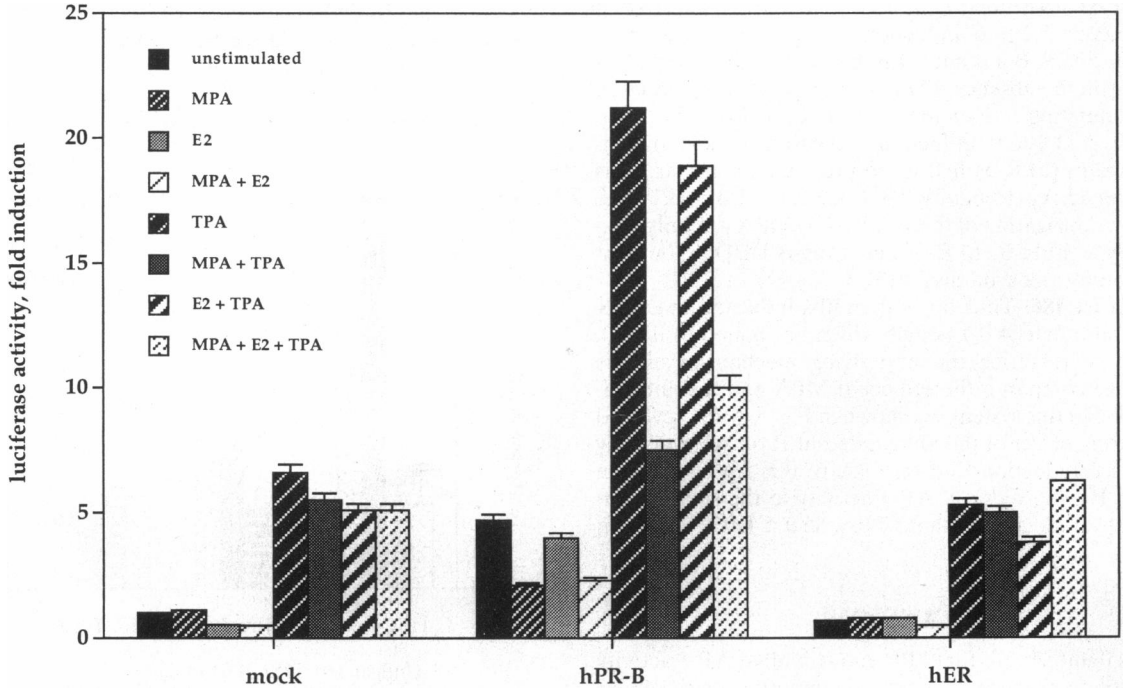

FIG. 2. hER does not induce AP-1 activity in HEC-1-B cells. All cells were transfected with ARRE-1-luc and cotransfected with either mock vector, hPR-B, or hER. Cells were treated with MPA, estradiol (E2), and/or TPA as indicated.

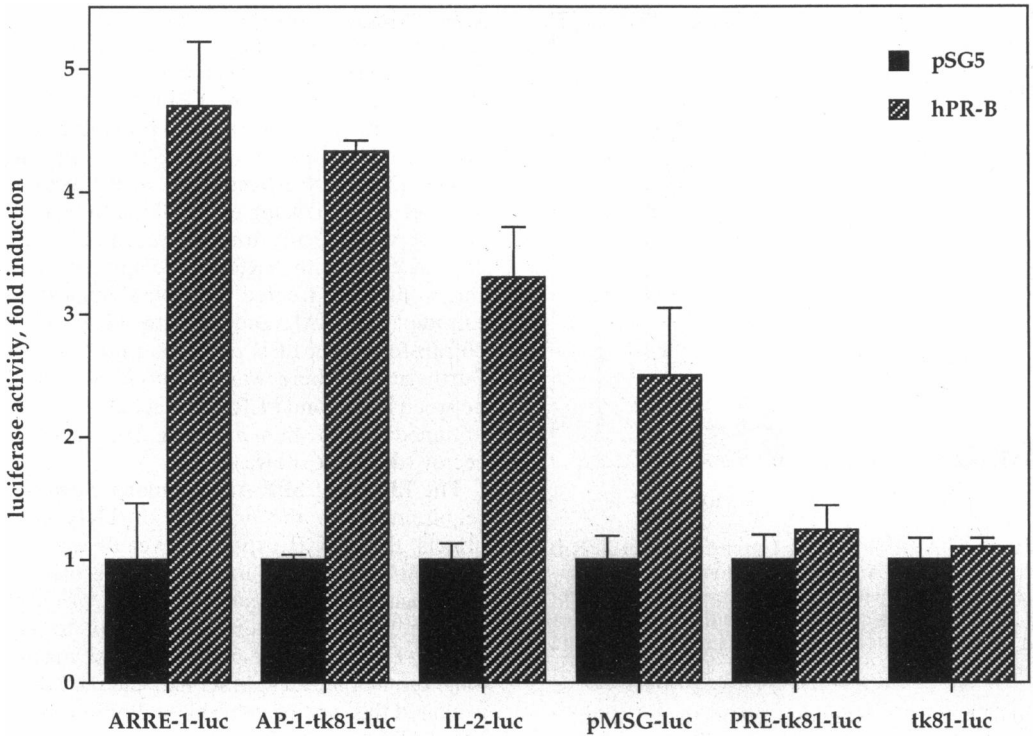

FIG. 3. The enhancing effect of unliganded hPR-B on AP-1 activity requires the presence of AP-1-responsive elements. HEC-1-B cells were cotransfected with the indicated promoter-luciferase constructs and either pSG5 or hPR-B. Only ARRE-1-luc, AP-1-tk81-luc, IL-2-luc, and pMSG-luc were inducible by hPR-B in the absence of ligand.

Effects of Cotransfected hPR Mutants on AP-1 Activity. To investigate which domains of hPR-B are required for the observed effects, we cotransfected HEC-1-B cells with the ARRE-1-luc reporter and different mutant hPR-B expression vectors. All three mutant receptors were able to induce AP-1 activity in the absence of ligand, albeit to a lesser extent than wild-type hPR-B (data not shown). In this set of experiments, wild-type hPR-B induced AP-1 activity 5.0 ± 1.0-fold in the absence of TPA and 42 ± 3.8-fold in the presence of TPA. Deletion of AF-1 (hPR-3) had the least consequences (3.8 ± 1.2 and 35.0 ± 5.2-fold induction, respectively). The non-DNA-binding hPR-B mutant (hPR-B-DBD_{cys}) exhibited wild-type activity in the absence of TPA and a moderate reduction of AP-1-stimulating activity in the presence of TPA (5.0 ± 0.8 versus 27.0 ± 3.3-fold induction). Deletion of the ligand-binding domain (hPR-5) had more pronounced effects. This mutant induced AP-1 activity 2.9 ± 0.6 and 21.0 ± 3.2-fold, respectively. Abrogation of these effects by MPA was only seen with wild-type hPR-B, hPR-3, and hPR-B-DBD_{cys}, but not with the nonhormone binding hPR-5.

Effects of RU486. Thus far, our results indicated that hPR could only stimulate AP-1 activity when not bound to ligand. To further characterize the underlying mechanism of this induction, we compared the effects of MPA and the antiprogestin RU486 in this system. As shown in Fig. 5, MPA reversed the stimulating effect of the unliganded hPR on AP-1 activity (61.0 ± 17.1% reduction without TPA, 67.0 ± 8.9% reduction with TPA). RU486 reduced AP-1 activity to the same extent (53.0 ± 16.9% reduction without TPA, 66.0 ± 6.0% reduction with TPA).

DISCUSSION

Our results demonstrate that hPR can stimulate AP-1 activity in the complete absence of ligand or any other extracellular stimulus. The magnitude of this effect is comparable to that induced by the phorbol ester TPA. In the presence of both unliganded hPR and TPA, AP-1 activity is stimulated in a synergistic manner. These effects are reversed at physiological concentrations of ligand.

The observed effects were hPR-specific, and could not be shown with either hER or human glucocorticoid receptor. They were also promoter-specific and strictly depended on the presence of TREs or TRE-like sequences. Thus, the ARRE-1-, AP-1-tk81-, IL-2-, and mouse mammary tumor virus-promoter constructs were inducible by unliganded hPR, whereas the PRE-tk81-luc and tk81-luc were not. Among the cell lines studied, induction of AP-1 activity by transfected hPR was observed only in HEC-1-B endometrial adenocarcinoma

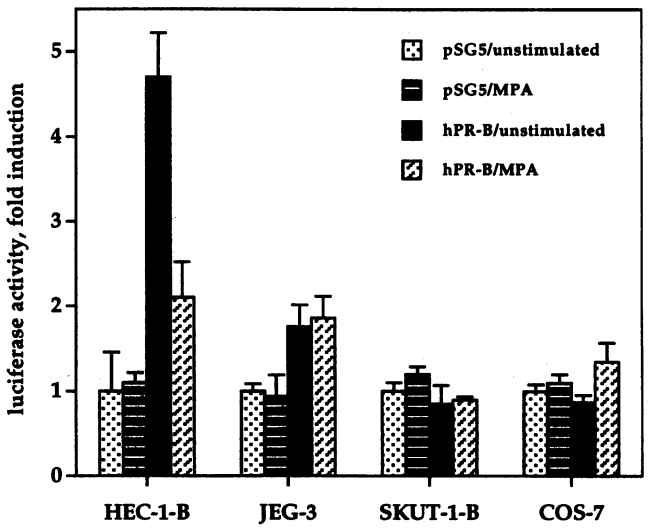

FIG. 4. Unliganded hPR-B stimulates AP-1 activity in a cell type-specific manner. HEC-1-B, JEG-3, SKUT-1-B, and COS-7 cells were transfected with ARRE-1-luc and either empty pSG5 or hPR-B expression vector.

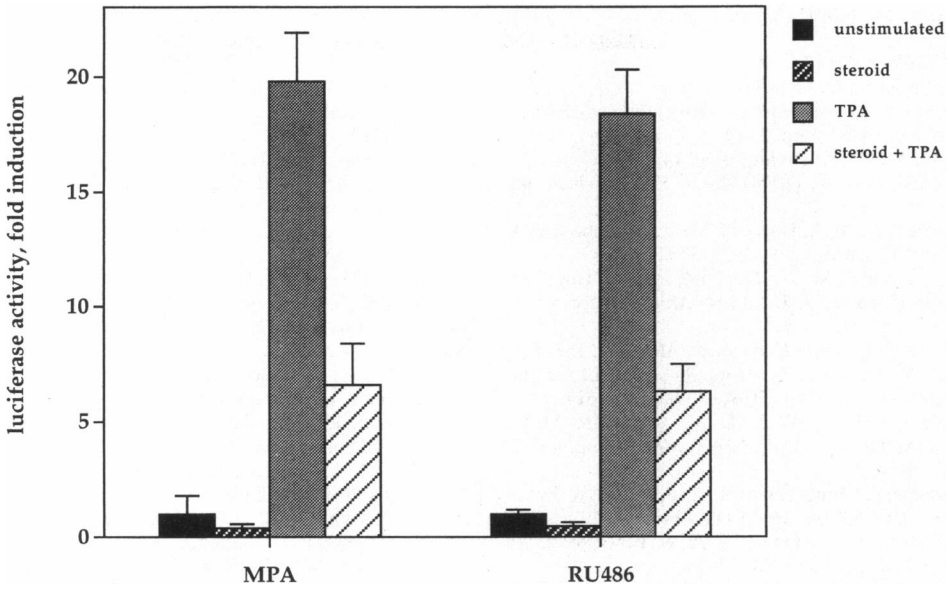

FIG. 5. RU486 exhibits agonist-like activity with respect to the reduction of AP-1 in HEC-1-B cells. All cells were cotransfected with ARRE-1-luc and hPR-B and treated as indicated.

cells. In SKUT-1-B cells, AP-1 activity was highly inducible by TPA, whereas transfection of hPR had no effect. In COS-7 and JEG-3 cells, neither TPA nor hPR induced AP-1 activity to a significant extent. The enhancing effect of the unliganded hPR on AP-1 transcriptional activity is, therefore, a cell type-specific phenomenon and has thus far only been observed in cells derived from the epithelial fraction of the human endometrium.

At this time, one can only speculate as to how the hPR could interfere with the complex cascade leading to AP-1 activation in the absence of ligand and other external stimuli (e.g., dopamine). With respect to the underlying mechanism, our results allow several interpretations. One possible mechanism would be that the unliganded hPR inactivates a putative inhibitor of AP-1 activity in HEC-1-B cells. This interaction could involve either the hPR molecule itself or the HSP complex, which is usually associated with hPR in the absence of ligand (16, 17). In this model, ligand binding would alter the conformation of the hPR molecule, thus abrogating its interaction with the inhibitory factor. However, the existence of a cell type-specific inhibitor of AP-1 action has not been reported as yet. Alternatively, the unliganded hPR could interact directly with the Jun/Fos complex to enhance its transcriptional activity. Again, the ligand-induced conformational change would reverse this interaction. Finally, the hPR molecule could be phosphorylated by protein kinase C, whose activity is exceptionally high in HEC-1B cells (unpublished observations). This could cause the hPR molecule to assume a conformation different from that induced by ligand, allowing it to enhance AP-1 transcriptional activity.

The other important finding is that progestins reverse the stimulating effect of the unliganded hPR. Anti-AP-1 activity of nuclear hormone receptors was previously reported for the glucocorticoid, androgen, thyroid hormone, and retinoic acid activity (25–31, 41). The estrogen receptor clearly behaved differently from the other receptors, as it only inhibited AP-1 activity in a limited number of cases, but had a stimulatory effect in most systems, including human endometrial cells (6, 42, 43). Previous evidence for hPR-mediated inhibition of AP-1 activity was presented by Shemshedini *et al.* (44) in HeLa and CV1 cells, where the progestin-activated hPR inhibited the activity of Jun. Indirect evidence was also presented by Marbaix *et al.* (45) who demonstrated that physiological concentrations of progesterone greatly reduced the release of collagenase from human endometrial explants. Transcriptional regulation of the collagenase gene is largely AP-1-dependent (25). The mechanism by which nuclear receptors inhibit AP-1 is still a matter of dispute. Ligand-induced release of a putative inhibitory factor from the receptor/HSP complex is likely to be hPR-specific. In addition, both hPR and other nuclear receptors could form inactive complexes with Jun and Fos family members. The latter mechanism is suggested by results from crosslinking studies, showing physical association of the glucocorticoid receptor and c-Jun *in vitro* (28). It is also interesting to note that the RU486-bound hPR-B inhibited AP-1 activity in this setting. This is in agreement with recent data showing inhibition of both AP-1 (46) and NF-κB (47) activity by RU486-GR. RU486, like progestin, promotes dissociation of hPR from the HSP complex (48).

The control of cellular proliferation and differentiation in the human endometrium largely depends on the coordinate activation of estrogen and PR (2, 32). In addition to their direct growth-modulating effects (49), both receptors have been shown to antagonize each other at multiple levels (2, 50, 51). Our findings, based on results obtained in the HEC-1-B adenocarcinoma cell line, if extrapolated to the normal endometrium, would suggest a novel type of interaction, through which these receptors could regulate proliferation and differentiation in the human endometrium: It was previously reported that estrogens stimulate the expression of hPR (14). In addition to the direct stimulatory effects of hER on AP-1 (6, 42, 43), this would further enhance AP-1 activity in the first half of the menstrual cycle, when the majority of hPR molecules is unliganded. Under the influence of progesterone, this effect would be reversed in the second half of the cycle. By permanently elevating cellular hPR levels, unopposed estrogenic activity would keep the endometrium in the proliferative phase and, thus, lead to the development of endometrial hyperplasia and, finally, tumor formation.

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