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Progestin Activation of Nongenomic Pathways via Cross Talk of Progesterone Receptor with Estrogen Receptor β Induces Proliferation of Endometrial Stromal Cells

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Uterine decidualization is characterized by stromal cell proliferation and differentiation, which are controlled by ovarian hormones estradiol and progesterone. Here we report that the proliferative response of UIII rat uterine stromal cells to a short treatment with progestins requires active progesterone receptor (PR) and estrogen receptor β (ERβ) as well as a rapid and transient activation of Erk1–2 and Akt signaling. The optimal R5020 concentration for the proliferative response as well as for activation of the signaling cascades was between 10 and 100 pM. UIII cells are negative for ERα and have low levels of ERβ and PR located mainly in the cytoplasm. Upon progestin treatment PR translocated to the cell nucleus where it colocalized with activated Erk1–2. Neither progestins nor estradiol transactivated the corresponding transfected reporter genes, suggesting that endogenous PR and ERβ are transcriptionally incompetent. A fraction of endogenous PR and ERβ form a complex as demonstrated by coimmunoprecipitation. Taken together, our results suggest that the proliferative response of uterine stromal cells to picomolar concentrations of progestins does not require direct transcriptional effects and is mediated by activation of the Erk1–2 and Akt signaling pathways via cross talk between PR and ERβ. (*Molecular Endocrinology* 19: 3023–3037, 2005)

THE PREEMINENCE OF progesterone in female reproductive biology has been highlighted by the phenotype of mice lacking the progesterone receptor (PR), which exhibit abnormalities in all aspects of reproduction including sexual behavior, mammary gland development, ovulation, and implantation (1). In the uterus, estrogen stimulates proliferation of the endometrial epithelium, whereas progesterone is the essential stimulus needed for *in vivo* proliferation of stromal cells and acts as the switch for their specific decidual program (2, 3). At d 4 of pregnancy in the rat, progesterone switches proliferation from the epithelial to the stromal compartment as a prerequisite for decidualization and implantation (4, 5).

The pattern of PR expression during the estrous cycle in rat endometrial epithelial cells is markedly different from that in the stromal cells. In immunocytochemical studies, the nuclei of the epithelial cells are intensively PR positive at diestrus, whereas those of the stromal cells stained deeply at proestrus; at metestrus, however, when the estrogen levels are low, the stromal cells invariably show low-intensity PR staining of the nucleus (6). Decidualization is normal in estrogen receptor-α (ERα)-deficient (ERα−/−) mice, indicating that ERα is not involved in this process (7). ERβ (ERβ) is present in the uterus, especially in the stromal cells (8). Moreover, ERβ-deficient mice have a poor reproductive capacity (9). Therefore, it is likely that estrogen effects on stromal decidualization are mediated by ERβ.

The molecular mechanisms underlying physiological action of progesterone on uterine function are unclear, although it has been assumed that they involve transcriptional activation of progesterone target genes mediated by binding of its nuclear PR to hormone-responsive elements (10). However, the proof for participation of this mechanism in the proliferative response of uterine stromal cells is missing.

In addition to their transcriptional effects, steroid hormones can act by nongenomic mechanisms involving cross talk with growth factor receptors and other cytoplasmic signaling pathways (11–13). In human breast cancer cells, rapid and transient activation of Src/p21ras/Erk and phosphatidylinositol 3-kinase (PI3-K)/

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Abbreviations: CAT, Chloramphenicol acetyl transferase; DCC/FBS, dextran-coated, charcoal treated fetal bovine serum; ER, estrogen receptor; ERID, ER-interacting domain; HSD, hydroxysteroid dehydrogenase; MMTV, mouse mammary tumor virus; PI3-K, phosphatidylinositol 3-kinase; PR, progesterone receptor; PR_B, PR-isoform B; PRE, progesterone response element; tk, thymidine kinase.

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Akt pathways has been shown to be important in estrogen and progesterone induction of cell proliferation (14, 15). This effect of progesterone is blocked not only by antiprogestins but also by antiestrogens, indicating a cross talk between the receptors for both ovarian hormones in T47D cells (14). This cross talk involves two domains on the N-terminal half of PR isoform B (PR_B), designated ER-interacting domains I and II (ERID-I and ERID-II), which interact with the ligand-binding domain of ERα and are required for progesterone activation of the c-Src/Ras/Erk pathway in breast cancer cells (16). The N-terminal region of the PR also contains a cluster of proline residues located between ERID-I and ERID-II, which interacts with the SH3 domain of c-Src and can activate its kinase domain (17), but this interaction is not required for progestin activation of the Src/Ras/Erk pathway (16). Instead binding of progestins to the cytoplasmic PR_B, which is part of a preformed PR_B-ERα complex, activates ERα in the absence of estrogens and leads to an interaction of the ligand-binding domain of ERα with the SH2 domain of c-Src and to activation of the cascade (16).

Here we explore the participation of activation of the Erk and the Akt pathways in the proliferative response of normal uterine stromal cells to progesterone, using as a model the established UIII cell line (18). UIII cells were derived from rat uterus by sequential enzymatic digestion and density fractionation on Percoll gradient and subcultured by trypsinization. They retained estrogen, progesterone, and prolactin receptors, and their proliferation is regulated by progesterone (18). We show that UIII cells cultured in serum-free medium respond to short exposure to picomolar concentrations of progestin with enhanced proliferation, and that this effect is preceded by transient activation of Erk and Akt signaling pathways. Moreover, the proliferative response required both active PR and ER and can be blocked with inhibitors of Erk or Akt activation. In the absence of hormone, UIII cells have low levels of PR located mainly in the cytoplasm and unable to transactivate a progesterone reporter gene. In response to low concentrations of progestins, PR translocates to the cell nucleus along with the activated Erk. UIII cells are negative for ERα and positive for ERβ, which is cytoplasmic in the absence of hormones and colocalizes with PR. The presence of a PR-ERβ complex was also evidenced by coimmunoprecipitation assays. Thus, our results demonstrate for the first time that a proliferative effect of picomolar progestin concentrations, mediated by Erk and Akt activation, needs the cross talk of PR with ERβ in a system in which nongenomic pathways are independent of transcriptional effects of steroids.

RESULTS

Progestin-Dependent UIII Cell Proliferation in Serum-Free Culture Conditions

UIII cells were described as immortalized stromal cells from normal rat uterus that retained progesterone and prolactin receptors and progesterone regulation of cell growth (18). To study the effects of progestin R5020 on cell proliferation, independently of serum components, we used FACscan to evaluate the percentage of cells in different cell cycle phases under serum-free culture conditions (Fig. 1A). During 3 d of serum starvation the percentage of cells in S and G2/M phases decreased from 45% to 19% (data not shown) Treatment with the synthetic progestin R5020 (10^{-8} M) for the last 24 h in the absence of serum increased the proportion of cells in S and G2/M phases from 22% to 36% (1.6-fold increase) (Fig. 1A). R5020 also increased the percentage of cells entering S phase in the presence of 10% dextran-coated charcoal (DCC)/fetal bovine serum (FBS) to a similar extent (from 28.2% to 48.3%, 1.7-fold increase; data not shown), although the absolute values are different. Thus, the proliferative response of UIII cells to progestins does not require additional serum factors.

Treatment (1 h) with Picomolar Concentrations of Progestins Induces Cell Proliferation

Next we tested whether a short time of exposure to progestins was sufficient to enhance cell proliferation or whether the continuous presence of the hormone was required. To this end we compared cells cultured either in 10% DCC/FBS or under serum-free conditions. In both cases, exposure to 10^{-8} M R5020 for 1 h increased the number of cells counted after 5 d to the same extent as in the continuous presence of 10^{-8} M R5020 (Fig. 1B) or progesterone (data not shown). Similar results were obtained when cells were treated with 10^{-6} M RU486 after washing out R5020 (data not shown). We conclude that 60 min of progestin exposure is sufficient to trigger the chain of events that eventually lead to increase cell proliferation.

Using this protocol of short-time exposure to R5020, we analyzed the dose-response curve. R5020 increased cell proliferation at a range of 10^{-12} to 10^{-8} M concentration. The statistical differences between the controls in the absence of hormone and in the presence of hormone are significant for 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} M. However, in various dose-response assays the effect on cell number was optimal at 10^{-11} to 10^{-10} M R5020, and the curves had a reproducible tendency to decrease at 10^{-9} and 10^{-8} M. In the experiment shown in Fig. 1C, the differences between both 10^{-11} M and 10^{-7} M ($P < 0.01$) and between 10^{-10} M and 10^{-7} M ($P < 0.001$) were statistically significant.

This unexpected finding is in contrast with the dose-response curves of transcriptional effects of R5020, which peak at 10^{-8} M concentrations (see below, Fig. 5).

To test whether the observed effects are mediated by the classical PR, we analyzed the effect of a well-established PR antagonist, RU486. UIII cells incubated with 10^{-6} M RU486 in serum-free medium did not change their proliferation behavior, but this concentration of antagonist completely abolished the effect of

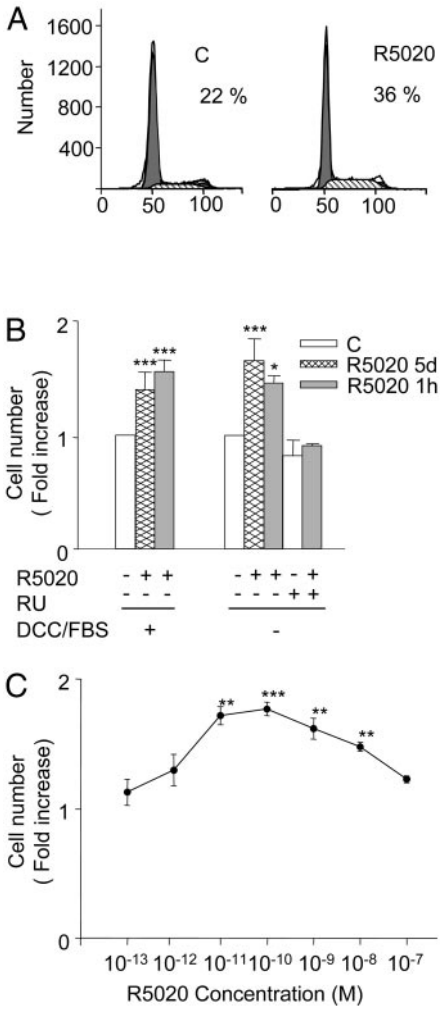

Fig. 1. Effect of Progestin R5020 on Cell Proliferation in Serum-Free Culture Conditions

A, Effect on cell cycle. UIII cells were plated in medium supplemented with 10% FBS. After 2 d of culture, the medium was removed and replaced by serum-free medium. After 2 d of serum starvation, the cells were treated for 24 h with vehicle (C, control) or 10^{-8} M R5020 (R5020), and the distribution of cells in the various phases of the cell cycle was evaluated by FACscan of a cell aliquot stained with Hoescht 33342. Fluorescent data were collected with an LSR detector Dickinson FACscan (area of FL5 channel), and DNA histograms were analyzed with ModFIT 3.0 software. The *numbers* show the corresponding quantitation of the percentage of cells in S and G_2/M. B, Short-term effect of picomolar concentrations of progestin R5020. UIII cells were plated in 35-mm dishes at about 1.2×10^5 cells/cm^2 in 10% DCC/FBS. After 48 h medium was removed, and cells were further cultured in medium with 10% DCC/FBS (DCC/FBS, +) or without serum (DCC/FBS, −). After an additional 48 h, medium was removed and cells were treated for 1 h with vehicle (*white columns*) or with 10^{-8} M R5020 (*gray columns*) in 10% DCC/FBS or without serum, respectively. Cells were then carefully washed six times (10 min each) with serum-free medium to remove any traces of hormone and further cultured in 10% DCC/FBS or without serum. Columns 2 and 5 (*striped*) show the number of cells after 5 d in the presence of 10^{-8} M R5020 in the absence and presence of 10% DCC/FBS, respectively (R5020, 5d). Columns 4–8 show the results obtained with cells incubated in serum-free medium for 30 min with vehicle (4, 5, and 6) or with 10^{-6} M RU486 (7 and 8), followed by 1-h incubation with either vehicle (*white columns*) or 10^{-8} M R5020 (*gray columns*). Media were removed, cells were carefully washed and further incubated without hormone, and cell numbers were determined after 5 d as in panel A. Data represent the media ± SEM of three independent experiments. ***, $P < 0.001$ *vs.* control (first column). C, Dose response. After 48 h of serum starvation cells were treated with vehicle or with various concentrations (10^{-13} to 10^{-7} M) of R5020 for 1 h in serum-free medium without additions. Medium was removed; the cells were carefully washed and further incubated without hormone. Cell numbers were determined after 5 d of culture as in panel B. Data represent the media ± SEM of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ *vs.* control (vehicle-treated cells). RU, RU486.

10^{-8} M R5020, and 10^{-9} M RU486 abolished 10^{-11} M R5020 (Fig. 1B *last column*, and data not shown). This finding indicates that the proliferative effects of low and high concentrations of progestin are mediated by the classic PR.

Rapid and Transient Progestin Activation of Erk1–2 Is Required for Cell Proliferation

In addition to their transcriptional effects, progestins are known to activate the Src/Ras/Erk pathway in breast cancer cell lines, and this activation is important for their effect on cell proliferation (14). We therefore analyzed Erk1–2 activation after progestin treatment in UIII cells. We found a stimulatory effect of 10^{-8} M R5020 on Erk1–2 activation that was maximum after 5 min treatment, decreased after 10 min (Fig. 2A), and returned to control values after 20–30 min (data not shown). These results are similar to those reported in breast cancer cell lines (14) and demonstrate a rapid and transient activation of the Erk1–2 cascade by progestins in immortalized uterine stromal cells. High activation (~5-fold) was already evident with 10^{-12} M R5020 but the 5 min Erk1–2 activation was maximal at 10^{-10} M (~10-fold) (Fig. 2, B and C). This dose-response curve is similar to that found for the progestin effect on cell proliferation (see Fig. 1C and *dotted line* in Fig. 2C), suggesting that Erk1–2 activation may be related to UIII cell proliferation.

We next tested the effect of PD98059, an inhibitor of Erk1–2 activation, on the enhancement of cell proliferation caused by 1-h treatment with R5020. Pretreatment of cells for 30 min with PD98059 on its own had no effect on cell proliferation but completely abolished the effect of R5020 treatment (Fig. 2D) or progesterone (data not shown). We conclude that Erk1–2 activation is required for the stimulatory effect of progestin on cell proliferation of uterine stromal cells.

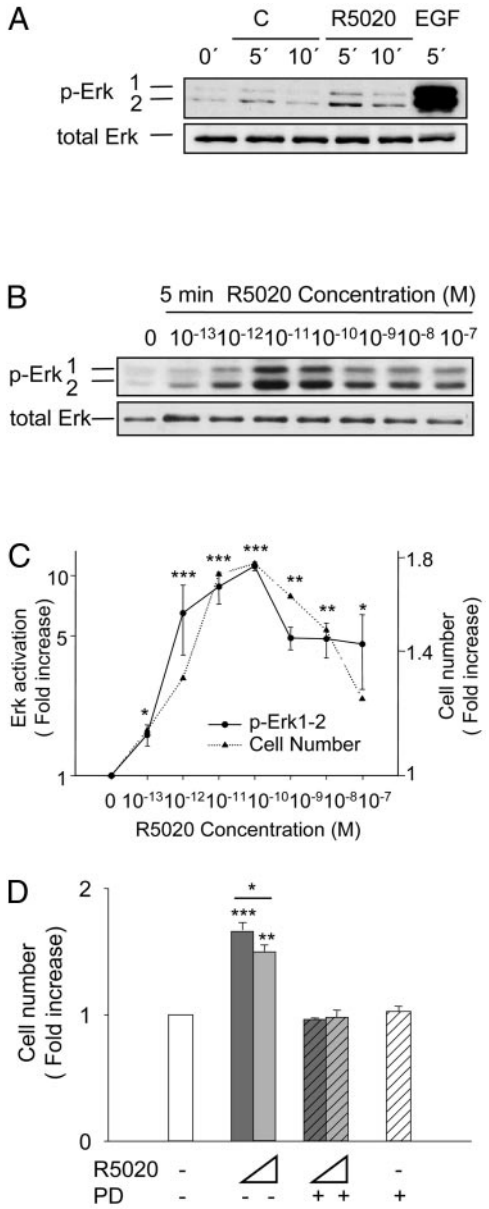

Fig. 2. Rapid and Transient Activation of Erk1–2 by R5020 Is Required for Progestin-Dependent Cell Proliferation

A, Time course of Erk1–2 activation. Western blot analysis of extracts from UIII cells incubated for 0, 5, or 10 min with either vehicle (C, control), or 10^{-8} M R5020 (R5020), or for 5 min with 100 ng/ml epidermal growth factor. The primary antibodies were antiphospho-Erk1–2 (*top panel*) or antitotal-Erk2 (*bottom panel*). B, Dose dependence. Western blot analysis using antiphospho-Erk1–2 (*top panel*) or antitotal-Erk2 antibodies (*bottom panel*) from extracts of UIII cells incubated for 5 min with either vehicle (0) or 10^{-13} M to 10^{-7} M R5020 as indicated. C, Quantitation of the dose-response curve. The arbitrary units corresponding to p-Erk 1 and p-Erk 2 were added up and divided by the arbitrary units corresponding to total Erk for each treatment. Each treatment value was divided by the vehicle control value and the mean ± SEM of two to four independent experiments were calculated after logarithmic transformation of data. Individual comparisons within a given group of treatments were made using an ANOVA and a Tukey-Kramer test for multiple comparisons. For comparison, the *dotted line* data represent the media ± SEM of three independent experiments shown in Fig. 1C. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control (0). D, Effect of an inhibitor of Erk activation. Serum-starved cells were incubated in serum-free medium during 30 min with vehicle or with 50 μM PD 098.059 (*dashed columns*) as indicated, followed by 1-h incubation with vehicle (*white column*), with 10^{-11} M R5020 (*dark gray columns*), or with 10^{-8} M R5020 (*light gray columns*). Media were removed, the cells were carefully washed and further incubated without hormone, and cell numbers were determined as in panel A. Data represent the media ± SEM of five independent experiments. ***, $P < 0.001$ *vs.* controls (*white column*). *, $P < 0.05$, 10^{-8} M *vs.* 10^{-11} M R5020 (*second vs. third column*). EGF, Epidermal growth factor; PD, PD 098.059.

The Effects of Progestins on Cell Proliferation and Erk1–2 Activation Are Blocked by an ER Antagonist

The rapid effects of progesterone on Erk activity can be mediated by either a direct interaction of PR with c-Src (17) or by an interaction of PR with the ER, which in turn interacts with c-Src (16). To test for an ER involvement in the progestin signaling in UIII cells, we analyzed the effect of the ER antagonist ICI182.780 on the response to progestins. Erk1–2 induction by R5020 was inhibited by ICI182.780 to the same extent as by the PR antagonist RU486 (Fig. 3A). The proliferative effect of R5020 was also blocked by ICI182.780, which alone had no effect on cell number (Fig. 3B). Therefore, we conclude that unliganded ER is required for progestin activation of Erk1–2 and cell proliferation in uterine stromal cells.

Effect of Progestin on Akt Activation

It has been reported that the PI3-K/Akt pathway mediates estrogen-induced S-phase entry in MCF-7 breast cancer cells (15). Because progestin action on UIII cell proliferation is mediated by ER, we analyzed phospho-Akt levels in cells treated with various concentrations of the progestin R5020, alone or in combination with PR or ER antagonists. Five minutes after R5020 treatment there was an increase in activated Akt, as measured by Western blotting with selective antibodies for the active phosphorylated protein. The maximum effect was obtained with the same low concentrations (10^{-10} to 10^{-11} M) as found in Erk1–2 activation assays (Fig. 4A). This effect was blocked by the PR antagonist RU486 as well as by the ER antagonist ICI182.780 (Fig. 4B).

Moreover, the progestin effect on cell proliferation was blocked by pretreatment of cells with wortmannin, a PI3-K inhibitor (Fig. 4C). This is the first evidence that progestin activation of the PI3-K/Akt pathway requires active PR and unliganded ER and is necessary for uterine stromal cell proliferation.

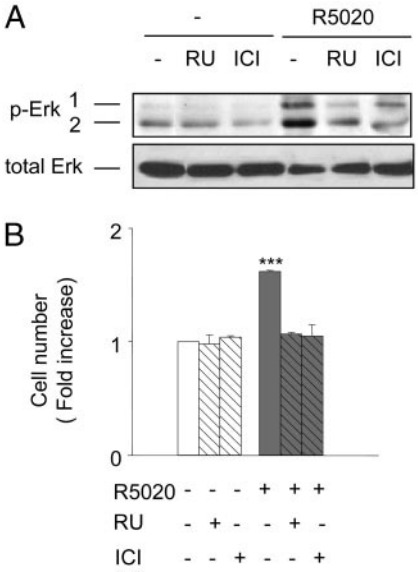

Fig. 3. Effect of the ER Antagonist ICI182.780 on Progestin Activation of Erk1–2 and on Progestin-Dependent Cell Proliferation

A, Erk activation. UIII cells were incubated for 5 min with either vehicle (−) or 10^{-11} M R5020 (R5020), alone or with the addition of 10^{-9} M RU486 (RU), or 10^{-9} M ICI182.780 (ICI), as indicated. The figure shows a Western blot analysis of cell extracts using antibodies against phospho-Erk1–2 (*top panel*) or against total Erk (*bottom panel*). B, Cell proliferation. Serum-starved cells were incubated in serum-free medium during 30 min with vehicle (−, *white columns*), or with 10^{-9} M RU486 (RU), or with 10 μM ICI182.780 (ICI), followed by 1-h incubation with vehicle or with 10^{-11} M R5020 (R5020) as indicated. The cells were carefully washed and further incubated without hormone, and the cell numbers were determined as in Fig. 1B. Data represent the media ± SEM of three independent experiments. ***, $P < 0.001$.

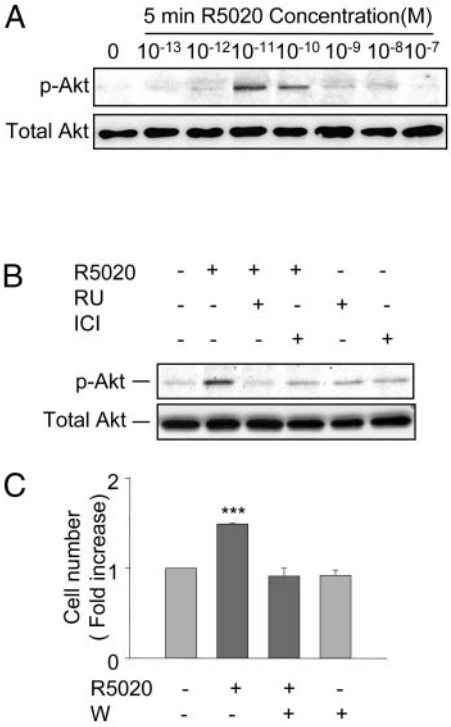

Fig. 4. Activation of Akt by R5020 Is Required for Progestin-Dependent Cell Proliferation

A, Dose dependence. UIII cells were incubated 5 min with either vehicle (0) or different concentration of R5020 (10^{-13} M to 10^{-7} M) followed by Western blot analysis using antibodies against phospho-Akt (*top panel*) or against total Akt (*bottom panel*). B, Effect of ER and PR antagonists. UIII cells were incubated for 5 min with either vehicle, 10^{-11} M R5020, 10^{-9} M RU486, 10^{-8} M ICI182.780, R5020 plus RU486, or R5020 plus ICI182.780, as indicated, followed by Western blot analysis using antibodies against phospho-Akt (*top panel*) or against total Akt (*bottom panel*). C, Effect of wortmannin on progestin-dependent cell proliferation. Serum-starved cells were incubated in serum-free medium for 30 min with vehicle, or with 10^{-9} M wortmannin (W), followed by 1-h incubation with vehicle (*light gray columns*) or with 10^{-11} M R5020 (R5020, *dark gray columns*) as indicated. Cells were carefully washed and further incubated without hormone, and the cell numbers were determined after 5 d as in Fig. 3A. Data represent the media ± SEM of three independent experiments. ***, $P < 0.001$ *vs.* controls. RU, RU486; ICI, ICI182.780.

UIII Cells Contain Levels of PR Insufficient for Transactivation of a Reporter Gene

To estimate the relative level of PR in UIII cells, we used Western blotting to compare extracts from UIII cells with extracts from the breast cancer cell line T47D, which is known to contain high levels of PR (19). The level of PR in UIII cells was considerably lower than in T47D cells (Fig. 5A). However, we cannot exclude differences in the affinity of the antibody for the rat *vs.* the human PR.

To have an independent evaluation of the relative levels of PR in UIII and T47D cells, we quantitated PR-mRNA by real-time PCR using as primers oligonucleotides that do not distinguish between PR_A and PR_B. We found that the level of PR-mRNA in UIII cells was 31-fold lower than in T47D cells (Fig. 5B). We also showed by RT-PCR that the levels of PR-mRNA in UIII cells were considerably lower than in rat uterus, the tissue from which they were derived (Fig. 5B, *inset*). This result is in accordance with the lower signal detected by Western blotting and suggests that the PR levels in UIII cells are very low.

To test the transcriptional competence of the endogenous PR levels of UIII cells, we performed transient transfection studies with a mouse mammary tumor virus (MMTV)-*luc* reporter gene. The reporter gene was not activated by treatment of the transfected cells for 36 h with concentrations of R5020 going from 10^{-11} M to 10^{-8} M (Fig. 5C, *white bars*), under conditions that activate the same reporter gene in PR-positive breast cancer cell lines (data not shown). The lack of response was not due to limiting factors in UIII cells other than PR, because the MMTV-*luc* reporter was induced by R5020 when an expression vector for PR_B was cotransfected (Fig. 5C, *gray bars*). Optimal induction was detected at 10^{-8} M, and no response was

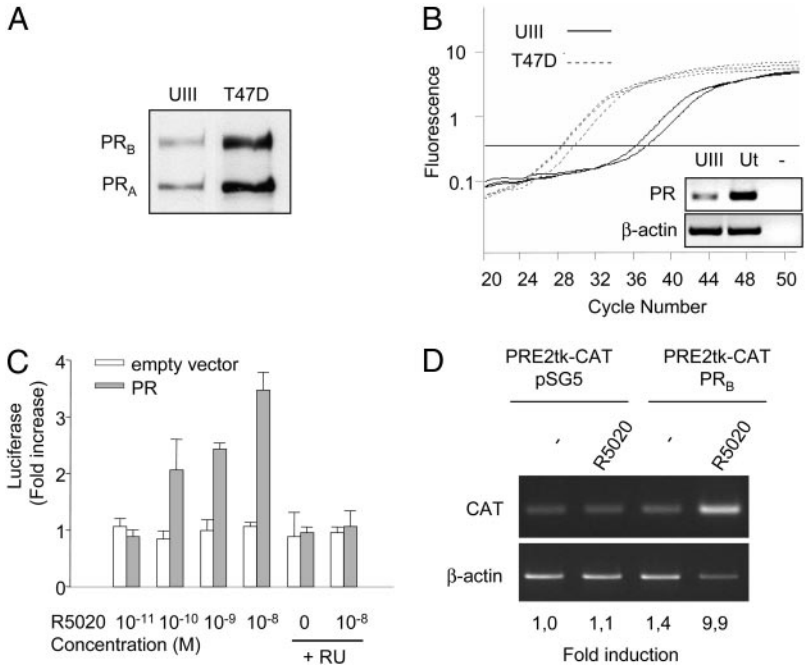

Fig. 5. UIII Cells Have Small Amounts of Transcriptionally Incompetent PR

A, Western blot analysis of PR isoforms. An antibody against PR that recognizes the isoforms A and B (Santa Cruz polyclonal antibody H190) was used for Western blot analysis of 100 μg serum-starved UIII cell extracts (UIII), or from 5 μg serum-starved T47D cell extracts (T47D). The positions where the A and B isoforms of PR migrate are indicated. B, Quantitation of PR mRNA in UIII and in T47D cells. The figure shows fluorescence intensity pattern of real-time PCR products for PR from three independent samples of serum-starved UIII mRNA (*solid line*), and from three independent samples of serum-starved T47D mRNA (*dotted lines*). *Inset*, Comparison of PR mRNA levels in UIII and in rat uterus. Ethidium bromide-stained gels of RT-PCR products for PR and β-actin from serum-starved cells (UIII), or from rat uterus (Ut), or from the PCR product performed without template (−). C, The PR of UIII is transcriptional incompetent. A MMTV-Luc reporter plasmid carrying the MMTV promoter linked to the luciferase gene was transiently cotransfected with the PR_B expression plasmid pSG5:PR (*gray columns*) or the empty plasmid pSG5 (*white columns*). Cells were incubated 36 h with vehicle, with the indicated concentrations of R5020 (10^{-11} M to 10^{-8} M), or with 10^{-7} M RU486 (RU) plus either vehicle (0) or 10^{-8} M R5020. Luciferase activity was determined in whole extracts. The average and SD of two independent experiments performed in duplicate are shown. D, A PRE2tk-CAT reporter plasmid was transiently cotransfected with the PR_B expression plasmid pSG5:PR_B or the empty plasmid pSG5. Cells were incubated 36 h with vehicle (−) or with 10^{-8} M R5020 (R5020). The figure shows ethidium bromide-stained gels of RT-PCR products for CAT and β-actin from the PCR product of transfected cells. Abundance of CAT mRNA relative to β-actin was expressed as fold induction over the relative level in cells cotransfected with the empty plasmid pSG5 of one representative experiment.

observed at 10^{-11} M R5020 (Fig. 5C), a concentration sufficient for optimal activation of kinase cascades. The induction by 10^{-8} M R5020 was blocked by the PR antagonist RU 486 (Fig. 5C). The lack of transcriptional activation by the endogenous PR was not promoter specific because it was observed in similar experiments with three other transfected reporters of PR; PRE2tk-CAT, consisting of two progesterone response elements (PREs) cloned upstream of the thymidine kinase (tk) promoter-CAT (chloramphenicol acetyl transferase) hybrid gene (20) (Fig. 5D); P4Bcl-X-Luc consisting of nucleotides from −3288 to −2652 from Bcl-X promoter cloned upstream of LUC (21) (Table 1); and hydroxysteroid dehydrogenase (HSD)11β2-Luc, consisting of nucleotides from −1778 to +117 of human 11β-HSD type II promoter cloned upstream of Luc (22) (Table 1). R5020 10^{-8} M did not transactivate these reporter genes unless expression vectors for PR_B were cotransfected (Fig. 5D and Table 1). We conclude that the level of endogenous PR in UIII cells is insufficient for transcriptional activation of a transfected reporter.

PR Is Mainly Cytoplasmic in UIII Cells and Translocates to the Cell Nucleus after Progestin Treatment

We next analyzed the subcellular localization of PR in UIII cells by immunocytochemistry. In the absence of the hormone, approximately 40–60% of the cells showed positive staining with PR antibodies, and the signal was mainly found in the cytoplasm (Fig. 6A, *top panels*, and C). This is in contrast with T47D cells, in which the large majority of the PR staining was localized to the cell nucleus even before treatment with progestins (Fig. 6A, *top panels*; T47D). After 30 min treatment with 10^{-11} M R5020, the cytoplasmic staining decreased and the remaining staining was mainly

Table 1. Transcriptional Incompetence of Endogenous PR

Reporter	Fold Induction			
	pSG5		pSG5:PR_B	
	—	R5020	—	R5020
MMTV-Luc	1	0.89 ± 0.21	1.00 ± 0.06	12.42 ± 0.48
P4Bcl-X-Luc	1	0.84 ± 0.01	1.25 ± 0.03	86.6 ± 0.54
HSD11β2-Luc	1	0.95 ± 0.01	1.05 ± 0.02	10.61 ± 0.37

MMTV-Luc, P4Bcl-X-Luc, and HSD11β2-Luc reporter plasmids were transiently cotransfected with the PR_B expression plasmid pSG5:PR_B or the empty plasmid pSG5. Cells were incubated 36 h with vehicle (−), with 10^{-8} M R5020 (R5020). Luciferase activity was determined in whole extracts. The average and SD of two independent experiments performed in duplicate are shown.

found near the cell membrane, whereas the staining in the nucleus increased in approximately 50% of the cells (Fig. 6A, *top panels*; R5020). As negative controls, we omitted the first antibody or preincubated the antibody with 100× molar excess of purified hPR_B (23). In both cases, staining disappeared (Fig. 6A, *bottom panels*; −αPR and αPR + hPR_B). We conclude that the unliganded PR of UIII cells localizes preferentially to the cytoplasm and partly translocates to the nucleus after R5020 binding.

Using antibodies against phosphorylated Erk1–2, we found that the levels of activated Erk increase 10 min after treatment with R5020 (Fig. 6B). A quantitation of the results (Fig. 6B, *histogram*) showed that 80% of the untreated cells exhibited low levels of p-Erk (<20 arbitrary units), whereas after progestin treatment more than 70% of the cells showed high levels of p-Erk (>20 arbitrary units).

Upon activation by phosphorylation, Erk1–2 is known to translocate to the cell nucleus, where it acts on nuclear targets. Only a small percentage of hormone-treated cells exhibited nuclei stained with anti-p-ERK antibody (Fig. 6B, α p-Erk). These Erk-positive cells also exhibited nuclear staining with antibodies against PR (Fig. 6C, α PR). An overlay of double staining showed colocalization of p-Erk and PR (Fig. 6C, merge).

UIII Cells Lack ERα and Their ERβ Does Not Transactivate a Reporter Gene

Next we used Western blot and RT-PCR to analyze the identity of ER in UIII cells. Western blots showed that UIII cells lack of ERα protein (Fig. 7A, *top panel*; see COS7 cells as negative control) and have low levels of ERβ protein (Fig. 7A, *bottom panel*), as compared with MDA-MB-231 cells. In agreement with these results, RT-PCR showed that UIII cells have low levels of ERβ-mRNA, as compared with rat ovary, but are negative for ERα-mRNA (Fig. 7A).

To test the transcriptional competence of endogenous ERβ of UIII cells, we performed transient transfection studies with an ERE-*luc* reporter gene. The reporter gene was not activated by treatment of the transfected cells for 36 h with 10^{-8} M estradiol (Fig. 7C, *white column*), under conditions that activate the same reporter gene in ER-positive breast cancer cell lines (data not shown). The lack of response was not due to limiting factors other than ER, because the ERE-*luc* reporter was induced by estradiol when an expression vector for either ERα or ERβ was cotransfected (Fig. 7C, *black and gray columns*, respectively). We conclude that the levels of endogenous ERβ in UIII cells are insufficient to mediate estradiol activation of a transfected reporter gene.

A Fraction of PR and ERβ Form a Complex in Absence of Hormones

To test whether PR and ERβ form a complex in UIII cells, as found for PR and ERα in breast cancer cells (14), we performed coimmunoprecipitation experiments. Cell lysates were immunoprecipitated with a polyclonal anti-PR antibody and the precipitates were analyzed by Western blots with anti-PR or anti-ERβ antibodies. We found that a small fraction of the ERβ was precipitated with anti-PR antibody (Fig. 8A). Quantitation of the bands showed that 6% of the total ERβ is associated with PR, a figure comparable to that found in breast cancer cells (16).

The existence of a complex of PR and ERβ is consistent with the results of immunofluorescences analysis of the intracellular location of PR and ERβ. The staining of untreated cells with antibodies to PR and to ERβ showed that both receptors colocalized mainly in the cytoplasm of UIII cells (Fig. 8B, *top panels*). Positive and negative controls confirm the specificity of the antibodies (Fig. 8B, *bottom panels*). We conclude that a fraction of the ERβ is associated with PR in the cytoplasm of UIII cells.

DISCUSSION

Serum-Free Progesterone-Dependent Uterine Stromal Cell Proliferation

In this study, we report a progestin-dependent proliferation of UIII rat uterine cell line cultured in serum-free medium. This is one of the novelties of our study because it excludes additional hormones or serum growth factors as necessary components of the pro-

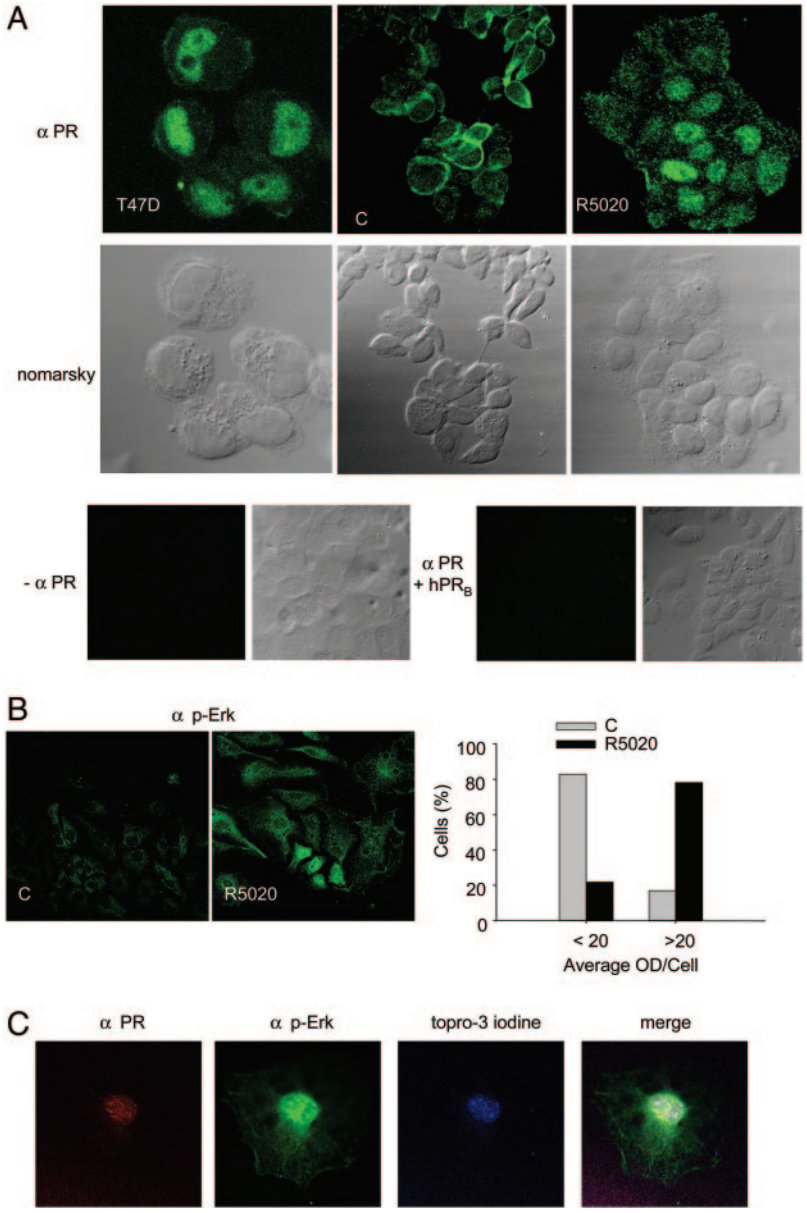

Fig. 6. Immunodetection of PR and Phospho-Erk

A, Identification of PR. UIII cells were plated over coverslips in 35-mm dishes at 1.2×10^5 cells/cm^2 in medium containing 10% DCC/FBS. After 48 h of serum starvation, cells were treated for 30 min with vehicle (C, control), or with 10^{-11} M R5020 (R5020). T47D cells were plated over coverslips under the same conditions and treated with vehicle after 48 h of serum starvation (T47D). Cells were fixed, permeabilized, and incubated with polyclonal antibody to PR (α PR). Fluorescence images were registered by confocal laser microscopy system. Nomarsky images are shown in the *middle panels* to visualize all cells. For negative controls of PR immunocytochemistry (*bottom panels*), UIII cells were treated in the absence of the first antibody (− α PR) or the polyclonal antibody was preincubated with 100-fold molar excess of purified hPR$_B$ (α PR + hPR$_B$). In both cases staining disappeared. B, Levels of Erk1–2 activation. UIII cells were plated as in panel A and treated, after 48 h of serum starvation, with vehicle (C, control) or with 10^{-11} M R5020 (R5020) for 5–10 min. Cells were fixed, permeabilized, and incubated with antibodies to phospho-Erk1–2 (α p-Erk). Fluorescence images were registered with a confocal laser microscopy system, and the optical density per cell was quantitated as described in *Materials and Methods*. The *histogram on the right* shows the percentage of cells with average OD per cell < 20 or >20 arbitrary units after treatment with vehicle (*gray bars*) or with 10^{-11} M R5020 (*black bars*) for 5–10 min. C, Nuclear translocation of PR and Erk1–2. UIII cells were plated as in panel A and treated, after 48 h of serum starvation, with 10^{-11} M R5020 for 5–10 min. Cells were fixed, permeabilized, and incubated with polyclonal antibody to hPR (α PR), or antibodies to phospho-Erk1–2 (α p-Erk), and with To-pro-3 iodine to stain nucleic acids. Fluorescence images were registered with a confocal laser microscopy system.

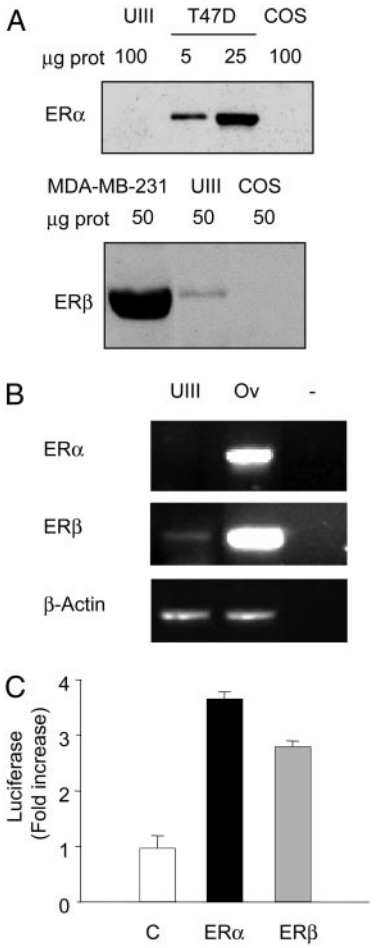

Fig. 7. Characterization of ER in UIII Cells

A (*Top panel*), Western blot analysis using anti-ERα antibodies of extracts from serum-starved UIII cells (UIII), T47D cells (T47D), and COS cells (COS). The amount of protein used is indicated at the *top of each lane*. *Bottom panel*, Western blot analysis using anti-ERβ (27) of extracts from serum-starved UIII cells (UIII) and MDA-MB-231 cells (MDA-MB-231). The amount of protein used is indicated. The positions to which the ERα and ERβ migrate are indicated. B, Expression of ERα and ERβ mRNAs in UIII. Ethidium bromide-stained gels of RT-PCR products for ERα, ERβ, and β-actin from serum-starved UIII cells (UIII) and from rat ovary (Ov). The PCR product performed without template (−) is shown as control. C, The ERβ of UIII is transcriptional incompetent. An ERE-*Luc* reporter plasmid was transiently cotransfected with an expression vector for ERα (*black columns*) or ERβ (*gray columns*), or with the empty plasmid pSG5 (*white columns*). Cells were incubated 36 h with either vehicle or 10^{-8} M 17β-estradiol, and luciferase activity was determined in whole extracts. The fold induction was calculated as the ratio between the values obtained with the estradiol-treated cells and the values of control cells treated with vehicle. The average and SD of two independent experiments performed in duplicate are shown. Prot, Protein.

liferative response to progestins of these endometrial stromal cells. In addition, the results show that UIII endometrial stromal cells do not require signals from epithelial cells to proliferate in response to progestins.

Transient exposure to progestins for 1 h was sufficient for a substantial proliferative response measured 5 d after hormone addition, even when an inhibitor of PR was added after the first hour. This suggests that all events needed for an irreversible commitment to proliferate are triggered by progestins within 60 min. Rapid and transient activation of Erk1–2 and Akt kinases is an essential part of the signaling pathway used by progestins, because inhibition of any of these kinases blocked subsequent cell proliferation. Thus, kinase activation could be involved in the generation of a strong positive feedback loop to produce the irreversibility of progesterone-dependent proliferation. A similar bistable memory module has been described to govern *Xenopus* oocyte maturation (24).

Cross Talk of PR and ERβ in Uterine Stromal Cells

Activation of the kinase cascades and enhanced cell proliferation in response to progestins can be inhibited by the PR antagonist RU486, demonstrating the need for the classical nuclear PR. Moreover, the progestin effect on cell proliferation and on kinase activation can be blocked by the ER antagonist ICI 182.780, thus excluding a participation of the newly described transmembrane ER GPR30 (25). From that we conclude that the classical ER mediates progestin activation of Erk1–2, Akt, and cell proliferation, A similar situation has been described in breast cancer cells, in which progestin activation of Erk1–2 is mediated by a preformed complex of PR_B and ERα (13). The lack of ERα and the presence of ERβ in UIII cells are consistent with the predominance of this isoform in uterine stromal cells.

There are three potential translational start sites at the 5′-end of rat ERβ mRNA, which, if used, would produce proteins 485, 530, and 549 amino acids (aa) in length, differing at their N termini (26). On Western blots with extracts of rat mammary glands using an antibody against the ligand-binding domain of ERβ, three bands were observed with the main bands in the 62-kDa range (27). The extracts from UIII cells yielded a similar main band using the same antibody for the Western blots.

Although ERβ can inhibit ERα-mediated gene transcription, in the absence of ERα, it can partially replace it (28). It has also been reported that ERβ can mediate nongenomic response to estradiol of endothelial cells, and that estradiol signaling via endothelial nitric oxide synthase can occur independent of ERα (29). Thus it is possible that in UIII cells, which lack ERα, the nongenomic response to hormones could be mediated by ERβ. Coimmunoprecipitation experiments showed that a fraction of PR and ERβ form a complex in UIII cells before hormone administration. The potential of ERβ to form a complex with another steroid receptor, the androgen receptor, has been reported using glutathione-*S*-transferase fusion proteins (30). Therefore, it is possible that the cross talk between proges-

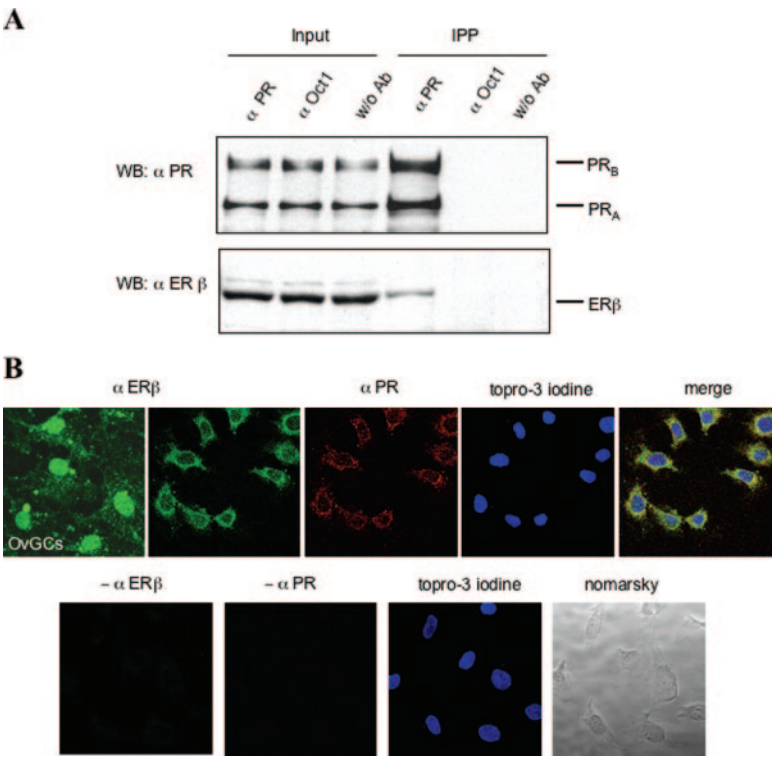

Fig. 8. Interaction between ERβ and PR

A, Coimmunoprecipitation. Cell lysates of serum-starved UIII cells were immunoprecipitated with anti-PR antibody (α PR) or the irrelevant antibody anti-Oct1 (α-Oct1) or without antibody (w/o Ab). The immunoprecipitates (IPP) were removed from the washed beads in SDS sample buffer, electrophoresed (lanes 4–6) along with the cell lysates (input, lanes 1–3), and analyzed by Western blotting with antibodies against PR (*upper panel*) and against ERβ (*lower panel*). ERβ and PR bands are indicated. Lanes 1–3 represent 20% of the amount of lysate used in the immunoprecipitation. B, Immunodetection. *Top panel*, Ovarian granulosa cells (OvGCs), known to be positive for ERβ, and UIII cells were treated as indicated in legend of Fig. 6A and incubated with polyclonal antibody against ERβ (α ERβ), or against hPR (α PR), or with Topro-3 iodine to stain nucleic acids (topro-3 iodine). *Bottom panel*, For negative controls of immunocytochemistry, UIII cells were treated in the absence of the first antibodies (− α ERβ and − α PR). Fluorescence images were registered by a confocal laser microscopy system. WB, Western blot.

tins and estrogens in UIII cells is mediated by a hitherto unreported interaction between PR and ERβ.

Estradiol, on its own, has no effect on cell proliferation under serum-free conditions (data not shown), as previously reported in the presence of serum (18). As compared with progesterone alone, a combination of progestins and estrogens does not enhance UIII cell proliferation, but rather induces decidual differentiation (Vallejo, G., D. Maschi, C. Ballaré, M. Beato, and P. Saragueta, manuscript in preparation). This situation is different from that reported in LNCaP prostate cells, where androgens and estrogens induce cell proliferation (30).

Subcellular Localization of PR and ERβ

In breast cancer cells with high levels of PR, the majority of PR is nuclear before hormonal treatment. In contrast, the immunocytochemical assays showed that the subcellular localization of the small amount of PR in UIII cells was mainly cytoplasmic in the absence of hormone. A large fraction of PR translocated to the nucleus after R5020 treatment, indicating that the fluorescence signal corresponds to an active PR. However, a fraction of PR remained close to the cell membrane even after hormone addition, suggesting a vicinity to the kinase pathways leading to activation of Erk1–2 and Akt (14).

ERβ has been reported to be localized in the cell nucleus before hormone administration in rat uterus (31) In contrast, in UIII cells the majority of ERβ is found in the cytoplasm before hormone addition. We do not know whether this is due to the low levels of ERβ in these cells or whether ERβ is retained in the cytoplasm by its interaction with PR.

Transcriptional Incompetence of Endogenous PR and ERβ

UIII cells with low PR content are reminiscent of uterine stromal cells during metestrus or of endometrial stromal cells from rats depleted of ovarian hormones (6). The levels of PR in UIII cells are indeed so low that they are not sufficient for progestin activation of a

transfected PR responsive gene. However, if the levels of receptor are increased by transient transfection of a PR expression vector, we observed transcriptional activation of a progesterone reporter gene in response to nanomolar progestin concentrations. Similarly, in fibroblasts transfected with low concentrations of AR, no transcriptional activation of a transfected androgen-responsive promoter was observed in response to hormone (32). We conclude that low receptor concentration is sufficient for activation of the kinase pathways by picomolar hormone concentrations, but they are not sufficient for mediating a transcriptional response.

Similar to PR, endogenous ERβ in UIII cells is unable to transactivate a transfected ERE reporter gene. This is not due to lack of coactivators, because cotransfection of an expression vector for ERβ or ERα leads to activation of the reporter in response to estrogens. The inability of endogenous ERβ to mediate transactivation may be due to its low cellular levels or to its interaction with PR in a complex that is transcriptionally incompetent. The nuclear translocation of PR after progestin treatment shown in Fig. 6 does not invalidate this argument because it is a prerequisite but not sufficient for gene activation. For instance, in several cell types RU486 induces nuclear translocation of PR and GR without leading to transcriptional activation of target genes (33, 34).

The mechanism that maintains low levels of the ERβ-PR complex in the cells remains to be established, but it is clear that a fraction of the transfected ER escapes this type of control.

Optimal Response at Picomolar Concentrations of R5020

The proliferative response of UIII cells reaches a maximum at picomolar progestin concentrations (10^{-10} to 10^{-1} M), which is also the optimal concentration for activation of Erk1–2 and Akt kinase activity. This finding is in apparent contradiction with the measured dissociation constant (K_d) of PR for R5020, which lies in the nanomolar range. Similarly, fibroblasts transfected with low amounts of an androgen receptor expression vector exhibit hypersensitivity to low doses of androgen in terms of androgen-dependent Erk1–2 activation (32).

A recent study in mice uterus has revealed the presence of high-affinity, low-capacity, progesterone binding sites (K_d for R5020, 43 ± 9 pM) in addition to the classical PR (K_d 9.2 ± 4.2 nM) (35). The high-affinity sites represent about 3% of the total progesterone binding capacity. As we have shown that less than 5% of the total PR in breast cancer cells exists in a complex with ER (16), it is possible that the PR in this complex exhibits a higher affinity for progestins. If this were the case, low concentrations of progesterone could activate the Erk pathway acting through this high-affinity receptor complex.

Suboptimal Effect at Nanomolar Concentrations of R5020

An unexpected finding is that nanomolar concentrations of R5020 have a less pronounced effect on cell proliferation and kinase activation than picomolar concentrations. One possible explanation would be that nanomolar concentrations of progestins favor PR dimerization and thus reduce the amount of PR in the monomeric conformation required for interaction with ER and kinase activation. Kinetic analysis of estradiol receptor homodimerization showed that formation of the receptor homodimer *in vitro* is markedly dependent upon the receptor concentration (36) and is promoted by hormone binding (37). Moreover, receptor homodimerization is required for nuclear translocation and ligand-dependent transcriptional activation *in vivo* (38). Therefore, differential tissue responses in different cells may be obtained depending on their steroid receptor content. T47D cells, the intracellular PR concentration of which is in the nanomolar range (19), may form a substantial amount of the activated dimer receptor in the absence of ligand, and respond to nanomolar concentrations of progestins with optimal nuclear translocation and transcriptional activation. In contrast, in UIII cells, with subnanomolar concentrations of PR, the majority of the receptor will be in the monomeric form in the absence of ligand and will localized to the cytoplasm, poised for cross talk with kinase cascades. We will test these hypotheses by constructing cell lines with different amounts of epitope-tagged PR and ER, allowing detection and biochemical analysis of minute amounts of the different forms of the receptor and their associated proteins.

Our results suggest that the proliferative response of uterine stromal cells to picomolar concentrations of progestins does not require direct transcriptional effects. A progestin response via integration of rapid intracellular signaling and PR that functions as a ligand-activated transcription factor (39) has been recently reported for gene regulation and proliferation in breast cancer cells. The UIII cell line is a useful model to study the effects mediated by the rapid signaling component.

The relevance of the cross talk between steroid hormones and signaling cascades for activation of cell proliferation in cells with very low receptor concentrations force us to reconsider our definition of progesterone target cells. By many classical criteria, including Western blot, steroid binding assays, and conventional immunocytochemistry, UIII cells would have been considered PR negative because their levels of PR are 1 order of magnitude lower than in classical breast cancer model cell lines. Nevertheless they exhibit a clear dependence on progesterone for growth and differentiation (Vallejo, G., D. Maschi, C. Ballaré, M. Beato, and P. Saragueta, manuscript in preparation) and are sensitive to very low concentrations of hormone. Thus, more accurate and sensitive

methods are needed to estimate the hormone receptor content of cells, and the spectrum of target cells for various steroid hormones will have to be expanded. This is especially important given the eminent physiological relevance of the nongenomic effects of steroid hormones, which are measurable in a whole variety of cell types not belonging to the traditional target tissues of these hormones (12, 13).

MATERIALS AND METHODS

Materials

Progesterone, epidermal growth factor, RU486, and wortmannin were from Sigma Chemical Co. (St. Louis, MO); FBS was purchased from Bioser (Buenos Aires, Argentina); R5020 was obtained from PerkinElmer (Norwalk, CT); ICI182.780 was from Tocris (Bristol, UK); PD98.059 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); and M199 and antibiotics were from Life Technologies (Gaithersburg, MD). Antibodies were as follows: monoclonal E10 against Erk1–2 phosphorylated at Thr202/Tyr204, polyclonal against Akt phosphorylated at Thr308, and polyclonal-anti Akt were from Cell Signaling Technology (Beverly, MA); polyclonal C14 anti-ERK 2, polyclonal C20 anti-hPR, polyclonal H190 anti-hPR and polyclonal HC-20 anti-ERα were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); polyclonal anti-ERβ DNA-binding domain and polyclonal anti-ERβ 503 were gifts of Jan-Ake Gustafsson. Antibodies To-pro-3 iodine and secondary antibodies, ALEXA 488 goat antirabbit, ALEXA 488 goat antimouse, and ALEXA 633 goat antimouse, were from Molecular Probes (Leiden, The Netherlands).

Plasmids

The pAGEMMTVLu (MMTV-Luc) plasmid, carrying the MMTV promoter linked to the firefly luciferase (*luc*) gene, PRE2tk-CAT carrying two synthetic PRE oligonucleotides cloned upstream of the thymidine kinase promoter-CAT hybrid gene, and P4Bcl-X-Luc encompassing nucleotides −3288 to −2652 from Bcl-X promoter in pGAW.Luc were described previously (20, 21, 40). pSG5:PR encoding the human PR_B, and pSG5:ERα (HEG0) encoding the human ERα were a gift from Pierre Chambon (41). pCXN2-ERβ encoding the human ERβ was a gift from Geoffrey Greene. HSD11β2-Luc encompassing nucleotides −1778 to +117 of human 11β-hydroxy steroid dehydrogenase type II promoter was cloned upstream of Luc gene in pGL3-Basic (22).

Cell Culture and Cell Proliferation Experiments

UIII rat normal uterine stromal cells were maintained in M199 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 C in humidified 95% air with 5% CO_2 (18). Culture media were changed every 2 d.

For hormone treatment experiments in the presence of serum, cells were first cultured in M199 supplemented with 10% dextran-coated charcoal-treated FBS (DCC/FBS). At the beginning of the treatment, media were replaced by fresh DCC/FBS medium with vehicle (ethanol) as control or with hormones. Viable cells were counted after 5 d of culture using the 0.1% trypan blue exclusion method.

For hormone treatment experiments in the absence of serum, cells were cultured in DCC/FBS, and 48 h later, media were replaced by fresh M199 without serum. After 2 d in serum-free conditions, media were replaced by fresh media with either vehicle or hormones. The effects of antagonists or inhibitors were tested in cells pretreated with antagonists, inhibitors, or vehicle during 30 min before hormone treatment. The cell numbers were determined after 5 d of culture in medium without serum.

For studying short-term (1-h) progesterone effect, media containing the hormone were removed after 1-h progesterone treatment, and cells were carefully washed six times (10 min each) with hormone-free medium. After the washings, media were changed every 2 d. The number of cells was determined after 120 h of treatment.

Flow Cytometry

To analyze cell cycle response to progestins, cells at 70–80% of confluence were incubated for 48 h in serum-free media and then treated for 24 h with 10^{-8} M R5020. After treatments, cells were trypsinized, washed, transferred to 15-ml tubes containing 0.9 ml PBS (1×10^6 cells per tube), and collected by centrifugation. The cell pellets were resuspended and incubated for 30 min at 37 C in 1 ml of freshly prepared analysis solution, containing 5 μg/ml Hoechst 33342. One million cells per time point and treatment were analyzed on a LSR detector flow cytometer (Becton Dickinson and Co., San Jose, CA). Fluorescent data were collected and DNA histograms were analyzed with the ModFit 3.0 software from Verity Software House, Inc. (Topham, ME).

Western Blots

SDS-PAGE and immunoblot analyses were used to identify the Erk1–2 and Akt proteins. Cell lysates were prepared in 1% sodium dodecyl sulfate, 25 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA plus protease and phosphatase inhibitors, and analyzed by Western blots with the antibodies described above. The band intensities were measured with ImageQuant 3.3 program (Amersham Pharmacia Biotech, Arlington Heights, IL).

Transient Transfections and Luciferase Activity

UIII cells were transiently transfected with the Lipofectamine Plus reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

For studying the transcriptional activity of PR, the MMTV-Luc plasmid (40) carrying the MMTV promoter linked to the luciferase reporter gene was cotransfected together with the PR_B expression vector pSG5:PR or the empty plasmid pSG5. RSV:βGal plasmid was also included in each transfection to normalize transfection efficiency. After transfection, cells were treated for 36 h with R5020 (from 10^{-8} to 10^{-11} M), or 10^{-8} M R5020 plus 10^{-6} M RU486, or 10^{-6} MRU486 or ethanol. Additional transfection experiments were also performed with PRE2tk-CAT, P4Bcl-X-Luc, and HSD11β2-Luc (20, 21, 22) cotransfected together with the PR_B expression vector pSG5:PR or the empty plasmid pSG5. After transfection, cells were treated for 36 h with R5020 (10^{-8} M) or ethanol.

For studying the transcriptional activity of ERα and β, the ERE-Luc plasmid (42) containing the luciferase reporter gene downstream from a minimum β-globin promoter and two estrogen-responsive elements was cotransfected together with the ERα or ERβ expression vector or the empty plasmid. RSV:βGal plasmid was also included in each transfection to normalize transfection efficiency. After transfection, cells were treated for 36 h with estradiol (10^{-8} M), alone or in combination with ICI182780 (10^{-6} M), or with ethanol.

Cell lysates were prepared and luciferase and β-galactosidase activities were determined with assay kits from Promega Corp. (Madison, WI), according to manufacturer's in-

structions. To correct for differences in transfection efficiencies, luciferase units were normalized for β-galactosidase activities. For detecting the transcriptional activity of PRE2tk-CAT, total RNA was isolated and the level of CAT mRNA was measured by semiquantitative RT-PCR using as forward primer: 5′-GCC TTT ATT CAC ATT CTT GC-3′ and as reverse primer: 5′-CAG GTT TTC ACC GTA ACA CGC-3′. PCR amplification reaction was carried out within the exponential range (28 cycles of 1 min at 52 C for CAT). Relative expression of CAT mRNA were normalized by β-actin mRNA content as described below.

RNA Extraction and RT-PCR

Total RNA isolation was performed according to the guanidinium thiocyanate-phenol-chloroform extraction single-step method (43). To determine hormone receptors' mRNA expression, cDNA was synthesized from equal amounts of total RNA (2.5 μg) with Superscript Reverse Transcriptase (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen), and amplified with Taq DNA polymerase (Invitrogen). The following primers were used: PR forward primer: 5′-CCC ACA GGA GTT TGT CAA GC-3′; PR reverse primer: 5′-TAA CTT CAG ACA TCA TTT C-3′; ERα forward primer: 5′-AAT TCT GAC AAT CGA CGC CAG-3′; reverse primer: 5′-GTG CTT CAA CAT TCT CCC TCC TC-3′; ERβ forward primer: 5′-GTC CTG CTG TGA TGA ACT AC, reverse primer: 5′-CCC TCT TTG CGT TTG GAC TA-3′; β-actin forward primer: 5′-GAA GGC CCC TCT GAA CCC CAA-3′; β-actin reverse primer: 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′. PCR amplification reaction was carried out within the exponential range (35 cycles of 1 min at 50 C for PR, 35 cycles of 1 min at 56 C for ERα and ERβ, and 24 cycles of 1 min at 62 C for β-actin). All primers used are specific for rat steroid receptors. In all cases the receptor mRNAs were compared with mRNAs from rat tissues.

All amplification products were routinely checked by gel electrophoresis on a 1.3% agarose gel and then visualized under UV light after staining with 0.05% ethidium bromide to confirm the size of the DNA fragment and that only one product was formed.

Real-Time PCR

Amplification mixtures were loaded into LightCycler glass capillary tubes containing 1 μl of cDNA, 2 μl of 10× LightCycler FastStart SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), 0.3 mM $MgCl_2$ solution, 0.2 μM of PR oligonucleotide forward primer, 0.2 μM of PR oligonucleotide reverse primer, and water to a final volume of 20 μl. PCR program was performed on the LightCycler (Roche Molecular Biochemicals) with an initial cycle of denaturation at 95 C for 300 sec, followed by a 55-cycle program consisting of heating at 20 C/sec to 95 C with a 2-sec hold, cooling at 20 C/sec to 50 C with a 10-sec hold, heating at 20 C/sec to 72 C with a 13-sec hold, and heating at 20 C/sec to 79 C with a 2-sec hold (single acquisition mode). The authenticity of the PCR products was verified by melting curve analyses and agarose gel electrophoresis. Fluorescent values of each capillary were measured at 530 nm. Crossing points were calculated with the fit point method (LightCycler software 3.5.3; Roche Molecular Biochemicals). Assuming that during the log-linear phase the efficiency is constant and that the fluorescence is proportional to the amount of template, these intensities were used to calculate the initial amount of target.

Coimmunoprecipitation of ERβ and PR

For the coimmunoprecipitation assay, cells were plated in M199 medium supplemented with 10% DCC/FBS and 48 h later medium was replaced by fresh M199 without serum. After 1 d in serum-free conditions, cells were lysed in 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 5 mM $MgCl_2$; 1% Triton X-100; 0.1% sodium deoxycholate plus protease and phosphatase inhibitors. Immunoprecipitation was conducted as previously described with some modifications (16). Briefly, cell extracts (2 mg/ml for each immunoprecipitation) were precleared 2 h with 30 μl antirabbit IgG beads (eBioscience, San Diego, CA). After centrifugation, aliquots of the supernatants were removed for later analysis (inputs), and the remaining supernatants were incubated overnight at 4 C with 6 μg anti-PR rabbit polyclonal antibody (H190, Santa Cruz), or with 6 μg of an unspecific control antibody (anti-Oct1 rabbit polyclonal antibody) or without antibody, followed by incubation with 60 μl antirabbit IgG beads for 2 h. Samples were centrifuged and the pellets were washed four times with washing buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 5 mM $MgCl_2$; 1% Triton X-100 plus protease and phosphatase inhibitors) and once with PBS plus protease and phosphatase inhibitors. The immunoprecipitated proteins were eluted from the beads by boiling in sodium dodecyl sulfate sample buffer. Inputs and immunoprecipitated proteins were analyzed for PR and ERβ by Western blot using anti-PR rabbit polyclonal antibody (H190, Santa Cruz) and polyclonal anti-ERβ DNA-binding domain, respectively.

Immunofluorescences

Cells were seeded onto coverslips in six-well multidishes as described above for hormone treatment experiments in the absence of serum. After 30 min of treatment with ethanol or 10^{-11} M R5020, cells were washed, fixed by incubation in 3% paraformaldehyde in 0.1% Tween-PBS for 5 min in ice, and permeabilized by incubation in 0.1% Triton X-100 for 10–15 min at room temperature. After rinsing three times for 5 min in 0.1% Tween-PBS, the coverslips were incubated for 2 h with 10% BSA in 0.1% Tween-PBS to reduce nonspecific staining. To detect PR, cells were incubated with polyclonal antibody (C20) from Santa Cruz Biotechnology, diluted 1:100 in 10% BSA 0.1%Tween-PBS at 4 C overnight. After several washes in Tween-PBS, coverslips were exposed to secondary antibody ALEXA 488 goat antirabbit (Molecular Probes), diluted 1:1000 in 0.1% Tween-PBS for 1 h at room temperature. Coverslips were mounted on slides with VectaShield mounting medium (Vector Laboratories, Burlingame, CA) and subjected to Leica DM IRBE inverted research microscope and Leica TCS SP2 spectral confocal module with a HCX PL APO 63× /1.32 Oil Ph3 CS objective (Leica Microsystems, Nussloch, Germany). Samples were excited at 488 nm and analyzed at an emission of 515 nm. Serial z-axis with a 40-nm resolution sections were collected as digitized fluorescent images.

For colocalization of phospho-Erk and PR immunofluorescence, the primary antibodies were diluted 1:400 for monoclonal antiphospho-Erk1–2 and 1:100 for C20 polyclonal antihPR in 10% BSA 0.1% Tween-PBS, and incubation was at 4 C overnight followed by a mixture of secondary antibodies from Molecular Probes: ALEXA 488 goat antimouse and ALEXA 633 goat antirabbit, both diluted 1:1000 in 0.1% Tween-PBS for 1 h at room temperature.

For colocalization of PR and ERβ immunofluorescence the primary antibodies, anti-PR rabbit polyclonal antibody (H190, Santa Cruz) and anti-ERβ 503 chicken polyclonal antibody, respectively, were diluted 1:100 in 10% BSA 0.1% Tween-PBS, and incubation was at 4 C overnight followed by a mixture of secondary antibodies, fluorescein isothiocyanate rabbit antichicken (Sigma) diluted 1:32 and ALEXA 633 goat antirabbit (Molecular Probes), both diluted 1:1000 in 0.1% Tween-PBS for 1 h at room temperature.

Nuclei were counterstained with To-pro-3 iodine in all cases (Molecular Probes). Samples were excited at 488, 632, and 645 nm and analyzed at an emission of 515, 647, and 661 nm, respectively. Serial z-axis with a 40-nm resolution sec-

tions were collected as digitized fluorescent images. Images were analyzed using Leica Confocal Software TCS SP: 3D, Physiology and Multicolor software (Leica Microsystems). Bright-field images (nonconfocal images) were recorded with a transmitted light detector (Leica Microsystems). Images for figures were resized with Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA).

Statistical Analysis

Results from progesterone's effect on the UIII cell proliferation experiment were analyzed with Kruskal-Wallis nonparametric test and Dunn's *t* multiple-comparisons test. Individual comparisons within a given group of treatments from all other experiments were made, using an ANOVA and Dunnett multiple-comparisons test. Differences were considered significant if $P < 0.05$.

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