Biphasic Regulation of Breast Cancer Cell Growth by Progesterone: Role of the Cyclin-Dependent Kinase Inhibitors, p21 and $p27^{Kip1}$

Steve D. Groshong, Gareth I. Owen, Bryn Grimison, Irene E. Schauer, Maria C. Todd, Thomas A. Langan Robert A. Sclafani, Carol A. Lange, and Kathryn B. Horwitz

University of Colorado Health Sciences Center
Departments of Medicine and Pathology (G.I.O., C.A.L., K.B.H.)
Department of Biochemistry, Biophysics and Genetics (I.E.S., R.A.S.)
Department of Pharmacology (M.C.T., T.A.L.)
The Molecular Biology Program (S.D.G., B.G., T.A.L., R.A.S., K.B.H.)
Denver, Colorado 80262

Depending on the tissue, progesterone is classified as a proliferative or a differentiative hormone. To explain this paradox, and to simplify analysis of its effects, we used a breast cancer cell line (T47D-YB) that constitutively expresses the B isoform of progesterone receptors. These cells are resistant to the proliferative effects of epidermal growth factor (EGF). Progesterone treatment accelerates T47D-YB cells through the first mitotic cell cycle, but arrests them in late G1 of the second cycle. This arrest is accompanied by decreased levels of cyclins D1, D3, and E, disappearance of cyclins A and B, and sequential induction of the cyclin-dependent kinase (cdk) inhibitors p21 and $p27^{Kip1}$. The retinoblastoma protein is hypophosphorylated and extensively down-regulated. The activity of the cell cycle-dependent protein kinase, cdk2, is regulated biphasically by progesterone: it increases initially, then decreases. This is consistent with the biphasic proliferative increase followed by arrest produced by one pulse of progesterone. A second treatment with progesterone cannot restart proliferation despite adequate levels of transcriptionally competent PR. Instead, a second progesterone dose delays the fall of p21 and enhances the rise of $p27^{Kip1}$, thereby intensifying the progesterone resistance in an autoinhibitory loop. However, during the progesterone-induced arrest, the cell cycling machinery is poised to restart. The first dose of progesterone increases the levels of EGF receptors and transiently sensitizes the cells to the proliferative effects of EGF. We conclude that progesterone is neither inherently proliferative nor antiproliferative, but that it is capable of stimulating or inhibiting cell growth depending on whether treatment is transient or continuous. We also suggest that the G1 arrest after progesterone treatment is accompanied by cellular changes that permit other, possibly tissue-specific, factors to influence the final proliferative or differentiative state. (Molecular Endocrinology 11: 1593–1607, 1997)

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INTRODUCTION

Progesterone is involved in the development, growth, and differentiation of the breast and breast cancers (1, 2), and presence of progesterone receptors (PR) identifies tumors likely to be hormone-dependent (3) and patients likely to have a favorable disease prognosis (4). Mice lacking PR exhibit incomplete mammary gland ductal branching and failure of lobulo-alveolar development (5). This phenotype is strikingly similar to that of mice lacking cyclin D1 (6). These and other studies (7–9) suggest important functional links among progesterone, cyclin D1, and breast cancer and implicate the mitotic cell cycle in progesterone-dependent differentiation of the breast.

Opposing views that progesterone is a proliferative hormone in the breast are currently reflected in clinical practice (10–12). Progestins are added to estrogens for hormone replacement therapy at menopause because they block the proliferative and tumorigenic effects of unopposed estrogens in the uterus. However, women who have been hysterectomized are not given progestins, to spare their breasts from the presumed proliferative effects of these hormones (13–15). This is defended by the prevailing notion that progesterone is

differentiative in the uterus but proliferative in the breast (1, 2).

It is now clear that control of proliferation and differentiation by many hormones and growth factors is linked by events that occur in G1 of the cell cycle (16–19). Recent studies implicate up-regulation of the cyclin-dependent kinase (cdk) inhibitors, particularly p21 ($p21^{Kin1,Waf1,Sdi1}$) (20), not only in inhibiting cell proliferation, but in promoting differentiation. In contrast, overexpression of cyclin D1 inhibits the differentiative program (21) and, in the breast, promotes cellular hyperplasia and tumor formation (9).

The molecular mechanisms underlying the proliferative and differentiative effects of progesterone at its target tissues have been difficult to assess for several reasons (2, 6). First, in most progesterone target cells the levels of PR are regulated by estradiol (22). Therefore, obligatory pretreatment with estradiol, itself a potent proliferative agent (23), confounds assessment of progesterone's role on growth and other cellular processes. Second, as Musgrove *et al*. (24) have shown, short-term progestin treatments have dual effects on the cell cycle: they inhibit reentry of cells from mitosis into G1, but stimulate progression of cells through G1 (24). This complicates analysis of the role of progesterone in regulating proliferation acutely. The mechanisms underlying its sustained effects are unknown. Third, progesterone target tissues contain two isoforms of PR, the 120-kDa B receptors and the N-terminally truncated 94-kDa A receptors (25), that have unequal transcriptional activities (26–28). Additionally, the two isoforms are dissimilarly regulated and expressed during development, after hormone treatments, and in different target tissues and tumors (29–32).

Our approach to reconciling these complexities, which limit studies of progesterone actions, has been to construct simpler model systems. To that end, we have used the $T47D_{CO}$ human breast cancer cell line, in which the two PR isoforms have escaped from estrogen controls and are constitutively expressed (33), to isolate the effects of progesterone and eliminate the confounding effects of estradiol. To define the role of each receptor isoform, we recently isolated and subcloned a PR-negative subline of $T47D_{CO}$ called T47D-Y and used it as the recipient into which either the B- or A isoform of PR was stably reintroduced to produce T47D-YB and T47D-YA cells (34).

In the present study, we used T47D-YB cells to analyze the effects of acute and sustained progesterone on the factors that regulate cell cycle progression and integrate growth-regulatory signals. This includes measurement of the protein levels of key cyclins (35–39); the levels and phosphorylation state of the retinoblastoma (Rb) tumor suppressor (40); the levels of cdk inhibitors (36, 41); and the kinase activity of cdk2 (17, 35, 37–39). Since T47D-YB cells are resistant to the proliferative effects of epidermal growth factor (EGF), we analyzed the role of cross-talk between the progesterone- and EGF-signaling pathways, on these proliferative events.

We find that after stimulating one round of cell division, a single pulse of progesterone arrests T47D-YB cells in late G1 of the second cycle, by sequentially raising the levels, first of p21 and then of $p27^{Kip1}$. This is accompanied by an induction followed by inhibition of cdk2 activity. A second progesterone dose augments the growth arrest. However, during the arrested state, the cells become responsive to the proliferative effects of EGF, which can cause them to resume cycling. This occurs only after a single progesterone pulse; a second progesterone dose delays the p21 fall and blocks EGF responsiveness. We propose that progesterone is neither inherently proliferative nor antiproliferative, but that its effects on growth depend on whether treatment is transient or continuous; the former is stimulatory and the latter inhibitory. Additionally, cell cycle arrest in G1 may be accompanied by progesterone-induced cellular changes that can be permissive for growth-stimulatory effects by other factors.

RESULTS

Progesterone Treatment Leads to Growth Resistance Despite Functional PR

To study the proliferative effects of progestins, parallel sets of PR-negative Y cells (Fig. 1, panel A), A receptor-containing YA cells (panels B and D), and B receptor-containing YB cells (panels C and E), were grown in control medium or in medium containing the following additions: the synthetic progestin agonist R5020 alone; the antiprogestins RU486 or ZK98299; or R5020 plus one of the antiprogestins. Sets of cells were harvested every 4 h for 48 h, and the percent in $S+G_2/M$ for each treatment group was determined by flow cytometry. Optimal concentrations for each hormone were determined in preliminary studies. As shown in Fig. 1, panel A, the percent of PR-negative Y cells in $S+G_2/M$ during 50 h of treatment with 30 nM R5020, 100 nM RU486, or 1 μM ZK98299 is no different than untreated controls, supporting the idea that proliferative responses to progestins and antiprogestins are dependent on the presence of PR and ruling out any effects through glucocorticoid receptors. These cells are also unresponsive to estradiol (not shown) and EGF (see below). Compared with controls (set at 0), in either A receptor (panels B and D) or B receptor (panels C and E) containing cell lines, the percent of cells in $S+G_2/M$ starts to climb approximately 12 h after R5020 is added to the medium, peaks at 20–24 h, and returns to basal levels approximately 36 h later, as previously reported (24). During this transient increase in mitotic activity, the percent of cells in $S+G_2/M$ rises from basal levels of approximately 15–20% in the untreated controls, to 45–50% in the R5020-treated sets. The two antiprogestins, RU486 or ZK98299, when each is given alone, consistently have a transient

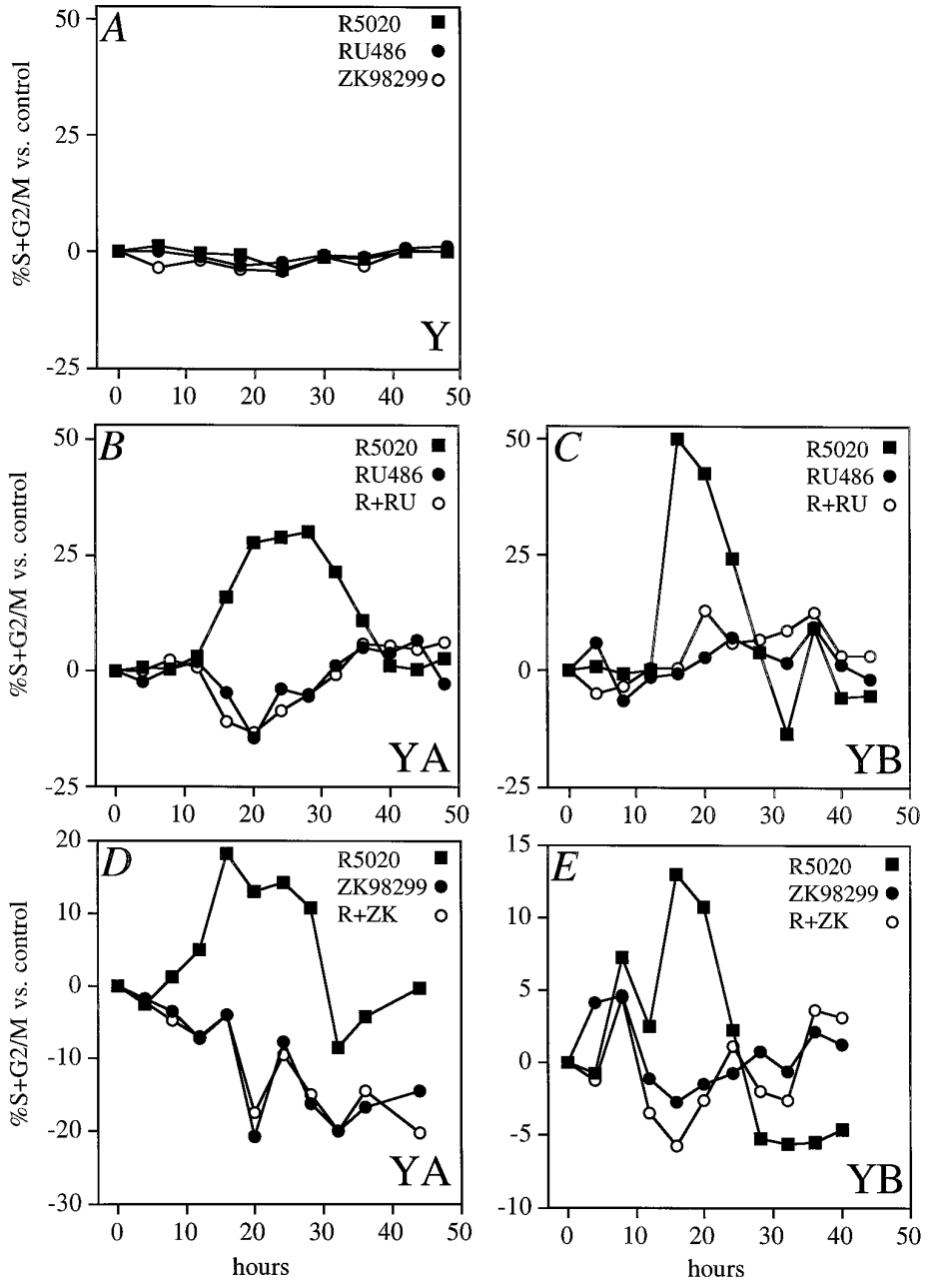

Fig. 1. The Synthetic Progestin R5020 Induces a Proliferative Burst in PR-Positive Cells That Is Inhibited by Antiprogestins

A, PR-negative T47D-Y cells were treated with the agonist R5020 (■) or the antiprogestins RU486 (●) or ZK98299 (○) for 48 h. Cells were harvested every 6 h, and the percent of cells in $S+G_2/M$ was measured by flow cytometry. B, T47D-Y cells stably expressing A receptors (YA) were treated with R5020 (■), RU486 (●), or both (○), and cells in $S+G_2/M$ were measured by flow cytometry over a 48-h period. C, T47D cells stably expressing B receptors (YB) were treated with R5020 (■), RU486 (●), or both (○). D, Same as panel B except that ZK98299 was the antiprogestin. E, Same as panel C except that ZK98299 was the antiprogestin. Change in percent of cells in $S+G_2/M$ is compared with controls, set at zero. In this study approximately 15–20% of control cells were in $S+G_2/M$.

growth-suppressive effect that is more pronounced in the YA than in the YB cells. However, in both cell lines, neither antagonist, when present alone, has long-term growth-suppressive effects (data not shown). In either cell line, the two antiprogestins completely block the R5020-induced proliferative burst, suggesting again that growth effects of progestins are PR-dependent. Since the two PR-containing cell lines did not differ significantly, the remaining studies described here used T47D-YB cells. Control of proliferation by antiprogestins in the presence of cAMP does exhibit PR isoform specificity (K. Horwitz, in preparation).

Although R5020 is known to be poorly metabolized in $T47D_{CO}$ cells (42), it was theoretically possible that

the transitory nature of the proliferative increase seen in Fig. 1 was due to degradation of the hormone during the 40 or more hours of cell culture. To determine whether additional hormone would produce a sustained rise in proliferation, T47D-YB cells were treated with R5020 at time 0, then given a second pulse of R5020 at 48 h, and the percent of cells in different phases of the cell cycle was measured for 96 h (Fig. 2A). Surprisingly, while the initial proliferative rise at 20 h was clearly evident, the cells were completely refractory to the second hormone challenge.

We postulated that the resistance to additional R5020 at 40 h was due to receptor down-regulation produced by the continuous exposure to hormone. Indeed, while untreated control cells have high levels of PR B receptors as measured by immunoblotting (Fig. 2B), 10 h after the start of R5020 treatment, B receptors are barely detectable. If the cell culture medium is not changed, there is little recovery of receptors for at least 50 h. However, as shown in Fig. 2B, after progesterone treatment, B receptor down-regulation is transitory: receptor levels are depressed 10 h after the hormone addition, but they rapidly replenish to control levels because this natural ligand is metabolized in $T47D_{CO}$ cells with a half-life of 2–4 h (42). We therefore postulated that T47D-YB cells are refractory to a second hormone challenge after initial treatment with R5020 (Fig. 2A) because PR are still down-regulated at 40 h, and we reasoned that if the cells were initially treated with progesterone, their progestin sensitivity would be restored at 40 h. Figure 2A shows that this is not the case. Like R5020, progesterone produces a transient increase in cell proliferation. Nevertheless, 40 h after the initial progesterone-induced growth, and despite adequate levels of receptors (Fig. 2B), the cells are completely refractory to subsequent treatment with R5020.

This result was surprising, and we wondered whether the replenished receptors were somehow functionally incompetent (Fig. 3). To test this, we measured the ability of replenished receptors to activate transcription of chloramphenicol acetyl transferase (CAT) driven by a PR-responsive promoter. Parallel sets of YB cells were pretreated with progesterone at zero time. Twenty-four hours later a subset of cells was transfected with the PRE_2-$TATA_{tk}$-CAT reporter, in which the proximal promoter of the thymidine kinase gene is controlled by two upstream progesterone response elements (PRE). The cells were glycerol shocked at 46 h to complete the transfection and immediately treated with a second dose of either progesterone or R5020 for an additional 24 (Fig. 3, *inset*)

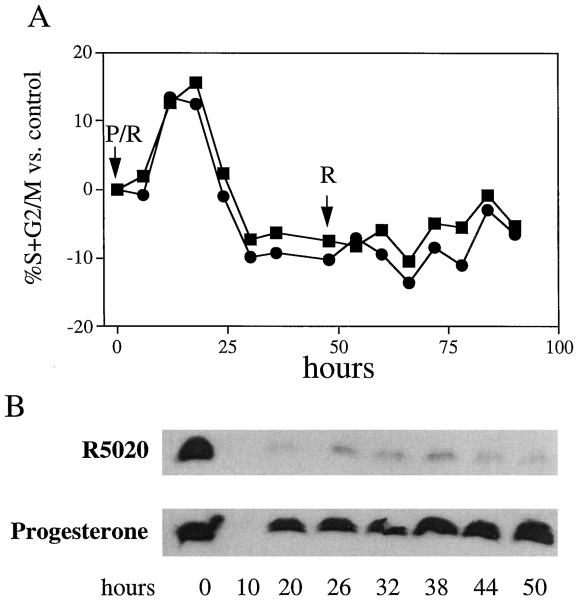

Fig. 2. After the Initial Proliferative Burst, T47D-YB Cells Are Resistant to Another Progestin Challenge Despite Adequate PR Levels

A, T47D-YB cells were untreated or were treated with progesterone (■) or R5020 (●) at 0 time, and again with R5020, 48 h later. Cells were harvested every 4–6 h for 96 h. Percent cells in $S+G_2/M$ were measured by flow cytometry. Changes in the percent $S+G_2/M$ of hormone-treated cells are compared with untreated controls, which were set at 0. B, T47D-YB cells were treated with R5020 or progesterone at 0 time and harvested periodically for 50 h. B receptor levels were measured by immunoblotting with anti-PR antibodies; hormone-untreated controls are shown at time 0.

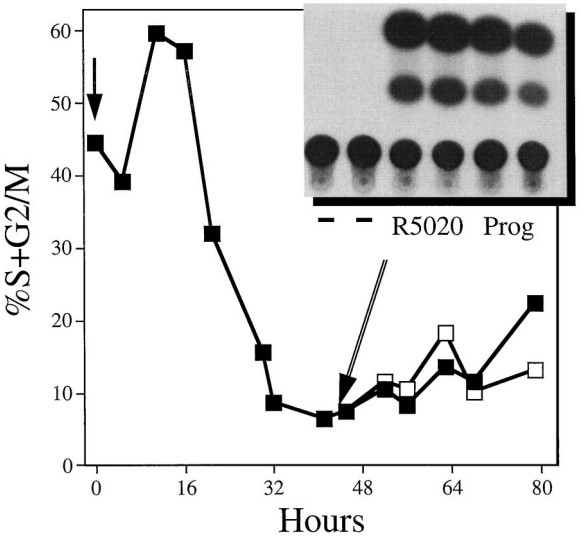

Fig. 3. T47D-YB Cells Resistant to the Proliferative Effects of Progestins Contain Transcriptionally Competent and Hormone-Responsive PR

T47D-YB cells were treated with progesterone at time 0 and retreated with progesterone at 40 h (□), or left untreated (■). Cells were harvested every 4–6 h for 80 h, and the percent of cells in $S+G_2/M$ was measured by flow cytometry. *Inset*, YB cells treated with progesterone at time 0 were transfected with the PRE_2-$TATA_{tk}$-CAT reporter at 46 h and left untreated (−) or treated with R5020 or progesterone. Cells were harvested 24 h later, lysates were prepared, normalized to β-galactosidase activity, and CAT activity was measured by TLC. Duplicates from separate transfections are shown.

or 48 h (not shown) before they were harvested, and CAT activity was measured in cell lysates. A control set of transfected cells was left untreated at 40 h (Fig. 3, *inset*). Additionally, parallel sets of untransfected cultures were treated with a second pulse of R5020 at 40 h, and the cells were harvested periodically over the next 48 h for analysis of cell cycle phases by flow cytometry (Fig. 3). As shown above, after the initial progesterone-induced proliferative burst, the cells enter a period of growth arrest, lasting at least 80 h, from which they cannot be rescued by retreatment with R5020 at 40 h. However as the *inset* (Fig. 3) shows, this inhibition is not due to incompetent receptors, since they can induce CAT transcription in the same endocrine setting. Thus, despite progesterone pretreatment at time zero, at 40 h R5020 and progesterone strongly induce transcription from the PRE-$TATA_{tk}$-CAT reporter. The control cells that received no second hormone dose confirm that 40 h after the initial progesterone dose, insufficient hormone remains in either the cells or the medium from the primary treatment to transactivate the promoter, so that the high levels of CAT activity seen in the 40-h R5020- and progesterone treated sets must be due to the second dose. We conclude that the replenished receptors are fully functional: they are capable of binding ligand, of binding DNA at cognate PREs, and of interacting with the requisite factors on a promoter to activate transcription (43).

Progesterone Produces G1 Phase Arrest by Up-Regulating p21 and $p27^{Kip1}$ and Inhibiting cdk2 Activity

Since recent studies suggest links among progesterone, breast cancer, and the cell cycle (Refs. 5–9 and others), we measured the protein levels of cell cycle-regulatory proteins during the dual progestin treatment regimen, in an attempt to explain the progesterone resistance. Figure 4 shows the changes in protein levels over a 70-h period, of cyclins D1, D3, and E, of the inhibitors p21 and $p27^{Kip1}$, and of Rb. These proteins regulate progression of cells through G1 (36, 39). The cells were treated with progesterone at time zero, followed 40 h later by no treatment or by R5020. The cells were harvested periodically, and aliquots of cell lysates were assayed for the cell cycle proteins by immunoblotting, and for the percent of cells in $S+G_2/M$ by flow cytometry. In Fig. 4, the flow cytometric data are shown by the *dashed line*, since they represent the same data points that are shown in detail in Fig. 3. After primary treatment with progesterone, the levels of cyclin D1 rise transiently and then fall as previously reported (24), coincident with the increased proliferative activity observed in the first 24 h. D1 expression increases within 2 h of progesterone addition (not shown). A second abortive peak is observed between 25–40 h, and levels then fall again after 40 h. This second peak is not always observed; it is markedly attenuated if the basal cell proliferation rate is relatively low. Note that the study shown in Fig. 4 involves cells that have a rapid basal proliferation rate, characteristically seen in late passages. The changes in D3 levels resemble those of D1, in which an initial rise is followed by a persistent fall, and retreatment with R5020 at 40 h produces an abortive rise. Cyclin E levels fall to low levels as the cells transit the cell cycle in the first 24 h after progesterone, then rise spontaneously to very high levels and remain high, in cells that receive no further treatment. This lack of cyclin E degradation suggests that the cells are blocked in late G1 and cannot enter S phase (44). Retreatment of the cells with R5020 at 40 h lowers cyclin E levels only minimally, and the flow cytometry data confirm that these cells do not resume cycling.

Because progesterone produces a prolonged growth-refractory state, protein levels of cdk inhibitors were also measured (Fig. 4A). We find that $T47D_{CO}$ cells and their descendents do not express p16 ($p16^{INK4,MTS1}$) (45), but they express p21 ($p21^{Cip1,Waf1,Sdi1}$) (20) and $p27^{Kip1}$ ($p28^{Ick1}$) (46). A rise in protein levels of the inhibitors begins as cyclin levels are declining. The levels of p21 increase first, peak approximately 36 h after the initial progesterone pulse, and then fall. This decline can be delayed approximately 18 h by retreatment of the cells with progesterone at 40 h. Thus elevated p21 levels are transiently stabilized by the second progesterone pulse. The levels of $p27^{Kip1}$ remain relatively unchanged for 36–40 h after the initial progesterone treatment and then start to climb as the cells arrest. Its levels eventually fall (not shown here, but see Fig. 7D) in the absence of a second hormone pulse, as the cells spontaneously recover their ability to proliferate. This recovery rate varies somewhat among experiments and appears to be related to the fall in p27. However, a second hormone dose produces even higher and sustained levels of both p21 and $p27^{Kip1}$ resulting in failure to recover and persistent growth suppression (see Fig. 6B).

Since the signals from the G1 cyclins and the cdk inhibitors are integrated into the Rb transcriptional regulatory pathway (40), the protein levels of Rb and its phosphorylation state were measured at 6-h intervals for 72 h following the two hormone pulses (Fig. 4B). In the first 6–12 h, levels of Rb are high but the protein is predominantly in its inactive hyperphosphorylated state. In this period, lacking sufficient amounts of inhibitory Rb, the cells undergo one round of cell division. However, coincident with the G1 arrest in the second cycle, 18–24 h after progesterone treatment, significant levels of the hypophosphorylated active repressor form of Rb are present. Thereafter, total Rb protein levels down-regulate by more than 90%, consistent with G1 cell cycle arrest in many systems (47). This decline occurs in the presence (not shown) or absence (Fig. 4B) of a second hormone pulse at 40 h.

Because progesterone regulates the levels of the cdk inhibitors, p21 and p27, in a complex manner, we examined the activity of the cyclin-dependent

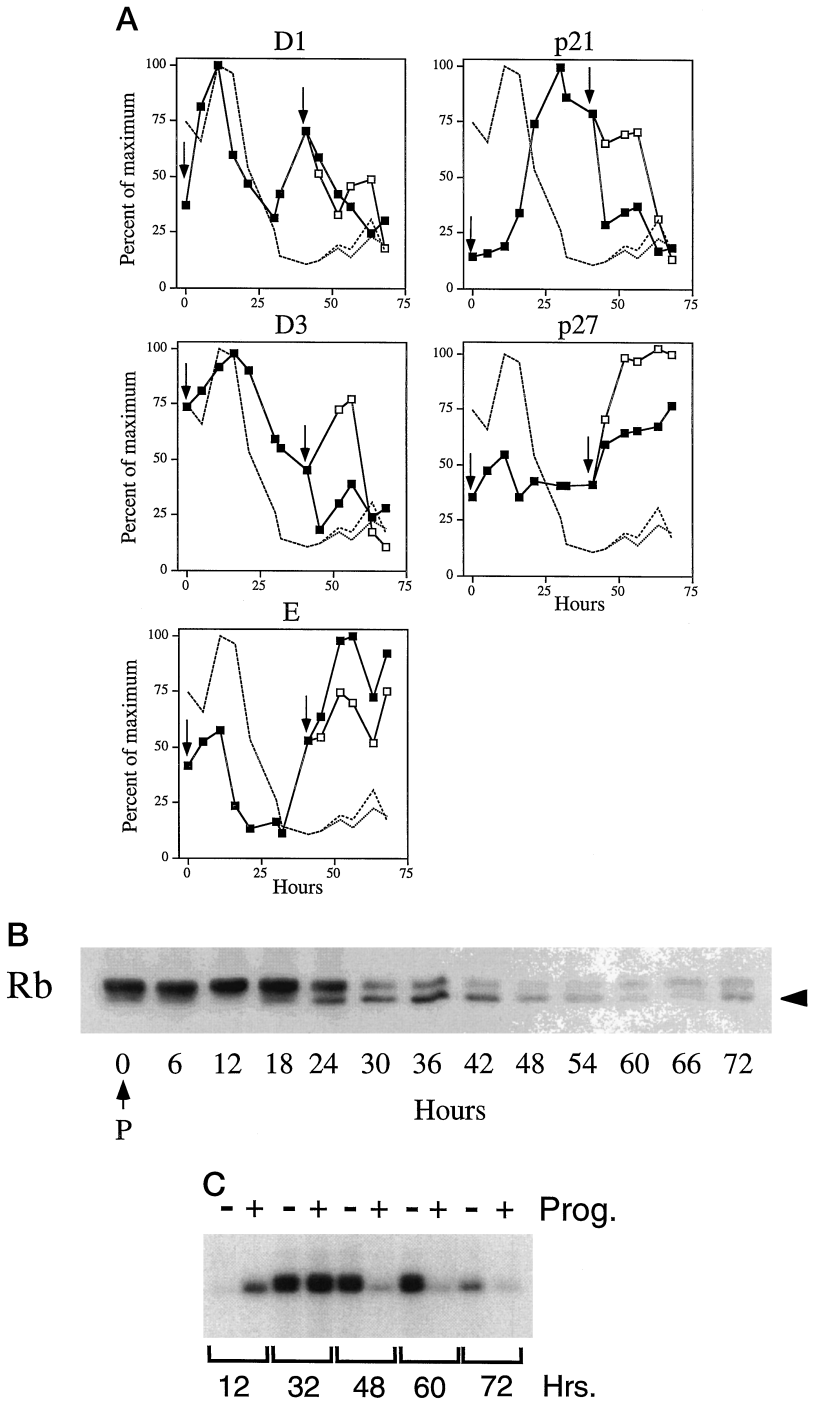

Fig. 4. Progesterone Treatment Arrests T47D-YB Cells in G1 of the Second Cycle by Up-Regulating p21 and $p27^{Kip1}$ and Down-Regulating cdk2 Activity

A, Growth, cyclin, and cdk inhibitor levels. T47D-YB cells were treated with progesterone at time 0 (*black arrow*), and again at 40 h (□) or left untreated (■) as shown in Fig. 3. Cells were harvested every 4–6 h for 80 h, and the percent in $S+G_2/M$ was measured by flow cytometry (*dashed lines*; see Fig. 3). Parallel sets of cells were lysed in Laemmli buffer, and extracts normalized to total protein were resolved by SDS-gel electrophoresis and immunoblotted with antibodies to the cyclins and cdk inhibitors shown: cyclins D1, D3, and E; Cdk p21 and $p27^{Kip1}$. Protein bands were detected by enhanced chemiluminescence and quantitated by densitometry, and their levels were normalized to pSTAIRE levels determined in parallel. B, Rb Immunoblot. T47D-YB cells were treated with progesterone at time 0, and parallel sets were harvested every 6 h for 72 h. Cells were lysed in Laemmli buffer, and extracts were normalized to total protein level, resolved by SDS-PAGE, and immunoblotted with an antibody directed against Rb. C, Cyclin-dependent protein kinase activity. T47D-YB cells were treated with progesterone (Prog.) at time 0 or untreated and harvested at the indicated times (Hrs.). cdk2 was immunoprecipitated from T47D-YB cell lysates, and immune complex kinase assays were performed using recombinant glutathione *S*-transferase-pRb fusion protein as a substrate as described (38).

protein kinase, cdk2, by its ability to phosphorylate purified recombinant pRb (Fig. 4C). cdk2 is regulated both by p21 and $p27^{kip1}$ (20, 40, 41, 46). In untreated control cells at early time points after cell plating, cdk2 activity is low and minimal changes in cell proliferation are observed (Figs. 6B and 7B). cdk2 activity rises as untreated cells begin to cycle (32 h) and then falls as cells reach confluence and become contact inhibited (72 h). Interestingly, progesterone up-regulates cdk2 activity early (12 h), then suppresses it at later time points (48–72 h) relative to untreated controls. Thus, progesterone exerts biphasic effects on cdk activity, consistent with its biphasic effects on cell proliferation.

Indeed, the profound quality of the arrest in G1 is further confirmed by analysis of cyclin A and B levels as shown in Fig. 5. These cyclins are produced in S- and G2-phase, respectively, in preparation for mitosis (35, 37, 38). In progesterone-treated T47D-YB cells, the levels of both cyclins reach a peak during the initial proliferative burst, but then fall precipitously to almost undetectable levels for at least 72 h (Fig. 5, A and B), and they cannot be rescued by a second hormone pulse at 41 h (Fig. 5C).

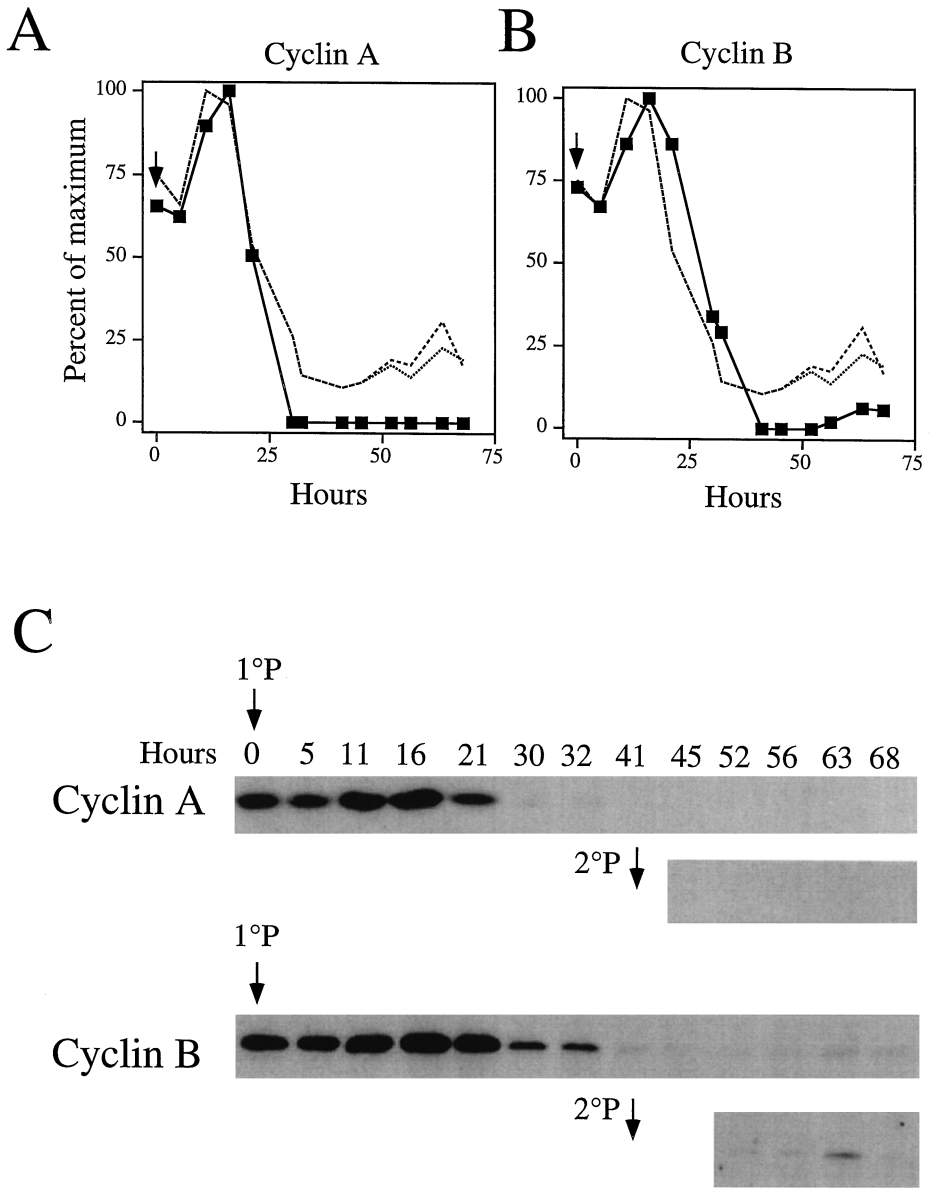

Fig. 5. Down-Regulation of Cyclin A and Cyclin B after Progesterone-Induced Growth Arrest

T47D-YB cells treated with progesterone at 0 time (■) as described in Fig. 3 were harvested at the times indicated, lysed in Laemmli buffer, resolved by SDS-gel electrophoresis, and immunoblotted with antibodies directed against cyclin A (panel A) or cyclin B (panel B). Percent of cells in $S+G_2/M$ are indicated by the *dashed line*, from the same sets shown in detail in Fig. 3. The highest cyclin protein level at any time point was set at 100%. Panel C, Immunoblots of the data shown in panels A and B that received no second progesterone dose (1°P) or that received a second dose of progesterone at 41 h (2°P).

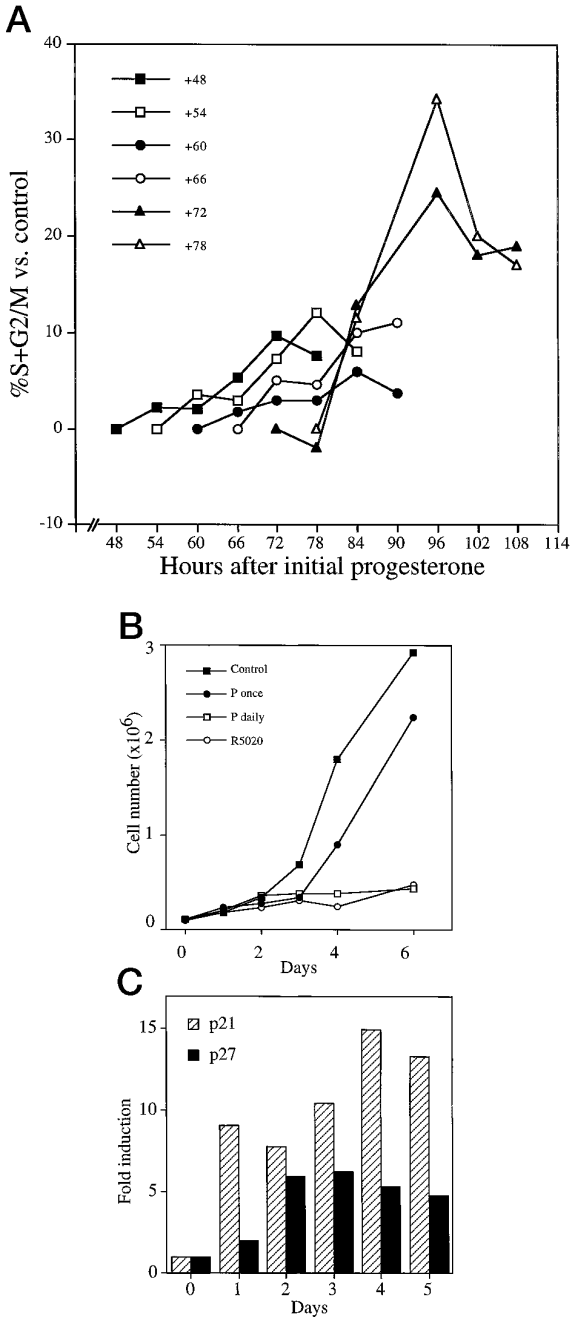

Fig. 6. A Brief Progesterone Pulse Leads to Prolonged Progesterone Resistance Accompanied by Elevated Levels of cdk Inhibitors

A, Time course for restoration of progestin responsiveness after a brief progesterone pulse. Parallel sets of T47D-YB cells were treated with progesterone at time 0 (not shown), and either 48 h later, or every 6 h thereafter up to 78 h later, they were again treated with progesterone. The ability of the second progesterone dose to induce a proliferative response was monitored for the subsequent 36 h. Cells were harvested at the time points shown and percent in $S+G_2/M$ were measured by flow cytometry and were compared with control cells ($\%S+G_2/M$ set at 0) that had received no second hormone treatment (not shown). B, Effects of single *vs*. daily progestin treatments on cell proliferation. Parallel sets of T47D-YB cells were untreated (■), treated once with progesterone at time 0 (●), or treated with progesterone (○) or R5020 (□) daily. Cells were harvested as shown over a 6-day period, and the average number of cells, in duplicate flasks, were counted. C, Levels of p21 and $p27^{Kip1}$ after chronic progesterone. Sets of cells from the daily progesterone treatment (panel B, *above*) were harvested and lysed in Laemmli buffer. Lysate concentrations were normalized to total protein levels, resolved on SDS-PAGE, immunoblotted with anti-p21 or anti-$p27^{Kip1}$ antibodies, and relative cdk inhibitor levels were quantitated by densitometry and normalized to pSTAIRE levels determined in parallel blots. *Bars* represent the average of duplicate assays.

When do T47D-YB cells regain sensitivity to progestins? To assess this (Fig. 6A), parallel sets of cells were treated with progesterone at time zero, then exposed to R5020 starting 48 h later, or every 6 h thereafter. For each set of time points, the ability of R5020 to induce proliferation was monitored for the subsequent 36 h. Each hormone-treated set was compared with control cells that had not received the second R5020 dose to monitor spontaneous recovery. Figure 6 shows that resistance to R5020 persists until approximately 72 h after the initial progesterone dose, at which point R5020 produces a brisk proliferative response (Fig. 6A), coincident with spontaneous recovery (not shown, but see Fig. 7C). Thus, only when they are poised to resume growing spontaneously, do the cells regain sensitivity to progestins. The extent and duration of this second proliferative burst resemble that of the initial response seen in naive, hormone-untreated cells (Figs. 1 and 2). The recovery time varies somewhat among experiments, depending on the basal cell proliferation rate at time zero; cells with a high basal rate, usually late passage cells, recover more quickly. In general, T47D-YB breast cancer cells remain in stasis for approximately 3 days after a brief pulse of progesterone (recall the $t_{½}$ is 2–4 h), but then resume growing at the same rate as controls (Fig. 6B). On the other hand, repeated exposure to progesterone or R5020 every 48 h produces permanent growth arrest (Fig. 6B). This arrest is associated with a 12- to 15-fold increase in the levels of p21 and a 6- to 7-fold increase in the levels of $p27^{Kip1}$ (Fig. 6C).

EGF Induces a Proliferative Response in Progesterone-Resistant Cells

To determine whether, during the progesterone-resistant state, T47D-YB cells are also resistant to other mitogenic signals, we tested the effects of EGF. This mitogen is an important growth factor in breast cancers, and clinically, an inverse relationship exists between steroid receptor loss (with concomitant hormone resistance) and the expression of EGF receptors (48–50). We therefore tested the relationship, if any, between progesterone resistance and EGF growth sensitivity (Fig. 7, A-E). Control T47D-YB cells express low levels of EGF receptors, which are functionally competent in their ability to signal to downstream cy-

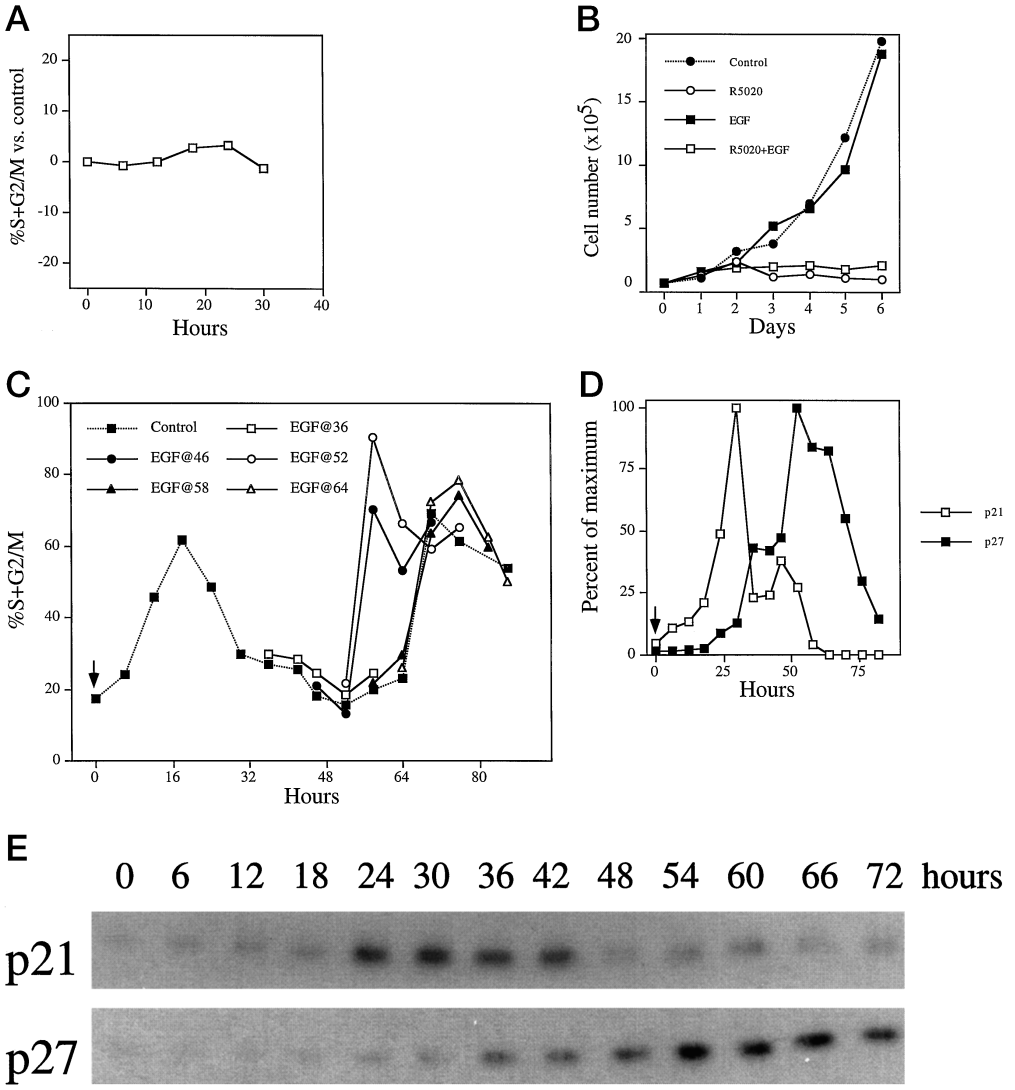

Fig. 7. Progesterone-Arrested Cells Can Mount a Transient Proliferative Response to EGF

A, EGF effects on naive T47D-YB cells. T47D-YB cells growing under control conditions without progesterone were treated with 10 nM recombinant human EGF at time 0. Cells were harvested every 6 h, and the percent of cells in $S+G_2/M$ was measured flow cytometrically and compared with EGF-untreated controls, set at 0. B, Chronic EGF and progestins. Parallel sets of T47D-YB cells were treated daily for 6 days with R5020 only (○), R5020 + EGF (□), EGF only (■), or left untreated (●). Cells were harvested daily and their number counted. C, Proliferative effects of EGF after a pulse of progesterone. Parallel sets of T47D-YB cells were treated with progesterone at time 0. Control cells were left untreated for the subsequent 80 h (*dashed lines*). Parallel sets received 10 nM EGF starting at 36, 46, 52, 58, and 64 h after time 0, and the percent of cells in $S+G_2/M$ was monitored flow cytometrically over the subsequent 36 h for each treatment group. D, p21 and $p27^{Kip1}$ levels after a pulse of progesterone. Cells from panel C, which received progesterone at time 0 and no further treatment, were harvested periodically as shown and lysed in Laemmli buffer. The lysates were normalized to total protein levels, resolved by SDS-PAGE, and immunoblotted with anti-p21 and anti-$p27^{Kip1}$ antibodies, and levels of the inhibitors were determined densitometrically and normalized to pSTAIRE levels in the same lysates. The data were plotted as a percent of the maximum level for each inhibitor, set at 100%. E, Immunoblot of p21 and $p27^{Kip1}$ levels after a pulse of progesterone. T47D-YB cells treated with progesterone at time 0 were harvested periodically as shown and lysed in Laemmli buffer. The lysates were normalized to pSTAIRE levels, resolved by SDS-PAGE, and immunoblotted as in panel D.

toplasmic effectors, since a 10 nM pulse of EGF strongly induces mitogen-activated protein (MAP) kinase activity (not shown). However, the naive cells are resistant to the growth-stimulatory effects of EGF (Fig. 7A). In this study, cells that had received no prior treatment were incubated with 10 nM recombinant human EGF or left untreated, and the percent of cells in $S+G_2/M$ was measured every 6 h for 30 h. As shown, the proliferation of cells treated acutely with EGF did not differ significantly from controls.

To determine whether chronic EGF treatment affects growth, cells received EGF continuously for 6 days in the presence or absence of continuous R5020, and their proliferation rate was compared with that of

untreated or R5020-treated cells (Fig. 7B). As shown, EGF alone does not accelerate growth above the control rate, and it cannot relieve the growth suppression produced by continuous R5020.

Surprisingly therefore, T47D-YB cells can be sensitized to the proliferative effects of EGF by a brief progesterone pulse (Fig. 7C). In this study, sets of T47D-YB cells that had been pretreated with progesterone at time 0 were challenged with EGF at various time points, starting at 36 h. Control sets received no second progestin treatment to monitor spontaneous recovery from the progesterone-induced arrest. While EGF given 36 h after progesterone was ineffective, the cells acquire sensitivity to EGF starting approximately 46 h after progesterone pretreatment and exhibit an extensive proliferative burst after a 6-h lag. Cells treated with EGF 52 h after progesterone respond even faster. Recall that at these time points the cells are insensitive to progesterone (Fig. 6) and have not recovered spontaneously (Fig. 7C, *dashed line*). These and other studies (not shown) suggest that there is a critical period during progesterone-induced growth arrest, in which T47D-YB cells acquire sensitivity to the proliferative effects of EGF, which accelerates their reentry into the cell cycle.

What accounts for this brief sensitivity to EGF? Figure 7D shows that after the progesterone pulse, p21 levels rise, peak at approximately 30 h, and then fall by 36 h, at a time preceding the rise in $p27^{Kip1}$ levels. Figure 7E shows a similar pattern from another experiment, in which it is evident that approximately 48 h after a single progesterone pulse, there is a brief period of time characterized by relatively low levels of both inhibitors, during which EGF can influence cell proliferation. This pattern occurs only after the initial signal produced by the short half-life of one progesterone pulse. More prolonged progestin exposure, produced either by repeated treatment with progesterone or by continuous treatment with a poorly metabolizable synthetic progestin such as R5020, prevents or slows the fall in p21 levels (Fig. 4) so that this event overlaps with, rather than precedes, the rise in $p27^{Kip1}$. We conclude that EGF cannot overcome the continuous inhibitor levels produced by a sustained progestin signal, as shown in Fig. 7B.

However, the brief window, characterized by low inhibitor levels after one pulse of progesterone, differs from the hormone-untreated condition in one respect. Namely, that EGF receptors are strongly up-regulated by the progesterone pulse (Fig. 8). These data, derived from immunoblots, show that progesterone treatment increases the number of EGF receptors by 3- to 5-fold between 24 and 48 h. Similar effects of progestins have been previously described (51, 52).

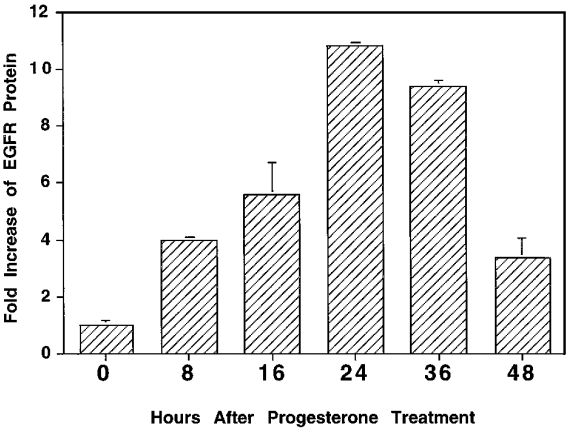

Fig. 8. One Dose of Progesterone Up-Regulates the Number of EGF Receptors per Cell

T47D-YB cells were treated with 30 nM progesterone at time 0 or left untreated. Sets of cells were harvested at the times shown and counted, and EGF receptor levels were measured by immunoblotting of cell membrane preparations normalized to cell number. The data represent the average of three experiments. Error bars depict the range among measures.

DISCUSSION

We demonstrate here, under conditions in which the proliferative actions of progesterone can be isolated from those of other mitogens, and in which PR levels are autonomously controlled, that this steroid hormone elicits a single round of mitosis in breast cancer cells, after which the cells chronically arrest in late G1 of the second cycle in a persistent progesterone-resistant state. Clearly, the cellular conditions that unmasked this property of progesterone are artificial (34). Nevertheless, they begin to allow study of a hormone whose proliferative effects have been intensely disputed in part because of their complexity (1). We believe that by simplifying the model we can dissect out the unique role that progesterone plays in physiological systems in which its actions are otherwise intricately regulated by cross-talk with other steroid hormones and growth factors.

Continuous Progesterone Is Autoinhibitory

The immediate response of proliferating breast cancer cells to progesterone is an acceleration of the cell cycle driven by increased levels of cyclins D1 (8), D3, and E and by accumulation of inactive forms of Rb in the face of persistent low steady state levels of p21 and $p27^{Kip1}$. Under these conditions cyclin D-cdk4 complexes accumulate above the inhibitory threshold of $p27^{Kip1}$ (53), allowing the cells to progress past the G1 restriction point and enter mitosis. Because the breast tumor cells used in these studies are proliferating rapidly initially (at a "low" proliferation rate, 15–25% of cells are in $S+G_2/M$ at any time), and progesterone more than doubles this rate (leading to more than 50% in $S+G_2/M$), the cells proceed through mitosis and reenter G1 of the second cycle in a partially synchronized state. At this point however, the cells are unable to adequately replenish the depleted stores of

cyclin Ds, Rb protein levels are extensively down-regulated, cyclin E levels rise sharply, followed first by rising p21, and later by rising $p27^{Kip1}$ levels, while cdk2 activity declines. Cyclin A, required for progression through S phase and cyclin B, the primary mitotic cyclin, are completely down-regulated. Taken together these data indicate that the cells are arrested late in G1 (44).

One surprising finding is that additional progesterone cannot override the growth suppression produced by the first progesterone dose, despite the presence of adequate levels of transcriptionally competent PR. Thus, the proliferative block is not at the level of the PR-signaling system. In fact, we propose that the growth arrest may actually require the presence of functional PR, since it appears to be due to sustained up-regulation of p21 and $p27^{Kip1}$ produced by a positive feedback loop initiated by at least two progestin treatments, given before the time at which the cells would spontaneously recover. Thus, we believe that sustained progesterone is autoinhibitory, in contrast to transient progesterone, which is stimulatory. This model has important implications for the scheduling of progestin treatments in clinical settings, since it predicts that the effects of continuously administered progestins (54) differ significantly from those of episodically or cyclically administered progestins (13, 14); the former would be growth inhibitory and the latter stimulatory. These data also suggest that the cyclical progesterone of the menstrual cycle can have different physiological consequences than the continuous progesterone of pregnancy. A model in which the rate and duration of progesterone treatment control the type of response observed would reconcile contradictory views that this hormone is either proliferative or differentiative. A similar model, in which a proliferative *vs.* differentiative end point is controlled by the duration of MAP kinase signaling was recently described (55).

The mechanism of progesterone-mediated induction of p21 and p27 is unknown. However, aside from its p53-dependent regulation in response to DNA damage, p53-independent transcriptional activation of p21 has recently been shown to be regulated by the MAP kinase pathway after stimulation of cells with growth factors (56). We are currently investigating the mechanism of p21 and p27 regulation by progesterone. Interestingly, in addition to its action as a cyclin-dependent protein kinase inhibitor and cell-cycle inhibitor, p21, at low concentrations, promotes the assembly of active cyclin/cdk/proliferating cell nuclear antigen complexes and exerts a positive influence on cell growth; kinase activity increases 3-fold upon the addition of low concentrations of p21 to lysates containing cyclin A and cdk2 (57). Thus, quaternary complexes containing one p21 molecule are fully active, while inhibition of cyclin kinases requires association of more than one p21 subunit. This may explain the biphasic effects of progesterone on breast cancer cell growth. In this model, the initial proliferative burst is supported by the assembly and activation of cdk/cyclin/proliferating cell nuclear antigen/p21 complexes. However, as p21 and p27 expression increases, these quaternary complexes become inactive due to the addition of multiple inhibitor subunits, and the cells are growth inhibited. A second exposure to progesterone produces sustained elevation of p21 and prolongs growth inhibition. Consistent with this hypothesis, Matsushine *et al.* (58) showed that cyclin D and cdk4 do not associate in serum-starved cells, but undergo association and activation upon serum stimulation, a condition that increases p21 levels in a p53-independent manner. Similarly, LaBaer *et al.* (59) found that the addition of low concentrations of p21 and p27 lead to a 35- and 80-fold increase in the assembly and activity of cyclin D-cdk4 complexes, respectively, while high concentrations inhibited activity, suggesting new roles for these inhibitors as adaptor proteins that assemble and program kinase complexes (59).

Progesterone Enhances Sensitivity to EGF and Up-Regulates EGF Receptors

Failure of progestins to reinitiate proliferation after a single progesterone pulse is not due to insensitivity of the cell-cycling machinery, since an alternate mitogenic signal emanating from the cell surface can transiently reactivate proliferation. The mechanisms by which one pulse of progesterone sensitizes the cells to the proliferative effects of EGF appears to be related to its ability to up-regulate the levels of EGF receptors, as previously described (51, 52). Untreated T47D-YB cells do not respond to proliferative signals by EGF (Fig. 7A), although the cells express low levels of immunoreactive EGF receptors (Fig. 8), and they respond to EGF by activating the downstream effectors, p42 and p44 MAP kinases (not shown). Thus, the EGF receptor-mediated signal transduction pathway leading to activation of cytoplasmic kinase cascades known to regulate cellular processes including growth (60), is functionally intact in these cells. At high EGF receptor levels, however, EGF appears to be able to induce the arrested cells to reenter the cell cycle, as long as the levels of the cdk inhibitors are relatively low. It is possible that at high levels, EGF receptors can engage novel signaling proteins (61) or be capable of associating with novel cell-surface partners (62) that are unavailable at low receptor concentrations. Similar, phenotypically different response, dependent on the number of active cell surface receptors, have been described (63–65).

On the other hand, our data suggest that the high levels of EGF receptors cannot overcome the inhibitory effects of high levels of p21 or $p27^{Kip1}$ produced by a second dose of progesterone. This may explain EGF-dependent proliferation after a single pulse of progesterone but resistance after a second pulse or during sustained progesterone treatment. We have shown that one progesterone pulse leads to a transient rise in p21 levels and that $p27^{Kip1}$ levels start to

rise as p21 levels are falling. Therefore we postulate that after a single progesterone pulse, there is a transient window of EGF responsiveness generated by increased EGF receptor levels and falling p21 levels, before the rise in $p27^{Kip1}$ levels. A second dose of progesterone delays the fall in p21 and increases the levels of $p27^{Kip1}$ (Fig. 4), eliminating the window to EGF responsiveness. This suggests that in breast cancer cells, proliferative sensitivity to EGF is dependent on the cell cycle state and progestational history of the cells.

There is considerable evidence linking the EGF and progesterone signaling pathways in breast cancer. This includes attenuation of progestin responsiveness and decreases in PR levels in cells treated with EGF (66); augmentation of the proliferative, differentiative, and transcriptional effects of progestins by cotreatment with EGF (67–69); and progestin-specific regulation of EGF and EGF receptor levels (Fig. 8 and Refs. 49, 51, and 66). There are also provocative clinical data linking enhanced expression of EGF receptors to acquisition of steroid hormone resistance in breast cancer (48, 50).

Is Progesterone Proliferative or Differentiative?

Proliferation and differentiation are complex processes governed by the concerted activity of multiple regulatory factors. Both processes appear to have the common requirement that cells stop in G1 to await appropriate directional signals (16, 18, 19). Our demonstration that progesterone can advance cells to this checkpoint, while it sensitizes the cells to the actions of a growth factor, provides a model that may reconcile opposing views that progesterone is either a proliferative or a differentiative hormone. We suggest that progesterone is neither, but that it is a competency factor necessary to drive cells into either pathway, and that breast cell proliferation and differentiation are intricately connected. In this model, progesterone accelerates cells to the G1 checkpoint in the second cycle, whereupon other, possibly tissue-specific, factors determine the fate of the cell. Therefore the final state of progesterone target tissues is determined by cross-talk between progesterone and growth or differentiative factors that remain to be defined. However, the progesterone treatment regimen may be a key factor in the ultimate response produced, with transitory progesterone being permissive of such cross-talk while sustained progesterone is inhibitory.

MATERIALS AND METHODS

Cell Lines and Reagents

Wild type PR-positive $T47D_{CO}$ breast cancer cell lines, their monoclonal PR-negative T47D-Y derivatives, and T47D-Y cells stably expressing either A- or B receptors (T47D-YA or T47D-YB cells) were previously described (34). Cells are routinely cultured in 75-cm^2 plastic flasks or six-well multiwell plates and incubated in 5% CO_2 at 37 C in a humidified environment. The stock medium consists of Eagle's Minimum Essential Medium with Earle's salts (MEM), containing L-glutamine (2 mM) buffered with sodium bicarbonate (4 μg/liter) and HEPES (4.8 μg/liter), insulin (6 ng/ml), and 5% FCS (Hyclone, Logan, UT) without antibiotics. For routine subculturing, cells are diluted 1:20 into new flasks once per week, and medium is replaced every 2–3 days. Cells are harvested by incubation in Hank's EDTA for 15 min at 37 C.

Antibodies were obtained from the following sources: anti-cyclin A, -cyclin B1, and -cyclin D1 were from Upstate Biotechnology (Lake Placid, NY); anti-cyclin D3 and E were from Pharmingen (San Diego, CA); anti-p21 and anti-$p27^{Kip1}$ were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-pRb was a gift from Wen-Hwa Lee (University of Texas Health Science Center, San Antonio, TX); anti-EGF receptor 20.3.6 was a gift from Roger Davis (University of Massachusetts Medical School, Worcester, MA); anti-PR AB52 and B30 were produced in our laboratories (49); and horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad Laboratories (Hercules, CA).

Flow Cytometry

Cells (2×10^5) were plated into duplicate wells of six-well plastic dishes with 3 ml of serum-containing medium. After 24 h, progesterone, or the synthetic agonist R5020 (Roussel-Uclaf, Romainville, France) or the antiprogestins RU486 (Roussel-Uclaf) or ZK98299 (Schering AG, Berlin, Germany) were added in ethanol, at final concentrations of 30 nM, 100 nM, or 1 μM, respectively. Control medium contained only ethanol. Some cells received 60 ng/ml (10 nM) human recombinant EGF (Upstate Biotechnology, Inc.).

Cells were harvested at the start of treatment (control, zero time) and every 4 or 6 h after hormone addition, into 1 ml of Hank's EDTA and vigorously pipetted. The cell suspension was pelleted, resuspended into 1 ml of Krishan's stain (70) containing propidium iodide and ribonuclease (RNase), and again vigorously pipetted. Samples were cooled to 4 C, and 10,000 cells were analyzed on an Epics 752 flow cytometer (Coulter Electronics, Hialeah, FL), using an incident beam from an argon laser at 488 nm, 500 mW. The cells were gated on forward angle *vs.* 90° light scatter to eliminate cellular debris and doublets. Red fluorescence, corresponding to DNA, was collected through a 590-nm longpass filter, and histograms of DNA content *vs.* cell number were constructed. Cell cycle analyses of the DNA histograms were performed using the ModFit Analysis program (Veritey Software House, Topsham, ME), which provides fits for the G_O/G_1, S and G_2/M fractions of the population. The S- and G_2/M-phase fractions were combined into a single growth fraction. In some figures, the percent of cells in $S+G_2/M$ in the hormone-treated sets were compared with the percent of cells in $S+G_2/M$ in the untreated controls, whose levels were set at 0. For long-term growth studies, cells were harvested into 1 ml of Hank's EDTA and pipetted vigorously to obtain single-cell suspensions, and aliquots were counted using a hemocytometer.

Immunoblotting

For measurements of PR, whole cell extracts were prepared in 0.4 M KCl as previously described (27, 34). Receptors were resolved by electrophoresis on an 11% polyacrylamide gel containing SDS and then transferred to nitrocellulose. After incubation with anti-PR monoclonal antibodies AB-52 and B-30 (71), the receptor bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). For cell cycle proteins, cells were harvested at 50–80% confluence and washed in PBS. Aliquots were removed for analysis by flow cytometry to simultaneously determine cell cycle distribution. The remaining cells were resuspended in Lae-

mmli sample buffer (72) at 1–4 $\times 10^7$ cells per ml, immediately boiled for 5 min, sheared through a syringe needle to reduce viscosity, aliquoted, and stored at −80 C. Volumes of cell extracts normalized to approximately 50 μg total protein, as measured by Ponceau S, were subjected to gel electrophoresis. For measurement of EGF receptors, cells were resuspended in RIPA buffer (0.1 M NaCl, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% deoxycholic acid, 1% NP-40, and 0.1% SDS) (61) for 10 min at 4 C, and centrifuged at 10,000 rpm to produce a membrane-containing pellet. The pellet was reextracted with RIPA and repelleted, and membranes (60 μg) were resuspended in four volumes of water, sheared through a 28-gauge needle, boiled in Laemmli sample buffer, and resolved by electrophoresis on an 8% polyacrylamide gel. Proteins were transferred for 45 min at 0.5 A to Immobilon P membranes (Millipore, Bedford, MA) using a Genie Electroblotter (Idea Scientific, Minneapolis, MN). After incubation with the appropriate antibodies, protein bands were detected by enhanced chemiluminescence (Amersham). Film exposures ranged from 2 sec to 1 h depending on the primary antibody. Bands were quantitated using a digital scanner and the Image program (NIH) and, where appropriate, normalized to levels of pSTAIRE sequence-containing cdks.

Transfection and Transcription

T47D-YB cells were plated and grown in 100-mm^2 cell culture plates in MEM supplemented with 5% FCS. Progesterone (30 nM) was added 40 h prior to completion of transfection. Transfection of plasmid DNA into cells was performed 24 h after the start of progesterone treatment by calcium phosphate coprecipitation using 1 μg of the PRE_2-$TATA_{tk-}$-CAT reporter (27), 3 μg of the β-galactosidase expression plasmid PCH110 (Pharmacia, Piscataway, NJ) to correct for transfection efficiency, and 15 or 16 μg Bluescribe carrier plasmid (Stratagene, La Jolla, CA) for a total of 20 μg DNA, as previously described (27). Sixteen hours later transfection was completed when the medium was aspirated, and the cells were shocked at room temperature for 4 min with 5 ml HBSS containing 20% glycerol. After the cells were washed twice with 10 ml serum-free MEM to remove the glycerol, 10 ml MEM containing 5% FCS were added per dish, either without or with 30 nM progesterone or R5020. Cells were harvested after an additional 24 or 48 h. Cells in duplicate plates were lysed by freeze-thawing in 200 μl of 0.25 M Tris, pH 7.8. Lysates (50 μl) were assayed for β-galactosidase activity, and normalized aliquots were assayed for CAT activity by TLC as described (27).

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Address requests for reprints to: Kathryn Horwitz, Department of Medicine, Division of Endocrinology, Box B151, University of Colorado Health Science Center, 4200 East Ninth Avenue, Denver, Colorado 80262.

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