Progestins Reinitiate Cell Cycle Progression in Antiestrogen-Arrested Breast Cancer Cells through the B-Isoform of Progesterone Receptor

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Abstract

Estrogen treatment of MCF-7 human breast cancer cells allows the reinitiation of synchronous cell cycle progression in antiestrogen-arrested cells. Here, we report that progestins also reinitiate cell cycle progression in this model. Using clonal cell lines derived from progesterone receptor (PR)–negative MCF-7M13 cells expressing wild-type or mutant forms of PRA and PRB, we show that this effect is mediated via PRB, not PRA. Cell cycle progression did not occur with a DNA-binding domain mutant of PRB but was unaffected by mutation in the NH_2-terminal, SH3 domain interaction motif, which mediates rapid progestin activation of c-Src. Thus, the progestin-induced proliferative response in antiestrogen-inhibited cells is mediated primarily by the transcriptional activity of PRB. Analysis of selected cell cycle targets showed that progestin treatment induced levels of cyclin D1 expression and retinoblastoma protein (Rb) phosphorylation similar to those induced by estradiol. In contrast, progestin treatment resulted in only a 1.2-fold induction of c-Myc compared with a 10-fold induction by estradiol. These results support the conclusion that progestin, in a PRB-dependent manner, can overcome the growth-inhibitory effects of antiestrogens in estrogen receptor/PR-positive breast cancer cells by the induction of cyclin D1 expression. The mediation of this effect by PRB, but not PRA, further suggests a mechanism whereby abnormal regulation of the normal expression ratios of PR isoforms in breast cancer could lead to the attenuation of antiestrogen-mediated growth arrest. [Cancer Res 2007;67(18):8942–51]

Introduction

Tamoxifen, a selective estrogen receptor antagonist (SERM), has been the endocrine therapy of choice for the treatment of breast cancer patients with estrogen receptor (ER) and progesterone receptor (PR)–positive tumors. A significant limitation of tamoxifen therapy is intrinsic and acquired resistance (1). This has led to the development of alternative and potentially more efficacious endocrine therapies, including pure antiestrogens and aromatase inhibitors. The pure steroidal antiestrogen, ICI 182780 (fulvestrant, "Faslodex"), displays no demonstrable estrogen agonist activity and is potentially more efficacious than tamoxifen (2), and is as effective as the aromatase inhibitor, anastrozole (3), in postmenopausal women with advanced breast cancer.

ICI 182780 is a potent inhibitor of cell proliferation in ER-positive, MCF-7 breast cancer cells, in which it induces quiescence (4). Growth arrest is preceded by a decline in c-Myc expression, a consequent decrease in cyclin D1 protein levels, and decreased cyclin-dependent kinase (Cdk4 and Cdk2) activity (4, 5). Studies investigating the role of ICI 182780 in the inhibition of breast cancer cell proliferation have focused mainly on ERα-mediated effects in breast cancer cell lines (6). However, ER has a functional relationship with PR in modulating many physiologic responses in target tissues, including the control of cell proliferation in both the breast and uterus (7), with evidence that progestins can enhance breast cancer risk (8, 9). Furthermore, high expression of ER and PR is a marker of therapeutic responsiveness and patient outcome in breast cancer (10, 11).

PR is expressed in most target tissues as two isoforms, PRA and PRB, transcribed from a single gene with distinct estrogen-inducible promoter regions (12). The first NH_2-terminal 164 amino acids of PRB are absent in PRA. This unique NH_2-terminal region of PRB mediates a transactivation function that contributes to the differential binding of cofactors and to the distinct properties of the two isoforms (13–16). PR is an estrogen-induced protein, and in some breast cancer cell lines, estrogen activation of ER preferentially stimulates PRB expression (17). Conversely, PRA can antagonize ER action and suppress the activity of PRB (13).

Target tissues for progesterone express both PRA and PRB (7, 18), but when expressed separately, PRA and PRB mediate both distinct and overlapping transcriptional responses (19), resulting in different physiologic effects in different target tissues (20). Selective ablation of PRA or PRB showed that PRB, but not PRA, was sufficient for normal mammary gland development in mice (21, 22). In the normal human breast, PRA and PRB have similar but low levels of expression (23), being expressed in ~30% of nonproliferating breast epithelial cells (24). In the progression from normal to malignant breast cells, the expression of PRA and PRB becomes more heterogeneous, with more aggressive breast cancers being associated with a predominance of either one of the two PR isoforms (23). Differential isoform expression is associated with different phenotypes. For example, overexpression of PRA in breast cancer cells resulted in an aberrant progestin response leading to loss of cell adhesion (25), whereas increased expression in the mammary gland of transgenic mice resulted in excessive side branching and disruption of normal gland architecture (26). Alternatively, increased PRB expression has been linked to cell proliferation in patients with breast cancer (27), whereas in mice,

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excess PRB induced lobulo-alveolar hyperproliferation (28). Consequently, altered PRA/PRB ratios might play a role in the etiology of breast cancer, and the detection of differential expression of PRA and PRB may be an important marker in breast cancer development, progression, and therapeutic responsiveness.

In the present study, we show that progestins can reinitiate cell cycle progression in antiestrogen-arrested cells. Furthermore, this effect is mediated by PRB and not PRA, and is dependent on a functional DNA-binding domain, implying that the effect is mediated primarily via PRB-mediated gene transcription.

Materials and Methods

Materials. Steroid solutions, prepared in absolute ethanol and stored at −20°C, were obtained from the following sources: ORG2058 [16α-ethoxy-21-hyhroxy-19-norpregn-4-en-3, 20-dione] (Amersham); R5020 [17α-21-dimethyl-19-norpregn-4,9-diene-3,20-dione] (Du Pont, Ltd.); RU486 [17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl)-etra-4,9-diene-3-one] (Dr. J-P. Raynaud of Roussel-Uclaf, France); ICI 182780 (Faslodex) [7α-[9-(4,4,5,5,5-pentafluropentyl-sulfinyl)nonyl]estra-1,3,5(10)-triene-3,17β-diol] and 4-hydroxytamoxifen (from Dr. Alan Wakeling, Astra Zeneca Pharmaceuticals, Alderley Park, Cheshire, United Kingdom).

Construction of the retroviral PR vectors. Constructs for retroviral expression were made using the pQCXIP vector (BD Biosciences) which was first converted to the Gateway system (Invitrogen) by cloning the Gateway Cassette RfA into the blunted *Bam*HI site of pQCXIP to generate pQCXIPgat. PRA and PRB and mutant constructs ($PRB^{\Delta SH3}$, $PRA^{\Delta SH3}$, PRB^{C587A}, PRA^{C587A}) were cloned into *Kpn*I/*Not*I sites of entry clone2b and subsequently transferred into pQCXIPgat via the LR reaction.

To block the expression of PRA the PRA transcriptional start site atg was mutated to gcg in all PRB constructs using the Quick-Change II kit (Stratagene; ref. 29). This also introduced an *Nru*I site at this position (PRB^{M165A}). The primer sequences used were *Nru*I minus (cttgcacccgg-cggctcgcgagcggggacaaca) and *Nru*I plus (tgttgtccccgctcgcgagccggt-ccgggtgcaag). All PR sequences were verified by sequence analysis to ensure the fidelity of the constructs.

Cell culture. MCF-7-PR and MCF-7-$B^{\Delta SH3}$ cell lines have been described previously (30). Stock cultures of MCF-7 and T-47D cells were maintained in RPMI 1640 supplemented with 10% FCS and insulin (10 μg/mL). MCF-7PR cell lines were supplemented with 0.5 μg/mL of zeocin and MCF-7M13-PR supplemented with puromycin (2 μg/mL; Invitrogen). For experiments investigating the effects of ICI 182780, six-well dishes were seeded at 1.5×10^5 cells/well in RPMI plus 5% FCS and supplemented with insulin (10 μg/mL). After 24 h, ICI 182780 (10 nmol/L) was added directly to the medium. Cells were treated with hormones and harvested at the times indicated.

Cell kinetic studies. At the completion of the experiments, cells were harvested for assessment of cell cycle phase distribution using flow cytometry as described previously (31). A minimum of 20,000 cells were counted for each sample. Analysis was done with ModFit software.

Retroviral infection of MCF-7M13 cells. MCF-7M13 cells, a PR-negative clone of the MCF-7 cell line, were transfected with the murine ecotropic receptor and selected for expression using geneticin (400 μg/mL; Sigma-Aldrich). Subsequently, MCF-7M13EcoR cells were infected with PRA, PRB, and various PR mutant retroviral constructs using methods described previously (31).

Immunoblot analysis. Cells were prepared for protein extraction as previously described (31). Protein was quantitated using the Bio-Rad assay and equal amounts of total protein (20 μg) were separated by SDS-PAGE then transferred to polyvinylidene difluoride membranes. Proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) after incubation with the following primary antibodies: 1294/H9, a mouse monoclonal antibody that recognizes both human PRA and PRB isoforms (32); cyclin D1 (DCS-6; Novacastra Laboratories, Ltd.); c-Myc-C-19 (Santa Cruz Biotechnology); anti–phospho-retinoblasma protein (Rb) (phospho-Ser^{780}; Sigma Aldrich); and Rb (G3-245; PharMingen). Protein abundance was quantified by analysis of autoradiographs using densitometry (Molecular Dynamics).

Indirect immunofluorescence. PR expressing MCF-7M13 cells were grown in RPMI 1640 supplemented with 5% FCS in four-well chamber slides and incubated for 24 h. The following day, cells were treated with ethanol or ORG2058 (10 nmol/L) for 8 h. After hormone treatment, cells were paraformaldehyde-fixed and permeabilized with 0.2% Triton X-100 in PBS. Cells were washed with PBS and blocked with 1% bovine serum albumin in PBS and incubated with a PR-specific monoclonal antibody (1294/H9) in 1% bovine serum albumin-FCS. The nucleus was defined by staining with 4′-6-diamidino-2-phenylindole, and the cytoskeleton (filamentous actin) was stained using TRITC-phalloidin (Sigma Aldrich). Slides were mounted using 80% glycerol.

Digital images were collected using a Zeiss fluorescence microscope (×40 objective). Using the "Image J" software package (33), a linear profile across the diameter of the cell measured the fluorescence intensity of PR in the cytoplasm and nucleus and the relative ratio of PR (nucleus/cytoplasm) for each cell treated with ORG2058 (10 nmol/L) or vehicle was determined. An average of 30 to 40 cells per cell line was analyzed.

Results

Progestins stimulate cell cycle progression in antiestrogen-arrested T-47D but not in MCF-7 breast cancer cells. Previous studies have shown that progestins can both stimulate and inhibit cell cycle progression in T-47D and MCF-7 cells (34, 35). Because ICI 182780 treatment induces growth arrest with features of quiescence (4), we initially assessed the effect of progestins on ICI 182780–arrested cells to determine whether the stimulatory effect of progestins could override antiestrogen-mediated growth arrest. T-47D and MCF-7 cells that express endogenous ER and PR were equally responsive to ICI 182780 treatment, with the proportion of S phase cells declining from 25% to 30% in control, vehicle-treated cells to 10% after 36 h of ICI 182780 treatment (Fig. 1*A* and *B*). ICI treatment alone substantially down-regulated PR in both cell types (Fig. 1*C*). Initial PR levels were higher in T-47D; thus, some PR remains detectable in these cells 21 h post–ICI 182780 treatment, whereas PR is down-regulated to undetectable levels in MCF-7 cells within 8 h (Fig. 1*C*).

Addition of the synthetic progestin, ORG2058 (10 nmol/L) to T-47D cells in the continued presence of ICI 182780 (10 nmol/L), resulted in an initial transient acceleration of cell cycle progression (increase in S phase from 10% to 20%) 12 to 18 h posttreatment, followed by a return to pretreatment levels at 24 h (Fig. 1*A*). The PR, still present in ICI 182780–treated T-47D cells, was further down-regulated 8 h post-ORG5058 treatment and was barely detectable by 21 h (Fig. 1*C*). The transient proliferative response was abrogated by the antiprogestin RU486, which alone had no effect on cell cycle phase distribution under these conditions (Fig. 1*B*). No further down-regulation of PR in ICI 182780–treated cells was observed with RU486, and RU486 abrogated ORG2058 down-regulation of PR (Fig. 1*C*), supporting the idea that cell cycle progression and down-regulation of PR were mediated through progestin binding. In contrast, no cell cycle response to ORG2058 was observed in ICI 182780–arrested MCF-7 cells (Fig. 1*B*). In these cells, ICI 182780 treatment down-regulated PR to undetectable levels (Fig. 1*C*), presumably precluding progestin action and the ability to initiate PR-mediated cell cycle progression in the presence of ICI 182780.

To test whether this phenomenon was shared by other structural classes of estrogen antagonists, we arrested T-47D cells with the SERMs, tamoxifen and clomiphene, and treated these cells with ORG2058 48 h later. As with ICI 182780–arrested cells, progestin treatment reinitiated cell cycle progression (Fig. 1*D*).

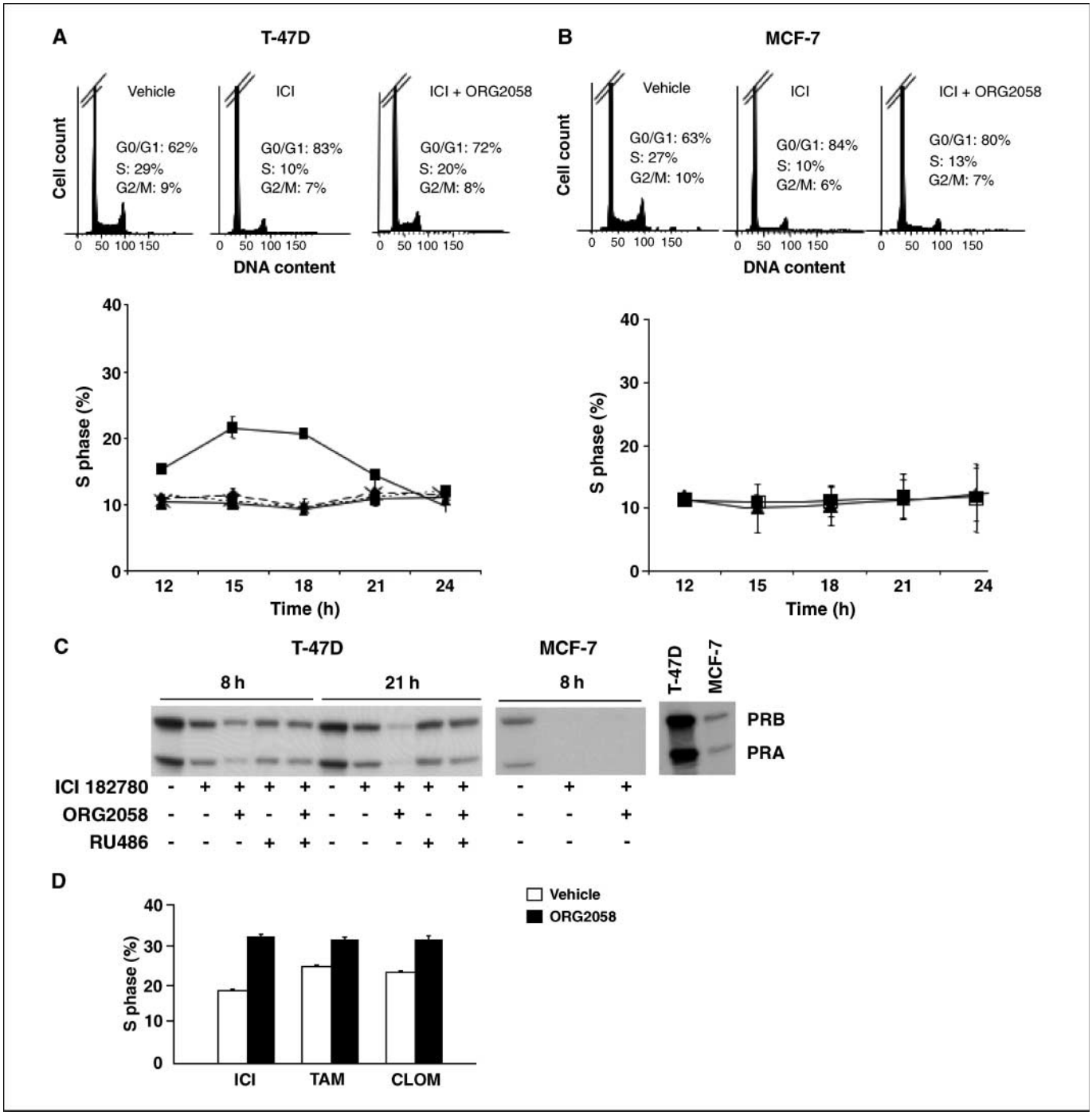

Figure 1. Progestins stimulate transient cell cycle progression in T-47D but not in MCF-7 breast cancer cells in the presence of antiestrogens. Exponentially growing T-47D (*A*) and MCF-7 (*B*) cells were treated with ICI 182780 (10 nmol/L) for 36 h in medium containing 5% FCS and insulin (1 μg/mL). Subsequently, cells were treated with ORG2058 (10 nmol/L) and/or RU486 (100 nmol/L) or vehicle and harvested at intervals for preparation of protein lysates and flow cytometry. Representative DNA histograms of the percentage of cells in each phase of the cell cycle in vehicle-treated and ICI 182780–treated cells at 36 h, or following subsequent treatment with ORG2058 for a further 15 h. *Points,* mean percentage of cells in S phase at time intervals after hormone treatment of three independent experiments; *bars,* SD: ORG2058, ■; vehicle, ▲; RU486, ◆; ORG2058 + RU486, ×. *C,* immunoblot of PR in T-47D and MCF-7 cells after the indicated treatments. Whole cell lysates (20 μg/sample) were subjected to SDS-PAGE and Western blots using a specific PR monoclonal antibody (1294/H9) and visualized by enhanced chemiluminescence. *D,* T-47D cells were arrested for 48 h with 1 μmol/L of 4-hydroxytamoxifen, 4-hydroxyclomiphene, or 10 nmol/L of ICI 182780 then treated with 10 nmol/L of ORG2058 for 18 h. *Columns,* mean of triplicate measurements of S and G_2-M phase cells before and after ORG2058 treatment; *bars,* SD.

Analysis of MCF-7 cell lines with stable wild-type and mutant PRA or PRB expression. To investigate the potential roles of PRA and PRB in progestin release from ICI 182780–induced cell cycle arrest, we stably expressed PRA and PRB independently in MCF-7M13 cells (a subline of MCF-7 cells which express high levels of ER and no detectable PR). To inhibit transcription of the PRA isoform, the second transcription start site which gives rise to the 165 amino acid–truncated hPRA, was mutated (Fig. 2*A*). Expression

of PRB only from this construct (PRB^{M165A}) was confirmed by Western blot (Fig. 2*B*). It was previously shown that progestins could mediate their effects via both "classical" PR-mediated transcription and rapid "non-classical" effects mediated via an interaction between a proline-rich domain in the NH_2 terminus (polyproline SH3 recognition motif, amino acids 421–428), and SH3-type 1 domains of selected signaling molecules including members of the Src family of tyrosine kinases (30). To distinguish the transcriptional effects of PR from c-Src (SH3)–mediated effects, we generated both ΔSH3 and zinc finger mutants (C587A) of PR (Fig. 2*A*) which were stably expressed in MCF-7M13 cells.

Protein expression and cellular localization of these ectopically expressed PRs in MCF-7M13 cells were determined by immunoblotting and indirect immunofluorescent microscopy. PR was not detected in vector-transfected MCF-7M13 cells (Fig. 2*B*), whereas wild-type PRA, PRB^{M165A}, and PR mutants were expressed at similarly high levels, comparable to endogenous PR in T-47D cells, one of the most widely employed *in vitro* models of progestin action. We verified that the ΔSH3 PR variants were transcriptionally active using a PRE-luciferase reporter construct (data not shown). Immunofluorescence analysis showed that >70% of cells expressed detectable levels of PR. As shown in Fig. 2*C*, PRA and PRB have distinct intracellular distribution in the absence of ligand: PRB^{M165A} and $PRB^{M165A/\Delta SH3}$ were localized to both the cytoplasmic and nuclear compartments, whereas PRA and $PRA^{\Delta SH3}$ proteins were predominantly nuclear. Interestingly, disruption of the DNA-binding domain (PRA^{C587A} and $PRB^{M165A/C587A}$) caused localization of PRB and PRA to the cytoplasm and nucleus with similar intensity (Fig. 2*C*). These data suggest that an intact zinc finger and the unique NH_2 terminus of PRB (amino acids 1–164) are important for receptor localization in the absence of ligand. Nevertheless, we observed nuclear translocation of all PR variants upon hormone binding (Fig. 2*C* and *D*).

The role of PRA/PRB in cell cycle reinitiation by progestins. Progestin treatment reinitiated cell cycle progression in MCF-7M13 cells pretreated with ICI 182780 and constitutively expressing PRB^{M165A} (Fig. 3*A*). In marked contrast, cells expressing wild-type PRA, mutant PRA isoforms, or no PR (vector) failed to reinitiate

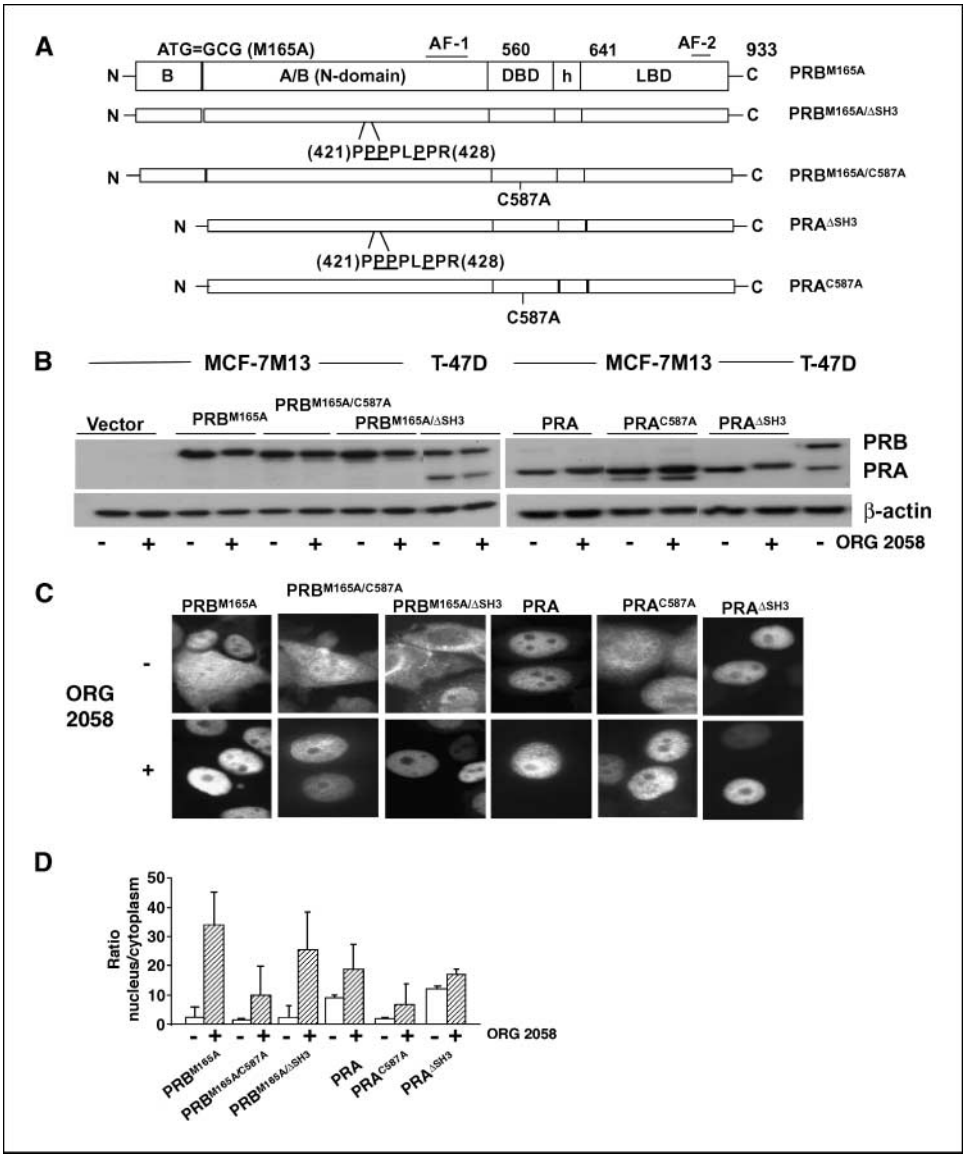

Figure 2. Characterization of MCF-7M13 cells expressing PRB and PRA. PR constructs were expressed in MCF-7M13 cells and pooled stable cell lines were selected with puromycin as described in Materials and Methods. *A,* schematic diagram of the PR structure and location of mutants. The PRA ATG (met) transcriptional start site was mutated to GCG (ala) to create an inactive PRA-ATG start codon (PRB^{M165A}). *LBD,* ligand-binding domain; *DBD,* DNA-binding domain; *h,* hinge; *AF-1,* transcriptional activation domain-1; *AF-2,* transcriptional activation domain-2. *ΔSH3,* polyproline motif mutation (421–428 amino acids); *C587A,* DNA-binding mutation. *B,* MCF-7M13 cell lines expressing these constructs were treated with ORG2058 (10 nmol/L) or vehicle (ethanol) for 8 h and harvested for protein analysis. Protein lysates were resolved by SDS-PAGE and immunoblotted with a specific PR monoclonal antibody (1294/H9). T-47D cells treated under the same conditions were used as a marker of levels of PR expression. β-Actin was used as a loading control. *C,* representative images of subcellular localization of PRB and PRA. Cells treated with ORG2058 (10 nmol/L) or vehicle for 8 h were fixed on chamber slides and visualized by indirect immunofluorescence staining using a specific PR monoclonal antibody (1294/H9). Fluorescence images were acquired using a Zeiss inverted fluorescence microscope (×40 objective). PR expression was analyzed by taking the arbitrary ratio of nuclear/cytoplasmic PR fluorescence using the Image J software package. *D,* the intensity of nuclear/cytoplasmic PR staining was analyzed for each cell line in the presence or absence of ORG2058 (10 nmol/L). *Columns,* relative ratios of nuclear/cytoplasmic PR; *bars,* SD.

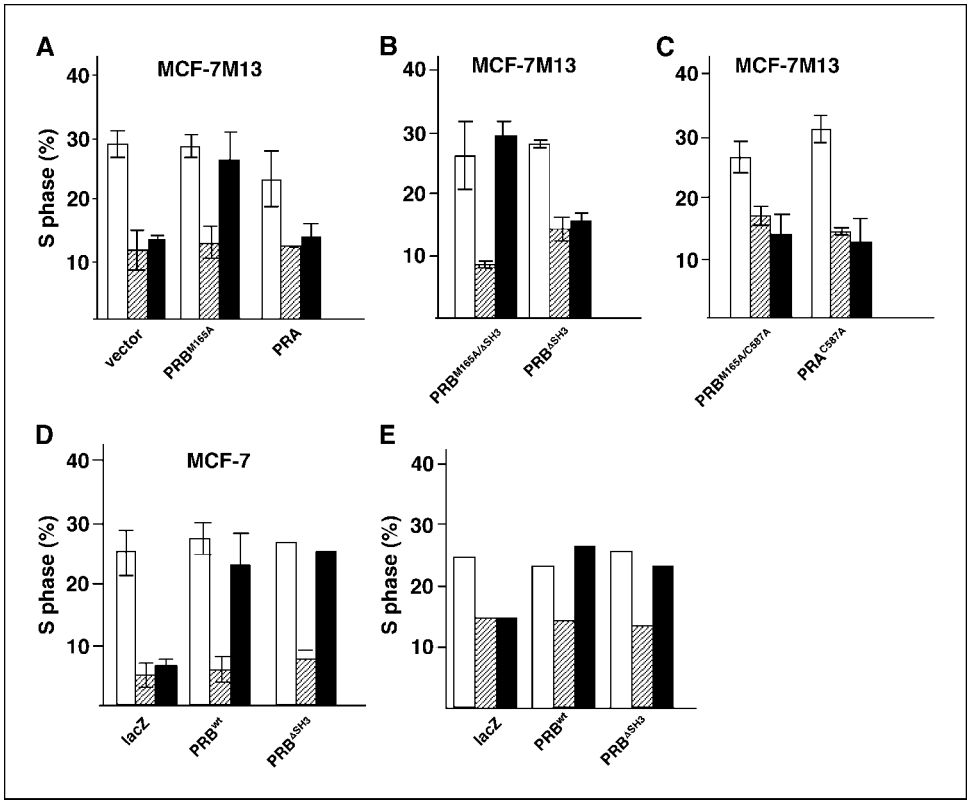

Figure 3. Reversal of antiestrogen-mediated cell cycle inhibition in MCF-7M13 cells expressing PR variants. *A* to *C,* MCF-7M13 cells stably expressing PRB^{M165A}, PRA, the indicated mutants, or vector control were treated with ICI 182780 (10 nmol/L) or ethanol vehicle for 36 h. Cells were subsequently treated with ORG2058 (10 nmol/L) or 17β-estradiol (100 nmol/L) or vehicle (ethanol) for a further 24 h and harvested for flow cytometry. MCF-7 cells ectopically expressing PRB, $PRB^{\Delta SH3}$, or lacZ (control) were pretreated with either ICI 182780 (*D*) or 1 μmol/L of 4-hydroxytamoxifen (*E*) and then treated with ORG2058 as described for MCF-7M13 cells, in parallel experiments. *Columns,* mean percentage of cells in S phase after ORG2058 treatment of three independent experiments; *bars,* SD (*D,* 24 h) or representative data from one of four experiments (*E,* 15 h). *Open columns,* vehicle; *hatched columns,* ICI 182780 (10 nmol/L) or 4-hydroxytamoxifen (1 μmol/L); *solid columns,* plus ORG2058 (10 nmol/L).

cell cycle progression under identical experimental conditions (Fig. 3*A–C*). Progestins also reinitiated cell cycle progression in cells expressing PRB mutated at the SH3 domain interaction motif ($PRB^{M165A/\Delta SH3}$). In contrast, MCF-7M13 cells expressing PRB with a mutation in the first zinc finger of the DNA-binding domain ($PRB^{M165A/C587A}$) were not released from cell cycle arrest following ORG2058 treatment (Fig. 3*C*). We confirmed these results with a previously described MCF-7 cell line constructed to stably express wild-type PRB, $PRB^{\Delta SH3}$, or empty vector (LacZ; Fig. 3*D*; ref. 30). In the absence of estrogen, endogenous PR is very low in these cells, as estrogen induction is required to elicit a progestin response (30). ICI 182780 inhibition was more effective in these cell lines than in MCF-7M13 cells (S phase = 5–10%) and progestin treatment again reinitiated cell cycle progression in cells expressing either wild-type PRB or $PRB^{\Delta SH3}$ but not in vector control MCF-7 cells that lack PR (Fig. 3*D*). Finally, to test that a similar effect was apparent when cells were arrested with tamoxifen, we conducted an experiment identical to that described in Fig. 3*D* following 48 h treatment with 1 μmol/L 4-hydroxytamoxifen. As described previously, tamoxifen was less potent than ICI 182780 in inhibiting S phase (36). However, the addition of ORG2058 to tamoxifen-treated MCF-7 cells expressing either PRB or $PRB^{\Delta SH3}$ reinitiated cell cycle progression but had no effect in empty vector cells (Fig. 3*E*). Hence, progestin reinitiation of cell cycle progression in antiestrogen-arrested cells was independent of the ability of PR to interact with and activate Src.

These data show that in the presence of structurally distinct antiestrogens, PRB (but not PRA) is sufficient to mediate stimulation of proliferation by ORG2058 in MCF-7 cells. Furthermore, the integrity of the first zinc finger in the DNA-binding domain is essential for progestin activation of cell cycle progression under these experimental conditions, suggesting that reversal of antiestrogen arrest is dependent primarily on the transcriptional activity of PRB.

Time course of progestin reversal of ICI 182780–mediated cell cycle arrest in MCF-7 and MCF-7M13 cells stably expressing PRA or PRB. We next measured the time course of progestin effects in ICI 182780–arrested MCF-7 and MCF-7M13 cells stably expressing different forms of PR to determine whether cells expressing PRA constructs initiated a transient proliferative response not evident at 24 h post-progestin treatment.

The distribution of cells in different phases of the cell cycle was determined by flow cytometry and the S phase fraction for each of the cell lines is presented in Fig. 4. In agreement with the data in Figs. 1 and 3, ICI 182780 treatment reduced S phase from ~30% to ~10% at 36 h in all cell lines and no significant changes were observed in these cells throughout the subsequent 24-h time course. Progestin did not stimulate MCF-7M13 PRA and $PRA^{\Delta SH3}$ cells, and combinations of RU486 and ORG2058 had no effect at the time points examined (Fig. 4*A*).

In contrast, at 12 h, the S phase was increased in PRB-expressing MCF-7 cells treated with ORG2058. By 21 h, S phase had increased to ~35%, and it remained significantly elevated at 24 h (Fig. 4*B*). Over this time period, cells did not show any evidence of an inhibitory response to progestin, as previously observed in T-47D cells at later time points (34, 35). In addition, the ORG2058-stimulated proliferative response was significantly enhanced in MCF-7 PRB cells compared with T-47D cells (~35–40% S phase compared with 20%, respectively; compare Fig. 1*A*–Fig. 4*B* and *C*). The antiprogestin RU486 abrogated this ORG2058-stimulated cell cycle progression. Progression from G_0/G_1 to S phase in ICI 182780–pretreated MCF-7 PRB cell lines was also observed 21 h after treatment with the synthetic progestin R5020 and this proliferative effect was repressed by RU486, as shown in

Fig. 4*C*. MCF-7 PRB$^{\Delta SH3}$ cells yielded results essentially identical to MCF-7 cells expressing wild-type PRB (Fig. 4*B* and *C*).

Effects of ICI 182780 on estrogen-regulated genes in breast cancer cells overexpressing PRB. The mechanisms of antiestrogen arrest of MCF-7 cells are well documented (4–6), as are the responses to estradiol (E_2) in antiestrogen-arrested cells (6). To elucidate the molecular mechanisms involved in progestin release from ICI 182780 inhibition, we first investigated whether the presence of unliganded PR could influence the effects of ICI 182780 on the levels of selected cell cycle regulatory proteins with a known role in estrogen/antiestrogen-regulated cell proliferation. Constitutive expression of PRB and PRB$^{\Delta SH3}$ in the absence of progestin did not affect the efficiency of ICI 182780–mediated growth arrest (Fig. 5*A*). Because early events in the growth-inhibitory response to ICI 182780 include down-regulation of ER and the cell cycle targets c-Myc and cyclin D1, we determined if increased PRB or PRB$^{\Delta SH3}$ expression influenced ICI 182780 regulation of these target genes.

As shown in Fig. 5*B*, ICI 182780 treatment resulted in the down-regulation of ER expression by 55% to 70% in MCF-7 and T-47D cells, and this was independent of constitutive expression of PR in MCF-7 cells. In agreement with previously published data (6), ICI 182780–treated cells showed a significant decrease in c-Myc protein levels (>90%) in both cell lines, which was also independent of constitutive PR expression (Fig. 5*B*). Cyclin D1 expression was also significantly reduced to 30% to 40% of control in MCF-7 and T-47D parent cell lines, but in MCF-7 cells, constitutive ectopic expression of PRB or PRB$^{\Delta SH3}$ attenuated ICI 182780 down-regulation of cyclin D1 (Fig. 5*B*). This did not seem to compromise the decrease in phosphorylation of Rb at Ser^{780} in MCF-7 and T-47D cells (Fig. 5*B*), characteristic of ICI 182780 treatment (37), or antiestrogen-induced growth arrest as assessed by decreased S phase (Fig. 5*A*).

Effects of estrogen and progestin on PR and ER expression. Because modulation of ER and PR expression, phosphorylation, and degradation are documented responses of breast cancer cells to estrogen and progestin agonists and antagonists (7), we assessed changes in ER and PR levels and phosphorylation in our models (Fig. 6*A* and *B*). Previous studies have suggested that in ER-positive cells, progestin-induced cell proliferation was mediated through an interaction between PRB and ER (38, 39). Thus, we also investigated the effects of ICI 182780, E_2, and ORG2058 on ERα levels. ORG2058 treatment of T-47D cells resulted in PR phosphorylation, as assessed by decreased electrophoretic motility, and subsequent degradation, whereas in MCF-7 cells expressing PRB or PRB$^{\Delta SH3}$, the characteristic upshift associated with phosphorylation was apparent without a subsequent decline in protein levels,

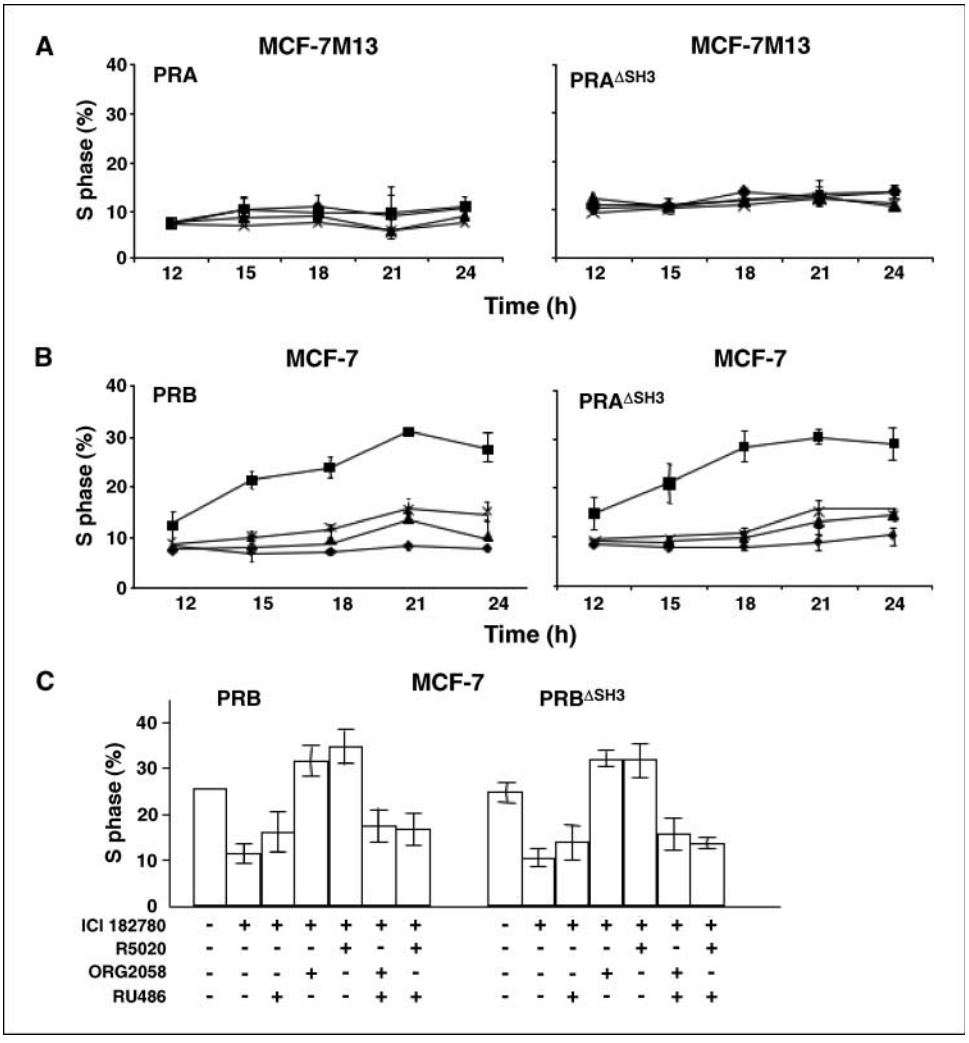

Figure 4. Kinetics of progestin-activated cell cycle progression in MCF-7M13 and MCF-7 cells stably expressing PRB or PRA constructs. *A,* MCF-7M13 cells expressing PRA or PRA$^{\Delta SH3}$. *B,* MCF-7 cells expressing PRB or PRB$^{\Delta SH3}$ were seeded at 1.5×10^5 cells in six-well plates in 5% FCS and insulin (1 μg/mL). Twenty-four hours after plating, cells were treated with ICI 182780 (10 nmol/L) or vehicle for 36 h. Cells were subsequently treated with ORG2058 (10 nmol/L), and/or RU486 (100 nmol/L), or vehicle for the time intervals indicated and harvested for flow cytometry. Percentage of cells in S phase at each time interval for each cell line: ORG2058, ■; vehicle, ◆; RU486, ▲; ORG2058 + RU486, ×. *C,* MCF-7 cells expressing PRB or PRB$^{\Delta SH3}$ were treated as described above, or with R5020 (10 nmol/L) as indicated. *Columns,* the mean percentage of cells in S phase at 21 h for at least four experiments; *bars,* SD.

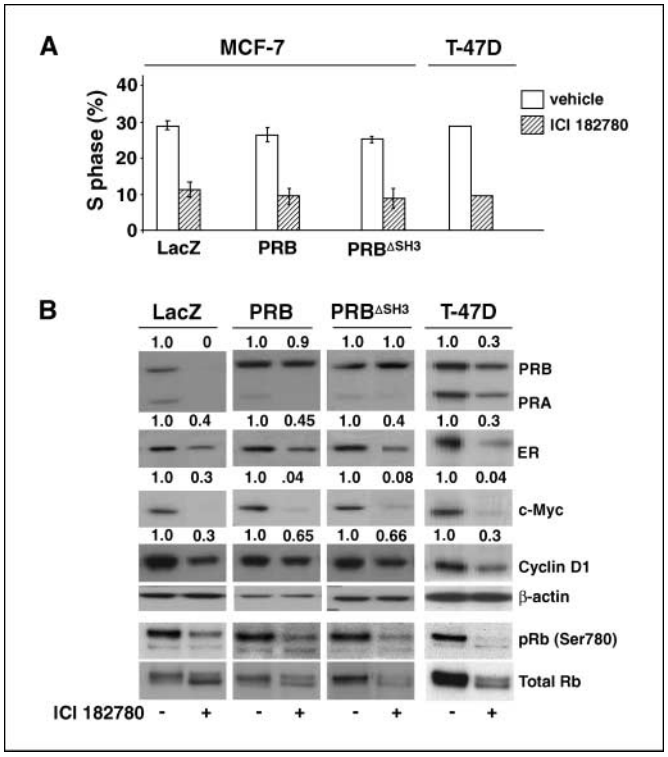

Figure 5. ICI 182780 down-regulates endogenous expression of PRB, ER, cyclin D1, and c-Myc proteins. MCF-7 cells constitutively expressing lacZ, PRB, or $PRB^{\Delta SH3}$ and T-47D cells were treated with ICI 182780 (10 nmol/L) or vehicle in the presence of 5% FCS and insulin (1 μg/mL), and harvested for flow cytometry and protein lysates at 48 h. *A,* percentage of cells in S phase for MCF-7 control cells (lacZ), cells expressing PRB or $PRB^{\Delta SH3}$, and T-47D: *columns,* means of triplicate experiments; *bars,* SD (*open columns,* vehicle; *hatched columns,* ICI 182780). *B,* representative Western blots of PR, ERα, c-Myc, and cyclin D1 for ICI 182780 or vehicle-treated cell lines; the relative change in protein levels is presented above each lane (vehicle treated cells = 1), using β-actin as a loading control. Relative changes in total Rb and Rb phosphorylated at Ser^{780} [pRb (Ser^{780})] expression for each treatment group and cell line are shown. All experiments were done in triplicate and showed similar expression profiles.

presumably as a consequence of continuous expression from the exogenous vector (Fig. 6*A* and *B*). In all cell lines, administration of E_2 resulted in a 2- to 3-fold increase in ERα (which depleted ERα, see Fig. 5) compared with ICI 182780 alone (Fig. 6*A* and *B*). This was unexpected, as in the absence of ICI 182780, E_2 down-regulated ERα (data not shown). In contrast, progestin treatment had no discernable effect on ERα levels in ICI 182780–pretreated cells. In summary, estrogen augmented ER expression in ICI 182780–pretreated MCF-7 and T-47D cells, whereas progestin had no effect in MCF-7 cell lines.

Both progestin and estrogen stimulate the cyclin D1-Rb pathway. Cyclin D1 is a key cell cycle regulatory molecule previously identified as a major target of progestin action in PR-positive T-47D cells (40). Cyclin D1 protein expression was increased >2-fold after ORG2058 treatment of T-47D cells pretreated with ICI 182780 and this was associated with an increase in Rb phosphorylation at Ser^{780} and in total Rb (Fig. 6*A*), consistent with previous data in the absence of ICI 182780 pretreatment (41). By 21 h, levels of cyclin D1 declined in these cells, and remained at levels similar to control cells pretreated with ICI 182780 alone. This was concomitant with the decrease in PR expression (Fig. 6*B*) and return of S phase to control levels (Fig. 1*C*). A minor, 1.2-fold increase in cyclin D1 was evident in E_2-treated cells at 8 h, and no change in Rb was observed at this time point (Fig. 6*A*). However, by 21 h, a >2-fold increase in cyclin D1 and Rb was evident (Fig. 6*B*). This delay in cyclin D1 induction corresponded with a delay in cell cycle re-entry from G_0/G_1 phase to S phase compared with progestin-treated cells (data not shown).

Cyclin D1 mRNA and protein were up-regulated by progestin treatment ~2-fold by 8 h in MCF-7 cells constitutively expressing ectopic PRB or $PRB^{\Delta SH3}$ (Fig. 6*A*; data not shown) and this increase was maintained at 21 h (Fig. 6*B*) in the presence of ORG2058 and ICI 182780. A similar 2-fold increase in cyclin D1 expression was observed in E_2-treated cells at 8 and 21 h in all cell lines examined (Fig. 6*A* and *B*). Cyclin D1 induction, through estrogen or progestin stimulation, was associated with increased phosphorylation of Rb at Ser^{780} (Fig. 6*A* and *B*). No significant increases in cyclin D1 expression or Rb phosphorylation were observed in progestin-treated MCF-7 cells that lack PR expression (LacZ control cells).

Differential regulation of c-Myc by progestin and estrogen. c-Myc is a key regulator of steroid-induced cell proliferation in both MCF-7 and T-47D cells (6, 40). In ICI 182780–arrested T-47D cells, treatment with ORG2058 increased expression of c-Myc by ~2.8-fold at both 8 and 21 h, whereas a >5-fold increase was observed following E_2 treatment at the same time points (Fig. 6*A* and *B*). This increase in c-Myc was maintained in ORG2058-treated T-47D cells (Fig. 6*B*), although the fraction of S phase cells had returned to ICI 182780–treated levels at this time (see Fig. 1*A* and *C*). In contrast, levels of c-Myc were maintained or slightly increased at 21 h in estrogen-treated cells concurrent with an increase in S phase (Fig. 6*B* and *C*).

In MCF-7 cells stably transfected to constitutively express PR, the addition of estrogen to ICI 187280–arrested cells significantly increased c-Myc levels as early as 2 h (data not shown) and these elevated levels (>10-fold) were maintained at 8 and 21 h in all MCF-7 cell lines (Fig. 6*A* and *B*). ORG2058 treatment caused only a minor increase of c-Myc expression in ICI 182780–arrested MCF-7 cells at 8- and 21-h time points (<2-fold; Fig. 6*A* and *B*). These data support a key role for cyclin D1/Rb in progestin reinitiation of cell cycle progression in ICI 182780–arrested breast cancer cells, whereas c-Myc seems to be less important. In contrast, estrogen-induced cell cycle progression of ICI 182780–arrested cells is associated with increased expression of both c-Myc and cyclin D1 (Fig. 6).

Discussion

Acquired resistance to antiestrogens is a major issue in breast cancer management, but the mechanisms underlying antiestrogen insensitivity are not fully defined (1). Normal cell proliferation in the breast is regulated by estrogen and progesterone, acting via their cognate receptors, and breast cancer is linked to the disruption of receptor expression and function. The pure antiestrogen ICI 182780 is effective in initiating and maintaining cell cycle arrest in breast cancer cells by rapid degradation of ER, decreased expression of c-Myc and cyclin D1, and exit from the cell cycle (4–6). Because PR is an estrogen-regulated gene, one of the possible positive outcomes of ER depletion is that endogenous PR is also depleted, and hence, PR-mediated activation of cell proliferation is reduced. However, our results clearly show that when endogenous PR was highly expressed, e.g., in T-47D cells, ICI 182780 did not totally deplete PR, and progestins could reinitiate cell cycle progression.

Of potential clinical importance is the observation that aberrant expression of PRA and PRB is symptomatic of hormone-dependent breast cancers (23). When we examined the effects of progestin in antiestrogen (ICI 182780 or SERM)–treated breast cancer cells, we observed that PRB, but not PRA, could mediate a significant proliferative response. Interestingly, constitutive expression of PRB in MCF-7 cells not only allowed the progestin-proliferative response in the presence of ICI 182780, but also enhanced and prolonged S phase entry compared with T-47D cells. We propose that this effect is a direct consequence of high PRB expression and is consistent with the observations that PRB is essential for mammary development, proliferation, and differentiation, whereas PRA is not (20) and that carriers of the PR +331 *A* allele, which leads to increased expression of PRB, are at increased risk of breast cancer (27). Hence, aberrant PRB expression may have a causative role in breast cancer development and/or progression, or in the context of this study, decrease the ability of antiestrogens and other endocrine treatments to maintain growth arrest in patients with breast cancer. The latter hypothesis is not consistent with a study of PR isoform expression in tamoxifen-treated patients in whom a high PRA/PRB ratio, predominantly caused by high PRA levels, was associated with early patient relapse (42). However, expression of high levels of PRA alone confer a more invasive phenotype on breast cancer cells which may, in part, explain these findings (25). Clearly a more extensive analysis of the potential role of PR isoform expression in breast cancer prognosis and response to therapy is warranted but is currently impeded by the lack of isoform-specific antibodies suitable for assessment of expression in the context of tamoxifen response.

A PR mutant with a disabled DNA-binding domain was unable to mediate the progestin-induced reinitiation of cell cycle progression in antiestrogen-arrested cells, whereas mutations in the NH_2-terminal, SH3 domain interaction motif had no effect on this response. Thus, progestin stimulation of antiestrogen-arrested cells is mediated primarily by the nuclear transcriptional activity of PR and not by its rapid activation of c-Src signaling. This result contrasts with previous studies (43, 44) showing that rapid progestin-induced activation of the Src/mitogen-activated protein kinase (MAPK) signaling pathway, mediated by PR interaction with SH3 domain of c-Src, was required for progestin induction of cyclin D1 expression and cell cycle progression. One possible explanation for this apparent discrepancy is that progestin treatment in the

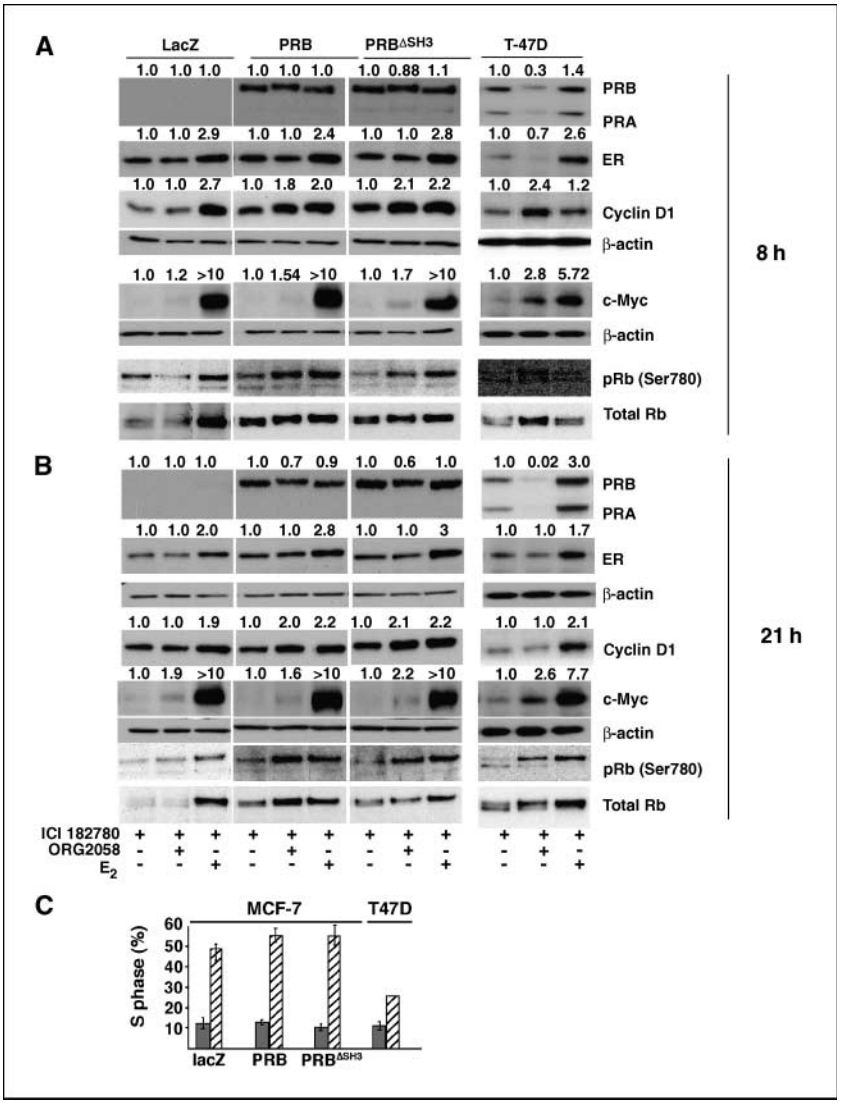

Figure 6. Effects of estrogen and progestin on PR, ER, and key cell cycle regulatory proteins (cyclin D1, c-Myc, and Rb) in the presence of ICI 182780. MCF-7 cells stably transfected to express lacZ, PRB or $PRB^{\Delta SH3}$, or T-47D cells were treated with ICI 182780 (10 nmol/L) or vehicle in the presence of fetal bovine serum and insulin (1 μg/mL) for 48 h and subsequently treated with ORG2058 (10 nmol/L), 17β-estradiol (E_2, 100 nmol/L) or vehicle, and harvested at 8 and 21 h for flow cytometry or for preparation of cell lysates. Representative Western blots of cell lysates for 8 h (*A*) and 21 h (*B*). Expression of PR, ERα, c-Myc, cyclin D1, total Rb, and Rb phosphorylated at Ser^{780} [pRb (Ser^{780})] for each treatment and cell line was visualized by Western blotting. The relative change in protein expression is shown above each of the lanes (ICI 182780–treated cells = 1). β-Actin was used as a loading control. Experiments were done in triplicate (MCF-7) or duplicate (T-47D) and showed similar expression profiles. *C,* cells were treated with ICI 182780 as described above and harvested for flow cytometry 21 h after treatment with 17β-estradiol. *Columns,* means of at least three independent experiments; *bars,* SD (*solid columns,* ICI 182780; *hatched columns,* ICI 182780 + 17β-estradiol).

present context occurred in a mitogen-rich environment (i.e., in the presence of whole serum, endogenous growth factors, and insulin), whereas the previous studies (43, 44) were conducted under growth factor–depleted conditions. Thus, progestin/PR-dependent induction of cell cycle progression may be mediated through multiple pathways and mechanisms dependent on the cellular environment. An alternative mechanism by which progestins can mediate rapid activation of Src/MAPK pathways has been reported to occur through a preformed PR/ER/Src complex, in which the progestin signal is transmitted to Src through unliganded ER (38, 39). However, in previous studies, cell lines were employed that lacked ER and only expressed various PRs, suggesting that PR activation of Src/MAPK through direct coupling of PR with SH3 domains is sufficient to mediate cyclin D1 expression and cell proliferation in the absence of ER (44). Because most target tissues express both ER and PR, and estrogens and progestins can each induce rapid activation of Src/MAPK (45), an important unresolved issue is how ER and PR integrate with cytoplasmic signaling pathways. In previous studies (38, 39), antiestrogens blocked progestin-induced activation of Src/MAPK and proliferation in cells that express both ER and PR. However, in the present study, progestin reinitiated cell cycle progression in the presence of antiestrogen in cells that express both ER and PR. Thus, the mechanism(s) and role that progestin/PR–induced rapid activation Src/MAPK plays in cell proliferation remains to be more completely defined.

Previous studies have shown that the regulation of cyclin D1 and cyclin E–activated cyclin-dependent kinases plays a critical role in exit from quiescence and the deregulated cell cycles of cancer cells (46), and that cyclin D1 is a critical element of progestin regulation of breast cancer cell proliferation (31, 40, 41). The introduction of PRB into MCF-7 cells compromised ICI 182780 down-regulation of cyclin D1; however, the phosphorylation of Rb decreased and cells ceased to proliferate. In concordance with previous reports, reactivation of the cyclin D1-Rb pathway was sufficient to reinitiate cell cycle progression. Moreover, progestin treatment had little effect on c-Myc expression and no effect on ER expression. In contrast, estrogen treatment was associated with significant increases in c-Myc and cyclin D1 expression and accelerated transition of cells from G_0/G_1 phase to S phase. Although the mechanisms underlying the progestin-induced release from antiestrogen inhibition are not well-defined and need to be further studied, we propose, based on the present results, the existence of two independent but interconnecting pathways of hormone-mediated cell cycle progression for progestins and estrogens in breast cancer cells. Progestins and estrogens both stimulate cyclin D1 expression, and hence, activate the cyclin D1-Rb pathway, whereas estrogen also stimulates cells by reactivation and maintenance of ER and c-Myc expression, which further activates the Rb pathway via cyclin E/Cdk2 (47).

In conclusion, we propose that the potential of breast cancer cells to become desensitized to antiestrogen inhibition may be dependent in part on the total and relative abundance of PR isoforms. If the responses documented here also operate *in vivo*, circulating hormones present in premenopausal women or as part of hormone replacement therapy regimens have the potential to stimulate cell cycle progression in cells that express high levels of PRB. Furthermore, high PRB in the presence of progestins can attenuate growth arrest induced by antiestrogens. This could have major clinical implications in the treatment of hormone-dependent breast cancer with endocrine agents targeting ER. However, our data showing that this effect of progestin is blocked by the progestin antagonist, RU486, suggests that some progestin antagonists (48, 49) might have therapeutic utility in this context.

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