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Elevated Expression of the Estrogen Receptor Prevents the Down-Regulation of $p21^{Waf1/Cip1}$ in Hormone Dependent Breast Cancer Cells

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Abstract Expression of an estrogen receptor α (ER) transgene in hormone independent breast cancer and normal breast epithelial cells arrests cell cycling when estradiol is added. Although endogenously expressed ER does not typically affect estradiol-induced cell cycling of hormone dependent breast cancer cells, we observed that elevated expression of a green fluorescent protein fused to ER (GFP-ER) hindered entry of estrogen treated MCF-7 cells into S phase of the cell cycle. In analyses of key cell-cycle regulating proteins, we observed that GFP-ER expression had no affect on the protein levels of cyclin D1, cyclin E, or p27, a cyclin dependent kinase (Cdk) inhibitor. However, at 24 h, p21 (Waf1, Cip1; a Cdk2 inhibitor) protein remained elevated in the high GFP-ER expressing cells but not in non-GFP-ER expressing cells. Elevated expression of p21 inhibited Cdk2 activity, preventing cells from entering S phase. The results show that elevated levels of ER prevented the down-regulation of p21 protein expression, which is required for hormone responsive cells to enter S phase. J. Cell. Biochem. 93: 619–628, 2004.

Key words: estrogen receptor; $p21^{Waf1/Cip1}$; cyclin dependent kinase 1

Estrogens are intimately linked to the development and progression of breast cancer. Proliferation of hormone responsive breast cancer cells is dependent upon estrogens. Estrogens bind to the estrogen receptor α (ER) which regulates the expression of several genes involved in cell-cycle progression [Charpentier et al., 2000; McKenna and O'Malley, 2002; Doisneau-Sixou et al., 2003]. The addition of estradiol to hormone responsive cells cultured under estrogen deplete conditions initiates a series of events resulting in cells transitioning from G_1 to S phase of the cell cycle. The expression of the immediate-early gene c-*myc* is increased followed by the delayed-early genes, cyclin D1, and cyclin E [Dubik and Shiu, 1988; Doisneau-Sixou et al., 2003]. Cyclin D1–Cdk4 activity, which phosphorylates Rb, is increased, while cyclin E–Cdk2 kinase activity is initially inhibited by Cdk inhibitors (CDKIs) $p21^{Waf1/Cip1}$ and p27. The CDKIs p21 and p27 have important roles in the maturation of cyclin D1–Cdk4 [Cheng et al., 1999; Roovers and Assoian, 2000]. Further, cyclin D1–Cdk4 sequesters p21 and p27 away from cyclin E–Cdk2, which has a key role in the ER signaling pathway in driving cells from G_1 to S phase of the cell cycle [Prall et al., 2001]. In breast cancer cells, $p21^{Waf1/Cip1}$ has a principal role in regulating cyclin E–Cdk2 activity [Prall et al., 2001].

Several studies have explored the role of ER in gene expression and cell proliferation by transfecting ER into human ER positive and negative breast cancer cells and nontumorigenic breast epithelial cells. Expression of the ER transgene did not typically affect the estrogen-induced cell-cycle progression of hormone responsive breast cancer cells. In contrast, estrogen arrested the proliferation of ER negative breast cancer cells and nontumorigenic breast epithelial cells expressing the ER transgene [Jiang and Jordan, 1992; Ince et al.,

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1993; Zajchowski et al., 1993; Jeng et al., 1994; Levenson and Jordan, 1994; Lundholt et al., 1996; Pilat et al., 1996; Wang et al., 1997; Planas-Silva et al., 1999].

We isolated an ER positive, hormone responsive breast cancer cell line stably expressing an inducible green fluorescent protein fused to ER (GFP-ER) transgene to study the subnuclear trafficking and turnover of ER in breast cancer cells [Zhao et al., 2002]. The fusion of the tracking protein GFP did not compromise the functional properties of ER, with GFP-ER functioning as a ligand activated transcription factor responding to agonist and antagonist ligands [Htun et al., 1999]. Further, the ligand dependent sub-cellular trafficking of GFP-ER to the nuclear matrix and the ligand dependent protein half-life of GFP-ER were identical to that of ER [Htun et al., 1999; Stenoien et al., 2000; Zhao et al., 2002]. During the course of these studies we observed that estrogen-induced cell-cycle progression at 24 h following the addition of estradiol was hindered in cells expressing high levels of GFP-ER. Cells expressing low levels of GFP-ER were not affected. These observations suggested a requirement for hormone responsive breast cancer cells to reduce ER levels to progress through the cell cycle.

In this study, we analyzed estrogen regulated proteins with roles in the control of G_1/S progression to identify the molecular event(s) being perturbed by elevated levels of ER in MCF-7 hormone responsive breast cancer cells. We report that GFP-ER expression did not alter the levels of p27 or the temporal estradiol-induced increase and subsequent decrease in c-*myc*, cyclin D1, and cyclin E protein levels. Elevated levels of GFP-ER, however, prevented the lowering of p21 protein levels, which hindered the entry of MCF-7 cells into the cell cycle.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions

MCF-7 (ER positive and hormone dependent) human breast cancer epithelial cell lines were prepared that stably express GFP-ER under regulation of the tetracycline-on system [Zhao et al., 2002]. Clone 89 was obtained by stably transfecting MCF-7 with the pUHD172-1 neo plasmid that has the sequence for the reverse tetracycline repressor (rtTA) fused to the activating domain of VP16 protein. Clone 11 was obtained by stably transfecting clone 89 with the plasmid, pUHD10-3, which has GFP-ER under the control of a tetracycline-inducible promoter.

Clone 11 and clone 89 were maintained as previously described [Zhao et al., 2002]. Cells were cultured in phenol red free Dulbecco's modified Eagle medium (PRF-DMEM) with 7% 2× charcoal stripped fetal bovine serum (2× CS-FBS). Doxycycline (Dox) at 0.1, 0.5, and 1.0 μg/ml was added in medium while cells were reseeded. The estrogen response pathway was initiated by the addition of estradiol (10 nM). For protein isolation, cells were lyzed on tissue culture plates. Alternatively for cell-cycle analyses, cells were released from the plates by trypsin digestion, fixed in 70% ethanol, digested with DNase-free RNase A and stained with propidium iodide before analysis by fluorescent-activated cell sorting [Chadee et al., 1999].

Gel Electrophoresis and Immunoblotting

Lysates from equal number of cells (0.5–1×10^5 cells) were resolved on SDS 10% polyacrylamide gels. Immunoblot analyses and ECL detection were done as previously described [Chadee et al., 1995, 1999]. The concentrations of antibodies were approximately 0.5 μg/ml in TTBS buffer. Antibodies used in this study were human cyclin D1 (PharMingen 14561C mouse monoclonal), human cyclin E (PharMingen 14591A mouse monoclonal), human Cdk2 (M2) (Santa Cruz sc163 rabbit polyclonal), mouse p27 (F-8) (Santa Cruz sc1641 mouse monoclonal), mouse p21 (F-5) (Santa Cruz sc6246 mouse monoclonal), human c-*myc* (C33) (Santa Cruz sc42 mouse monoclonal), and phospho-histone H1b (rabbit polyclonal; a kind gift from Dr. David Allis). Quantification of immunodetected bands was done as described previously [Zhao et al., 2002].

Immunoprecipitation

About 2×10^7 cells were lysed in 0.5 ml lysis buffer containing 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% (v/v), Triton X-100, 10% glycerol, 1 mM PMSF, and 1 tablet/50 ml complete EDTA-free protease inhibitor cocktail (Roche, Laval, Canada). The cells were sonicated in lysis buffer 3×15 s and insoluble cell debris was removed by centrifugation. Anti-Cdk2 antibody (5 μg/ml in PBS buffer, Santa Cruz M2-SC-163) was incubated with

25 μl of protein A (Pierce) and 25 μl of protein G (Pierce) at 4°C for 2 h with gentle rocking. The beads were washed three times with PBS. Cell lysate (3 mg) was pre-cleared with 25 μl of protein A (Pierce) and 25 μl of protein G (Pierce) at 4°C for 30 min. The pre-cleared cell lysate was incubated with the anti-Cdk2–bead complex at 4°C for 4 h with gentle rocking. The beads were washed three times with lysis buffer. The bound proteins were eluted from the beads by adding 50 μl of 2× SDS loading buffer and boiling for 5 min. The beads were collected by centrifugation, and the protein analyzed by SDS–polyacrylamide gel electrophoresis.

RESULTS

Elevated Expression of GFP-ER Hinders Cycling of Estradiol-Stimulated Breast Cancer Cells

MCF-7 clone 11 breast cancer cells stably express GFP-ER regulated by the Dox-on system [Zhao et al., 2002]. In the absence of Dox, there was no detectable expression of GFP-ER (Fig. 1). GFP-ER expression was elevated in response to increasing concentrations of Dox with maximal expression of GFP-ER being obtained at 1.0 μg/ml Dox [Zhao et al., 2002] (Fig. 1B). Twenty-four hours following the addition of estradiol to MCF-7 clone 11 cells resulted in the reduction of GFP-ER and endogenous ER levels (Fig. 1). This observation is in accord with our previous observations that estradiol reduced the stability of GFP-ER and ER proteins [Zhao et al., 2002]. Independent of ligand, increasing the level of GFP-ER reduced the expression of ER (Fig. 1). Our previous report demonstrated that enhanced expression of GFP-ER lowered not only the levels of ER protein but also that of ER mRNA, suggesting a negative feedback loop to regulate ER levels [Zhao et al., 2002].

To stimulate the entry of MCF-7 cells into the cell cycle, estradiol was added to the clone 11 cells which had been cultured under estrogen deplete conditions (Fig. 2). A large population of cells had moved into S phase following 24 h in the presence of estradiol. However, estradiol failed to stimulate the cell-cycle progression of Dox treated clone 11 cells. To determine whether Dox was affecting estradiol induced cell cycle entry, clone 89 cells, which express the rtTA-VP16 fusion protein but not GFP-ER, were analyzed. Figure 3 shows that Dox did not affect the estrogen stimulated cycling of clone 89 cells.

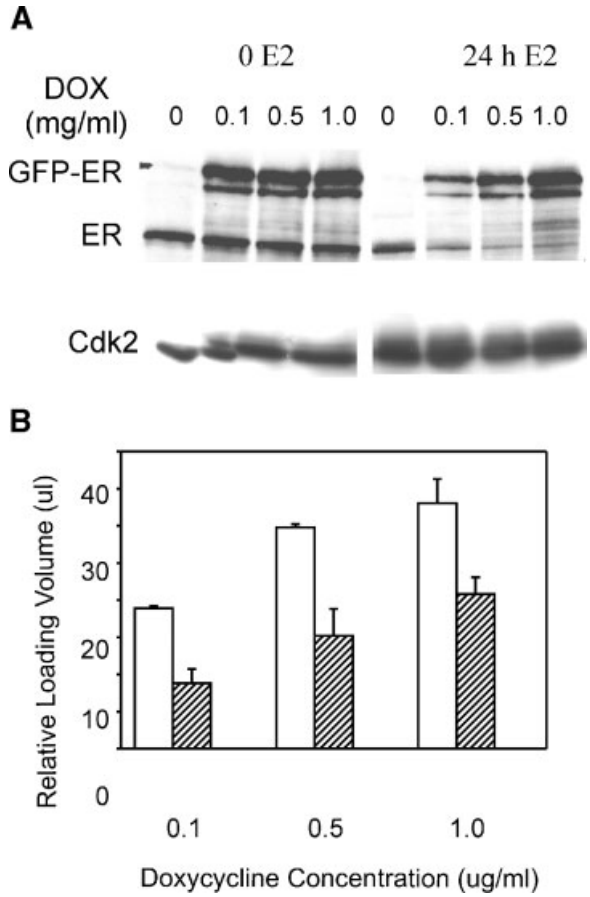

Fig. 1. Green fluorescent protein fused to ER (GFP-ER) protein levels in MCF-7 human breast cancer cells. **Panel A**: Clone 11 cells were cultured in phenol red free Dulbecco's modified Eagle medium (PRF-DMEM) containing 7% 2× charcoal stripped fetal bovine serum (2× CS-FBS). Different concentrations of DOX were added to induce GFP-ER expression. Estradiol (10 nM) was added for 24 h before harvesting. Proteins in whole cell lysates from equal number of cells were resolved by 10% polyacrylamide SDS gel electrophoresis followed by immunoblot analyses with a mouse anti-ER antibody. **Panel B**: Intensities of the immunoreactive bands detected by ECL were captured on a Image Station 440CF (Kodak Digital Science™) and the net intensities of the GFP-ER ECL signals were determined using 1 D Image Analysis Software (Kodak Digital Science™). Open bars represent GFP-ER levels in cells grown with different concentrations of DOC without estradiol. Hatched bars represent GFP-ER levels in cells grown with different concentrations of DOC with estradiol for 24 h. The results shown are the means ± standard error of the mean for three separate experiments.

Further analyses of the effect of Dox on estradiol stimulated clone 11 S phase progression revealed a relationship between the amount of Dox induced GFP-ER expression (Fig. 1B) and the extent of inhibition of cell cycling at 24 h post-estradiol administration (Fig. 3). Under estrogen deplete growth conditions, manipulating the expression levels of

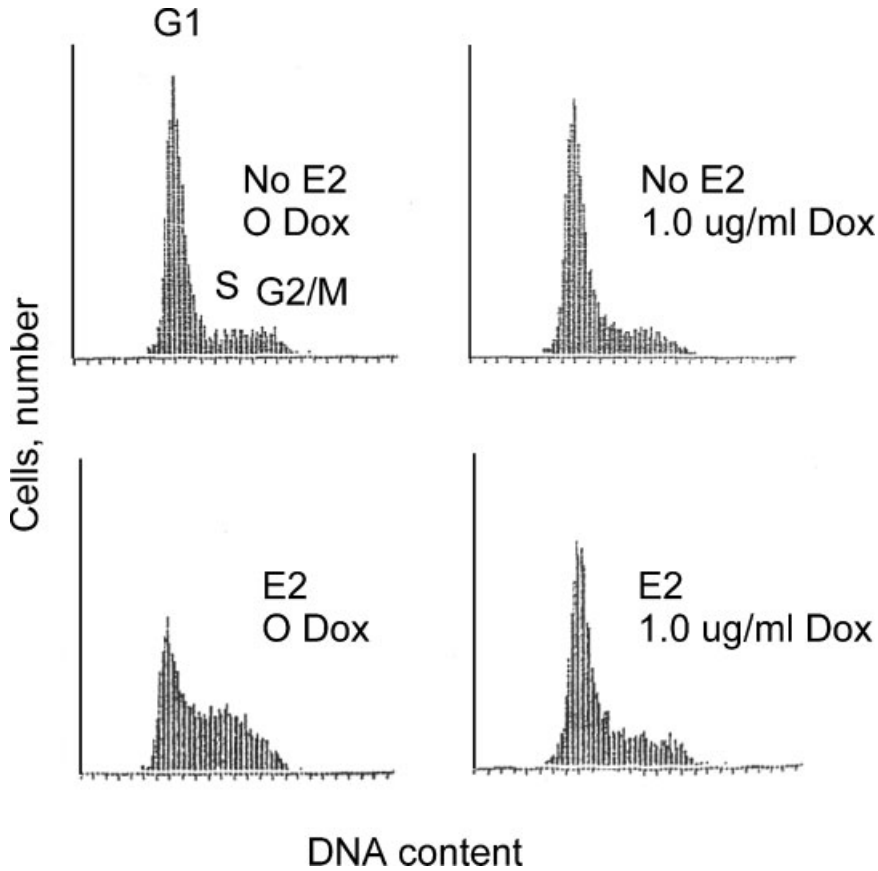

Fig. 2. Effect of GFP-ER expression on cell-cycle progression. Clone 11 cells incubated with and without 1.0 μg/ml doxycycline (DOX) were cultured with and without 10 nM estradiol (E2) for 24 h. DNA content in the propidium iodide-stained cells was determined by fluorescent-activated cell sorting. The number of cells is represented on the y axis, and the amount of DNA (fluorescence intensity) is shown on the x axis.

GFP-ER had no significant affect on the clone 11 cell-cycle distribution (Fig. 3).

The maximal expression of the Dox-inducible GFP-ER varied among the different clone 11 cultures, and occasionally we had a cell culture that expressed a low level of GFP-ER in response to 1.0 μg/ml Dox. The S phase entry of these GFP-ER poorly expressing cells treated with estradiol and 1.0 μg/ml Dox was minimally affected (data not shown). This is in marked contrast to clone 11 cells expressing high levels of GFP-ER. These observations suggest that elevated levels of GFP-ER is responsible for the stalled entry of the estradiol-stimulated clone 11 cells into S phase of the cell cycle.

Manipulation of GFP-ER Levels Does Not Alter c-*myc*, Cyclin D1, and Cyclin E Biphasic Expression

To determine how elevated expression of GFP-ER was perturbing the cell cycle of estradiol-stimulated clone 11 cells, we studied the expression of proteins with key roles in the cell cycle. C-*myc* and cyclin D1 are estradiol induced genes, the products of which are involved in cells progressing from G_1 to S phases of the cell cycle. In immunoblot experiments, the relative levels of these gene products at 0, 8, and 24 h post-estradiol addition to clone 89 and clone 11 cells cultured with 0, 0.1, 0.5, and 1.0 μg/ml Dox were determined. Figure 4A shows that c-*myc* levels were increased at 8 h and reduced at 24 h in clone 89 and clone 11 cells independent of Dox levels. Similarly, the levels of cyclin D1 were increased at 8 h and lowered at 24 h in clone 89 and clone 11 cells (Fig. 4B).

Cyclin E levels in clone 89 and clone 11 cells were also investigated. The levels of cyclin E were slightly increased at 8 h post-estradiol addition relative to the level of this protein in cells grown in estradiol deplete conditions

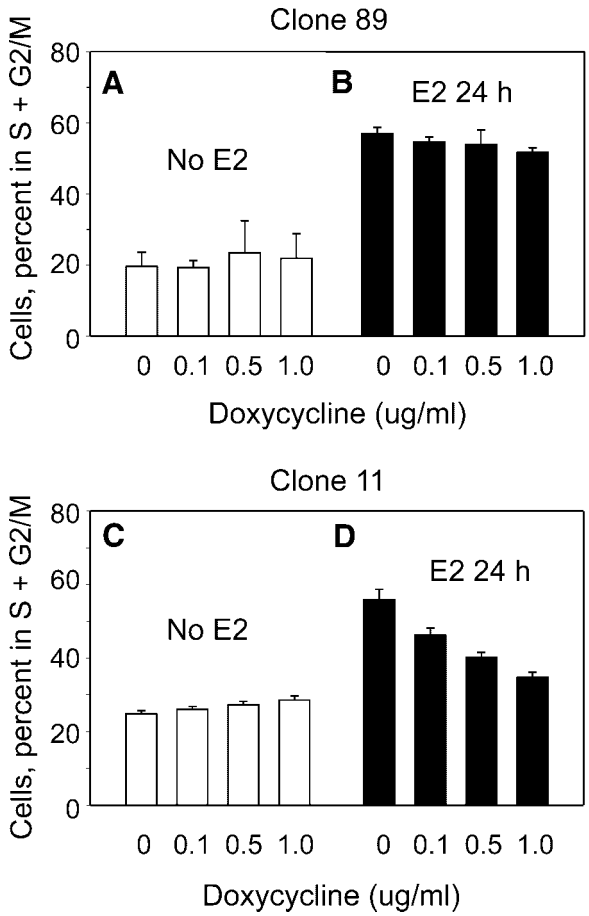

Fig. 3. Effect of increased GFP-ER expression on cell cycling. MCF-7 clone 89 and clone 11 cells were cultured in PRF-DMEM containing 7% 2× CS-FBS with increasing concentrations of Dox. Cells were cultured in the absence or presence of 10 nM estradiol (E2) for 24 h. DNA content and cell-cycle distribution in the propidium iodide-stained cells were determined by fluorescent-activated cell sorting. Addition of 10 nM E2 for 24 h results in an increase of cells into $S + G_2/M$ phase (**A** and **B**). The number of clone 11 cells in $S + G_2/M$ phases decreases as Dox concentration and GFP-ER expression were increased (**C** and **D**). The results shown are the means ± standard error of the mean for three separate experiments.

(Fig. 4C). At 24 h post-estradiol introduction, cyclin E levels declined in the clone 11 and clone 89 cells. Treatment of clone 11 cells with Dox did not affect the temporal expression of cyclin E.

The biphasic expression patterns observed for c-*myc*, cyclin D1, and cyclin E are similar to those previously described [Prall et al., 1997; Lai et al., 2001]. Our analyses revealed that elevated GFP-ER expression did not alter the temporal expression of these proteins following the stimulation of clone 11 cells with estradiol.

Elevated GFP-ER Expression Alters the Biphasic Expression of $p21^{Waf1/Cip1}$

The cyclin dependent kinase (Cdk) inhibitors p27 and p21 have key roles in the transition of cells from G_1 to S phase of the cell cycle. In immunoblot experiments, we determined the levels of p27 and p21 as a function of time following the addition of estradiol to clone 89 and clone 11 cells. In agreement with the results of others, Figure 5A shows that p27 levels declined following the addition of estradiol [Prall et al., 1997]. Elevated expression of GFP-ER in clone 11 cells (1.0 μg/ml Dox) did not affect this decrease in p27 levels.

Analyses of p21 showed that p21 levels were increased in clone 89 and clone 11 at 8 h post-estradiol addition (Fig. 5B). In clone 89 and Dox untreated clone 11 cells p21 levels dropped markedly at 24 h. However, the decline in p21 levels at 24 h in clone 11 cells was reduced as the Dox levels increased. At 24 h, the levels of p21 in the 1.0 μg/ml Dox treated clone 11 cells was similar to that of the 8 h levels.

The analyses of the Cdk inhibitors demonstrated that elevated GFP-ER expression affects p21 but not p27 levels. This analyses shows that elevated GFP-ER expression does not prevent estradiol-induced expression of p21, but it does prevent reduction in p21 levels.

Levels of Phosphorylated H1 Are Reduced in Cells Expressing Elevated Levels of GFP-ER

Others and we have reported that H1 histones are substrates for cyclin E–Cdk2 kinase [Bhattacharjee et al., 2001; Chadee et al., 2002]. In immunoblot experiments with an antibody against anti-phosphorylated H1 the levels of phosphorylated H1 at 0, 8, and 24 h post-estradiol addition were determined. Figure 6A shows that in clone 89 and clone 11 cells not treated with Dox phosphorylated H1 levels steadily increased as time with estradiol progressed. With 0.1 μg/ml Dox phosphorylated H1 levels increased at 8 h and increased slightly more at 24 h. In contrast, with clone 11 cells incubated with 0.5 or 1.0 μg/ml Dox there was an increase in phosphorylated H1 followed by a marked decline in phosphorylated H1 levels.

The levels of Cdk2 were also investigated in immunoblotting experiments. Figure 6B shows that the levels of Cdk2 were not altered by estradiol or Dox in clone 89 and clone 11 cells.

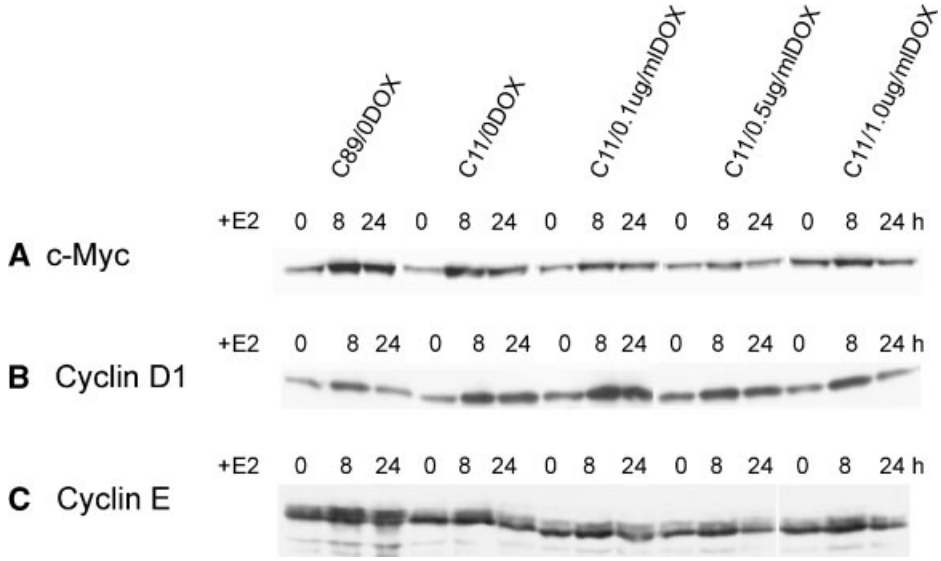

Fig. 4. Effect of GFP-ER expression on endogenous c-*myc*, cyclin D1, and cyclin E protein levels. MCF-7 clone 89 and clone 11 cells were cultured in PRF-DMEM containing 7% 2× CS-FBS. Increasing concentrations of Dox were added to induce GFP-ER expression. Cells were cultured in the absence or presence of 10 nM estradiol (E2) for 8 and 24 h. Proteins in whole cell lysates from equivalent cell numbers (0.5×10^5) were resolved by electrophoresis on 10% polyacrylamide SDS gels, immunoblotted and immunostained with an anti-c-*myc* antibody (**panel A**), an anti-cyclin D1 antibody (**panel B**) or an anti-cyclin E antibody (**panel C**). The figure shows a representative result of three separate experiments.

This result also serves as a control for the uniformity of protein loads in the blots shown in Figures 4–6.

The results demonstrated that phosphorylation of H1, an indirect measure of cyclin E–Cdk2 kinase activity, was reduced at 24 h post-estradiol addition when cells were expressing elevated levels of GFP-ER. Cdk2 levels remained constant. However, elevated expression of GFP-ER did not prevent the increased phosphorylation of H1 at 8 h post-estradiol administration in clone 11 cells.

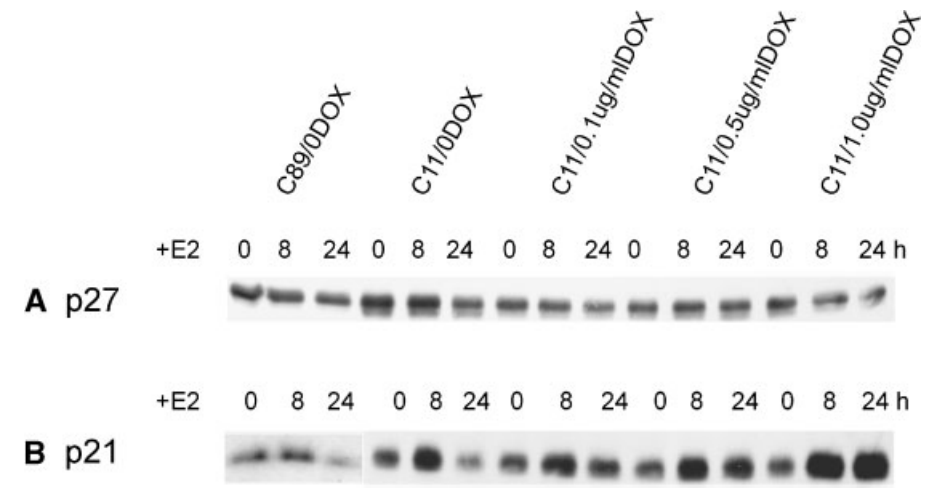

Fig. 5. Effect of GFP-ER expression on endogenous cyclin dependent kinase (Cdk) inhibitors p27 and p21. MCF-7 clone 89 and clone 11 cells were cultured in PRF-DMEM containing 7% 2× CS-FBS. Increasing concentrations of Dox were added to induce GFP-ER expression. Cells were cultured in the absence or presence of 10 nM estradiol (E2) for 8 and 24 h. Proteins in whole cell lysates from equivalent cell numbers (0.5×10^5) were resolved by electrophoresis on 10% polyacrylamide SDS gels, immunoblotted and immunostained with an anti-p27 antibody (**panel A**) or an anti-p21 antibody (**panel B**). The figure shows a representative result of three separate experiments.

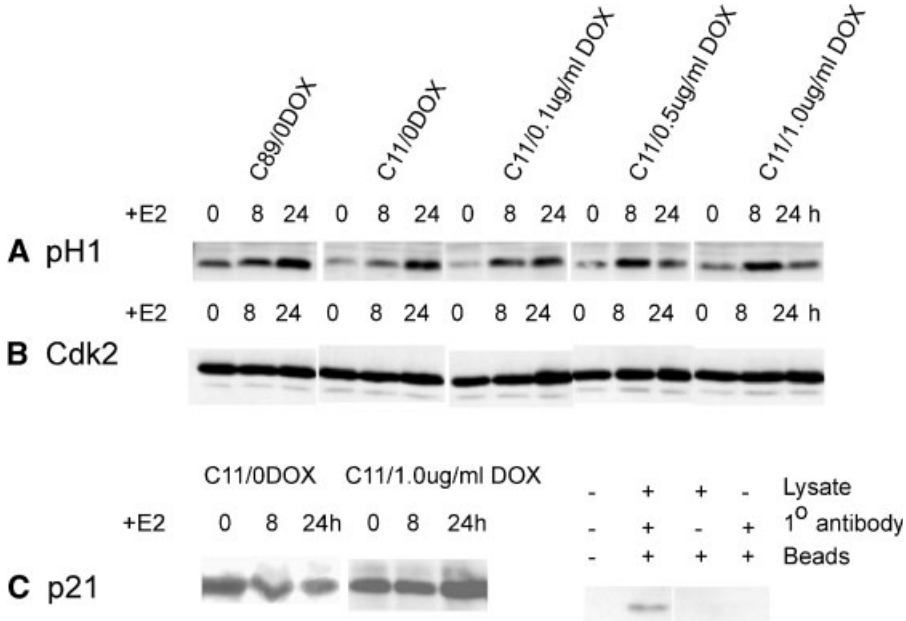

Fig. 6. Effect of GFP-ER expression on endogenous phosphorylated H1 and Cdk 2 protein levels. MCF-7 clone 89 and clone 11 cells were cultured in PRF-DMEM containing 7% 2× CS-FBS. Increasing concentrations of Dox were added to induce GFP-ER expression. Cells were cultured in the absence or presence of 10 nM estradiol (E2) for 8 and 24 h. Proteins in whole cell lysates from equivalent cell numbers (0.5×10^5) were resolved by electrophoresis on 10% polyacrylamide SDS gels, immunoblotted and immunostained with an anti-phosphorylated H1 antibody (**panel A**) or an anti-Cdk2 antibody (**panel B**). The figure shows a representative result of three separate experiments. **Panel C**: Cdk2 complexes were immunoprecipitated from cell lysates with an anti-Cdk2 antibody. Immunoprecipitated proteins were resolved by electrophoresis on 10% polyacrylamide SDS gels, immunoblotted and immunostained with an anti-p21 antibody. Control experiments in which cell lysate from cells cultured in absence of estradiol and Dox, primary antibody to Cdk2, and/or protein A/G beads was included or not are shown in the right hand panel. The figure shows a representative result of three separate experiments.

Cdk2 Complexes Are Associated With p21 in GFP-ER High Expressing Cells

The previous results demonstrated that p21 levels were increased and cyclin E–Cdk2 kinase activity were decreased at 24 h post-estradiol addition of the 1.0 μg/ml Dox treated clone 11 cells. To determine whether p21 was associated with Cdk2 in the clone 11 cells expressing high amounts of GFP-ER at 24 h post-estradiol addition, Cdk2 was immunoprecipitated from clone 11 cells treated with 0 or 1.0 μg/ml Dox at 0 and 24 h following addition of estradiol. The level of p21 associated with the Cdk2 complexes at 24 h was reduced relative to the estradiol untreated clone 11 cells cultured in the absence of Dox (Fig. 6C). In contrast the levels of p21 associated with Cdk2 complexes were elevated at 24 h in the clone 11 cells cultured with 1.0 μg/ml Dox. In control experiments in which the primary anti-Cdk2 antibody was not added p21 was not detected by immunoblotting of the proteins retained on the protein A/G beads.

The results suggested that the elevated levels of p21 in clone 11 cells expressing high amounts of GFP-ER inhibited Cdk2 activity at 24 h post-estradiol addition by forming enzymatically inactive p21-cyclin E–Cdk2 complexes.

DISCUSSION

Our results demonstrate that the inability of estradiol-stimulated MCF-7 breast cancer cells expressing elevated levels of GFP-ER to lower p21 protein levels at 24 h prevents their entry into the cell cycle. The estradiol-induced increased expression and subsequent reduction in the levels of c-*myc*, cyclin E, and cyclin D1 were not affected in the GFP-ER expressing cells. Consistent with the inhibition of cyclin E–Cdk2 activity by p21, we observed that the level of phosphorylated H1, a substrate of cyclin E–Cdk2 activity [Bhattacharjee et al., 2001;

Chadee et al., 2002], was reduced 24 h post-estradiol addition in GFP-ER expressing MCF-7 cells. Further, at 24 h cells expressing elevated levels of GFP-ER had cyclin E–Cdk2 complexes associated with p21, while the level of p21 associated with the cyclin E–Cdk2 complexes was low in cells not expressing GFP-ER.

At 8 h post-estradiol addition, phosphorylated H1 levels were elevated in the GFP-ER expressing and non-expressing cells. At this time, cyclin D1 and E levels have increased which would increase the abundance of the cyclin D1–Cdk4 and cyclin E–Cdk2 complexes. Previous studies show that the cyclin D1–Cdk4 complex will preferentially bind to p21, preventing p21 from inhibiting the activity of cyclin E–Cdk2 [Planas-Silva and Weinberg, 1997; Prall et al., 1997; Sherr and Roberts, 1999]. At later time points, p21 levels increase while cyclin D1 levels decrease, resulting in the inactivation of cyclin E–Cdk2 activity in cells expressing high levels of GFP-ER.

While the biphasic response of c-*myc*, cyclin D1, and cyclin E in response of MCF-7 cells to estrogen has been observed by others, this is not the case with p21. Sutherland et al. did not observe an estrogen-induced increase in p21, with only a steady decline in p21 protein levels being found following addition of estradiol to MCF-7 cells [Lai et al., 2001; Prall et al., 2001]. The discrepancy in results may be a consequence of tissue culture conditions. To deprive MCF-7 cells of estrogen, we culture cells in charcoal-stripped fetal bovine serum. Under these conditions ER levels are greater than those in cells cultured in the presence of estrogen [Zhao et al., 2002]. In contrast Sutherland's group cultures MCF-7 cells in the presence of the antiestrogen ICI 182780 which will decrease ER to very low levels, and it is to these cells that estradiol is added. In support of our observations, there is considerable evidence that p21 is an estrogen-induced gene [Thomas et al., 1998; Levenson et al., 2002, 2003; Licznar et al., 2003; Margueron et al., 2003]. Although the p21 promoter does not have a consensus ERE, it does have several half ERE-like sequences (GGTCC) that are nestled among Sp1 sites [Thomas et al., 1998]. ER may bind to the half-site ERE or to Sp1 [Stoner et al., 2004].

Expression of an ER transgene in a hormone independent breast cancer cell line or immortalized breast epithelial cell line resulted in the arrest of cell cycling when estradiol was added [Levenson et al., 2003]. This is not typically observed in hormone responsive cells expressing an ER transgene unless ER transgene expression is high [Lazennec et al., 1999]. As with GFP-ER expressing MCF-7 cells, the explanation for the arrest of the hormone-independent cells was elevated p21 protein levels [Thomas et al., 1998]. An apparent difference between ER negative, hormone-independent and ER positive, hormone-dependent breast cancer cells is the ability of the latter to down-regulate p21 protein levels, allowing cells to enter S phase of the cell cycle. The mechanism for p21 down-regulation is thought to be principally at the transcription level [Bottazzi et al., 1999; Prall et al., 2001]. In preliminary experiments we observed that p21 mRNA levels did not decline in the high GFP-ER expressing clone 11 cells at 24 h post-estradiol addition as was the case with clone 11 cells not expressing GFP-ER. Further, the turnover of p21 protein is similar in estrogen treated and untreated breast cancer cells [Prall et al., 2001]. The mechanism of p21 down-regulation is not known. It is conceivable that elevated c-*myc* competes with ER binding to Sp1, resulting in repression of the p21 promoter [Gartel et al., 2001; Doisneau-Sixou et al., 2003; Gartel and Shchors, 2003]. Overexpression of GFP-ER may compete for c-*myc* binding to Sp1 at the p21 promoter, preventing repression of p21 expression. Chromatin immunoprecipitation experiments are underway to test this model. Further research to determine the mechanisms regulating chromatin remodeling and transcriptional activity of the *p21* gene will present opportunities to develop strategies to prevent its repression and arrest proliferation of breast cancer cells.

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