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PRECLINICAL ANTIESTROGENS

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Antiestrogens and the Cell Cycle

*Colin K.W. Watts, Owen W.J. Prall,
Jason S. Carroll, Nicholas R.C. Wilcken,
Eileen M. Rogan, Elizabeth A. Musgrove,
and Robert L. Sutherland*

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1. INTRODUCTION

Recognition of the involvement of estrogen in the growth of breast cancer stemmed from observations made a century ago, when it was shown that ovariectomy in cases of pre-menopausal breast cancer could lead to tumor regression (1). Subsequent research in experimental models of carcinogen-induced mammary cancer revealed that estrogen was essential for both the initiation and progression of the disease. These observations, together with the demonstration that some breast tumors had a specific binding protein for estrogen, the estrogen receptor (ER), and that ER status was correlated with response to endocrine therapy, provided the rationale for the introduction of the antiestrogen tamoxifen in the treatment of breast cancer (2). Tamoxifen is currently the

treatment of choice for hormone-dependent breast cancer both in advanced disease and as an adjuvant to surgery in early breast cancer. Recent overviews of the outcome of randomized clinical trials of adjuvant tamoxifen therapy demonstrate significant reductions in risk of recurrence, increased overall survival, and reduced incidence of contralateral breast cancer (3,4). In addition to tamoxifen and other nonsteroidal antiestrogens, steroidal antiestrogens have been described (5,6) that generally exhibit pure antagonist activity, in contrast to the partial antagonist properties of tamoxifen. Such compounds are potentially more potent therapeutically than tamoxifen and early experience in the clinic shows efficacy in cases where tumors are resistant to tamoxifen. Thus antiestrogens of various structural classes with differing tissue-specific estrogen agonist/antagonist properties have an established and expanding role in the treatment of breast cancer. The accepted basis of their clinical efficacy in breast cancer is inhibition of estrogen-induced mitogenesis but the molecular basis of this action has not been fully elucidated. This chapter summarizes research from this laboratory aimed at understanding the mechanistic basis for estrogen/antiestrogen control of breast cancer cell-cycle progression.

2. EFFECTS OF ANTIESTROGENS ON CELL-CYCLE PROGRESSION

2.1 Cell-Cycle Effects In Vitro

Initial insights into mechanisms of antiestrogen action as growth inhibitory agents came from studies on the effects of antiestrogens on breast cancer cell proliferation in vitro. Early experiments showed that the relative cell number and rate of thymidine incorporation into DNA of ER-positive (but not ER-negative) breast cancer cells were markedly reduced by antiestrogen treatment (7,8). These compounds are predominantly cytostatic rather than cytotoxic in vitro and this is associated with arrest of cells in the G₁ phase of the cell cycle, with a resulting decrease in the relative proportion of cells synthesising DNA (S phase, Fig. 1A) (9–13). A typical response to antiestrogens of all structural classes is shown in Fig. 1B where MCF-7 breast-cancer cells growing exponentially in 5% fetal calf serum (FCS) with a doubling time of 28 h are treated with the steroidal pure antiestrogen ICI 182780. Little change is apparent over the first 8 h of exposure, but the proportion of cells in S phase then falls continuously to reach a minimum by 24 h. These decreases are mirrored by increases in the proportion of cells in G₁ phase. Experiments with cells synchronized by mitotic selection demonstrate that only those cells in early-to-mid G₁ phase are susceptible to growth arrest (9,11). Cells in plateau phase, where the proportion of proliferating cells is reduced, are relatively insensitive, suggesting that only actively cycling cells are sensitive to antiestrogen (13,14). Compatible with an antiestrogen-mediated, reversible inhibition of cell-cycle progression

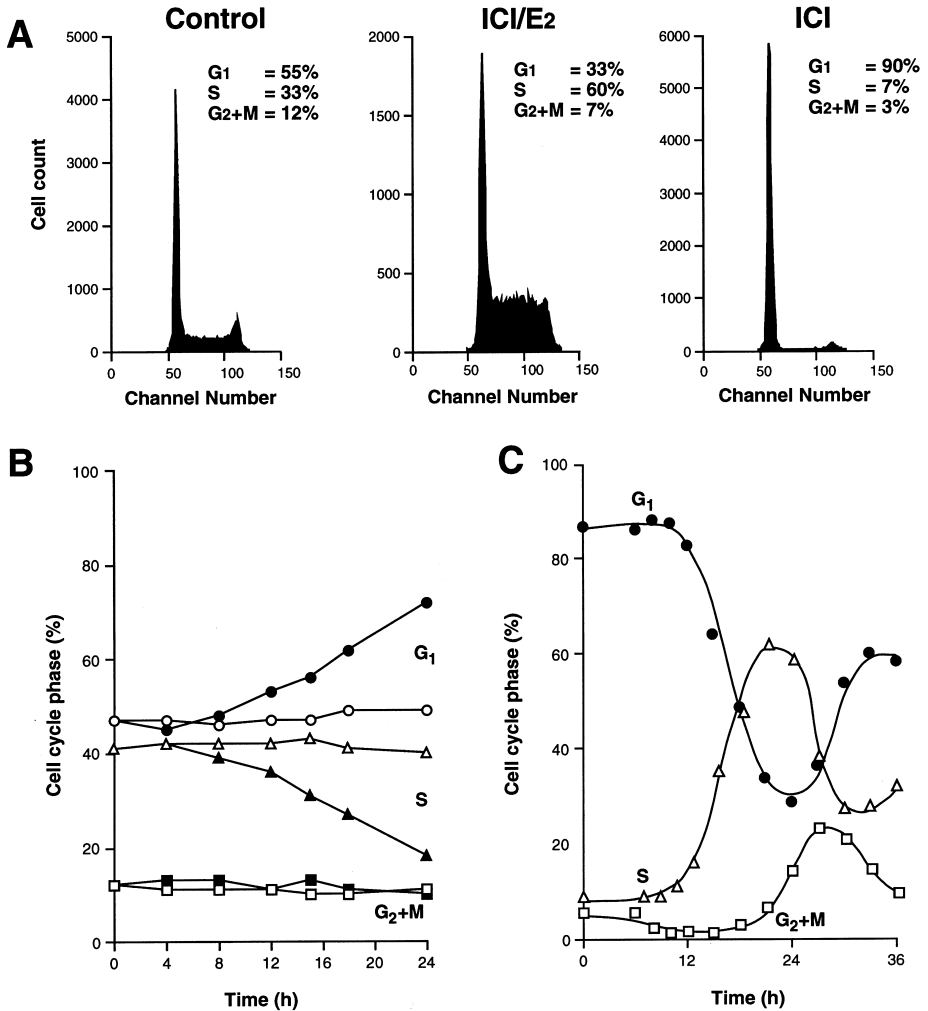


Fig. 1. Effects of estrogen and antiestrogen on MCF-7 cell-cycle phase distribution. MCF-7 cells were growth-arrested for up to 48 h with 10 nM ICI 182780 and then treated with 100 nM estradiol or vehicle (ethanol). Harvested cells were stained for DNA content and the proportion of cells in G₁, S and G₂ + M phases of the cell cycle determined. (A) Representative DNA histograms for untreated, exponentially growing cells (Exp), cells treated with ICI 182780 (ICI) for 59 h and cells treated with ICI 182780 for 48 h and then rescued by estradiol for 21 h (ICI +E₂). (B) Antiestrogen inhibition. Cells were treated with 10 nM ICI 182780 (solid symbols) or with 0.1% ethanol vehicle (open symbols) between 0–24 h. G₁ (J, E); S (H, C); and G₂ + M (B, G) phase. From ref. (62). (C) Estrogen rescue. After 48 h of ICI 182780 cells were treated with estradiol. At intervals thereafter, cells were harvested and the proportion of cells in G₁ (J), S (C), and G₂ + M (G) phases determined. From ref. (17).

in G₀/G₁ phase are data demonstrating semi-synchronous progression into S phase following estrogen “rescue” of antiestrogen-treated cells (Fig. 1A, 1C). This phenomenon was first noted by Lippman and Bolan (7) and has recently been exploited by us and others to gain new insights into estrogen control of cell-cycle progression (15–17).

These data provide strong evidence that antiestrogens inhibit breast cancer cell proliferation in culture by inhibiting cell-cycle progression in early to mid G₁ phase. The exact state at which they are arrested, i.e., in G₀ or G₁, has yet to be defined but this does not involve permanent exit from the cell cycle as estrogen (but not several growth factors, e.g. insulin-like growth factor [IGF]-1, epidermal growth factor [EGF] and heregulin) can re-initiate cell cycle progression.

Because the response to antiestrogens can be modulated by interactions with steroids and growth factors, such as those present in FCS (*see ref. 18 and references therein*) we have also defined the growth-regulatory actions of antiestrogens in estrogen-free, serum-free medium (19–21). Under these culture conditions the proliferation of MCF-7 and T-47D cells was markedly inhibited by nonsteroidal or steroidal antiestrogens, both being essentially cytostatic after 24 h exposure. The concentration-dependence of growth inhibition of these antiestrogens appears to be little affected by the absence of serum and estrogens. As in serum-containing medium, the changes in cell-cycle phase distribution that accompanied growth inhibition were similar for both classes of antiestrogens with the proportion of cells in S phase falling rapidly after 9 h to reach a minimum within 24 h. Other studies have also shown that antiestrogens are able to inhibit proliferation under steroid-depleted conditions (10,22–26) and that antiestrogens inhibit cells stimulated to proliferate by insulin (10,26), IGF-I (10), EGF (26), or transforming growth factor (TGF- α) (10). The diversity of mitogenic stimuli inhibited by antiestrogen treatment suggests that the molecular targets of inhibition are common to estrogen- and growth factor-activated pathways.

Although the mechanisms by which antiestrogens inhibit growth factor-induced proliferation in the apparent absence of estrogen are unknown, several potential mechanisms for antiestrogen inhibition of gene expression have been suggested that might operate under such conditions (27–29). These include: inhibition of the function of unoccupied ER bound to DNA; DNA binding of antiestrogen-ER complexes to estrogen-response elements resulting in transcriptional interference of basal expression or expression driven by other promoter elements; and inhibition of AP-1 activity, possibly resulting from protein-protein interactions between the antiestrogen-ER complex and Fos/Jun.

2.2 Cell-Cycle Effects In Vivo

Although growth inhibition by antiestrogens in vitro is primarily due to cell-cycle arrest in G₀/G₁ phase, data obtained from breast cancer cell lines grown

as solid tumors in nude mice are less clear-cut. Antiestrogen inhibition of tumor growth *in vivo* has been reported to result primarily from cell loss that is not cell-cycle-phase specific (30). To further address this issue we examined the effects of tamoxifen on the growth and cell cycle-phase distribution of MCF-7 cells grown as tumors in nude mice (19). Under conditions of estradiol (E_2) stimulation tumors grew rapidly. This estrogen-stimulated growth was almost completely abolished by the simultaneous administration of tamoxifen (Fig. 2A). However, at longer tamoxifen treatment times, slow tumor growth became apparent, consistent with results seen in other studies in which large MCF-7 tumors eventually re-grew in the presence of tamoxifen (31,32). Flow cytometric analysis showed control tumors had an S phase fraction of more than 20%, consistent with their rapid growth rate (Fig. 2B). While the differences in cell cycle-phase distribution were not as large as those observed *in vitro*, tamoxifen treatment resulted in significant decreases in the proportion of cells in S phase with a corresponding increase in cells in G_1 phase (Fig. 2C). These data clearly demonstrate that cell cycle phase-specific cytostatic effects of tamoxifen can occur both *in vitro* and *in vivo* but do not rule out other concurrent mechanisms of growth inhibition *in vivo*. Although we saw no evidence for tumor regression on tamoxifen treatment in agreement with others (31,32), Br  nner et al. (30) found tamoxifen led to growth inhibition and shrinkage of MCF-7 tumors in nude mice and concluded its effects *in vivo* were not mediated through a G_1 phase block but rather through non-cell cycle-phase-specific cell loss. If tamoxifen has this activity, it may be equivalent to estrogen withdrawal, which has also been shown to result in apoptosis and tumor regression of E_2 -stimulated MCF-7 tumors (33). There is some direct evidence for apoptotic effects of antiestrogens in xenograft models (34,35) and in primary breast cancer (36). However, it should also be noted that the extent of tumor shrinkage induced by tamoxifen treatment in another study (32) was no different from placebo controls suggesting tamoxifen has no cytotoxic effect *per se*. Thus the relative contributions of decreased cell proliferation and increased cell death to the antitumor activity of antioestrogens *in vivo* is a major unanswered question.

3. MOLECULAR MECHANISMS OF ANTIESTROGEN INHIBITION OF CELL-CYCLE PROGRESSION

Despite current knowledge of the effects of antiestrogens at the whole-cell level, a precise understanding of the molecular events underlying estrogen and antiestrogen action is not yet available, particularly with regard to effects on cell proliferation. The effects of antiestrogens at submicromolar concentrations can generally be reversed by the simultaneous or subsequent addition of estrogen (8,12,13,37). Antiestrogen action is therefore believed to be medi-

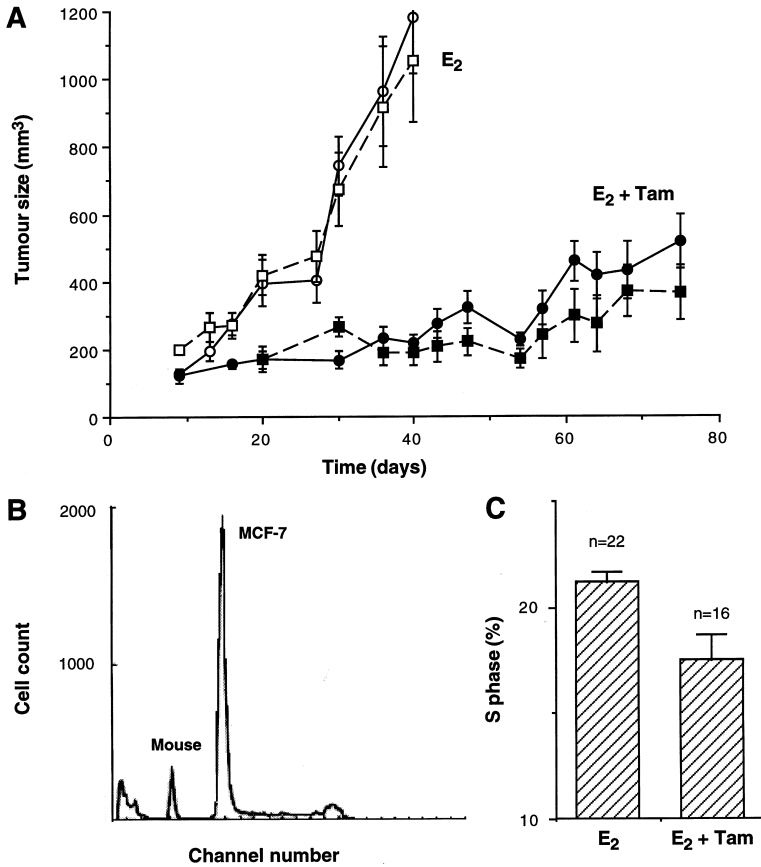


Fig. 2. Effects of tamoxifen on growth and cell-cycle phase distribution of MCF-7 tumors in nude mice. Nude mice bearing MCF-7 tumors were treated twice weekly with either estradiol or estradiol plus tamoxifen dissolved in peanut oil to a final dose of 20 (squares) or 50 (circles) mg/wk estradiol and 200 mg/wk tamoxifen. Tumor volumes were measured at intervals (Fig. 2A). At the conclusion of the experiment, tumors were harvested for DNA analysis. Figure 2B shows a representative DNA histogram of an estradiol treated tumor, where 'Mouse' indicates cells originating from the host animal. Figure 2C shows pooled results for the S-phase content of estradiol-treated tumors (both doses) and estradiol plus tamoxifen-treated tumors. From ref. (19).

ated primarily through competitive binding to the ER, with direct effects on the transactivation of estrogen-responsive genes, which in turn can subsequently alter the expression and activity of numerous additional gene products. The identity of the set of such genes specifically involved in antiestrogen control of cell-cycle progression has yet to be fully defined, although the action of antiestrogens to arrest cells at a point within the G₀/G₁ phase of the

breast-cancer-cell-cycle focuses the search for antiestrogen target genes on those with known activities in controlling progression through G₁ phase. Restriction of antiestrogen sensitivity to cells in early to mid G₁ phase further defines the potential genes that are the initial targets of antiestrogen action. To date most attention has been focused on the cyclin-dependent kinases (CDKs), their inhibitors and substrates, and the proto-oncogene *c-myc*.

3.1. Control of Cell-Cycle Progression

Progress through the cell cycle is governed by the sequential activation of a family of CDKs with the consequent phosphorylation of specific substrates to allow progression through checkpoints in the cell cycle. Since normal physiological regulation of cell-cycle progression by extracellular stimuli, including growth factors and steroid hormones (38–40), is mediated during G₁ phase (41), the major interactions controlling G₁ progression are a central focus and these are illustrated in Fig. 3. Key substrates of the CDKs with G₁-phase specific actions (i.e., Cdk2, Cdk4, and Cdk6) include the retinoblastoma gene product, pRB, and the related pocket protein p107, although it is likely that other important substrates remain to be identified (38). The consequence of inactivation of the pRB protein by phosphorylation is the release of a number of bound and functionally inactive factors including the E2F family of transcription factors (42–45). Upon release from pRB complexes, these transcription factors activate transcription of genes whose products are required for S-phase progression (42–45). CDK activity is subject to multiple levels of regulation. Since CDKs are inactive in the absence of cyclin binding, cyclin abundance is a major determinant of cyclin-CDK activity (46). Each cyclin is thus typically present for only a restricted portion of the cell cycle, and cyclin induction is an integral part of mitogenic signaling. Alteration of cyclin abundance is sufficient to alter the rate of cell-cycle progression since overexpression of the principal G₁ cyclins, cyclins D1-3 or E, accelerates cells through G₁ and conversely, inhibition of their function by antibody microinjection prevents entry into S phase (38,39,41). An essential role for cyclin D1 in normal mammary-gland development and breast cancer is indicated by the absence of lobular-alveolar compartments in transgenic mice with disruption of the cyclin D1 gene (47,48), and evidence that cyclin D1 overexpression is an early (49) and common (50) event in human breast cancer.

CDK activity is also regulated by a network of kinases and phosphatases so that cyclin binding is sufficient only for partial activation (46,51). Phosphorylation by the CDK-activating kinase (CAK) on a conserved threonine residue is necessary for full activity (46,51). However, even in the presence of phosphorylation at this residue and cyclin binding, CDKs can be inhibited by phosphorylation of N-terminal threonine and tyrosine residues within the catalytic cleft (51). The dual specificity Cdc25 phosphatases activate CDKs by dephosphorylating these inhibitory residues (51).

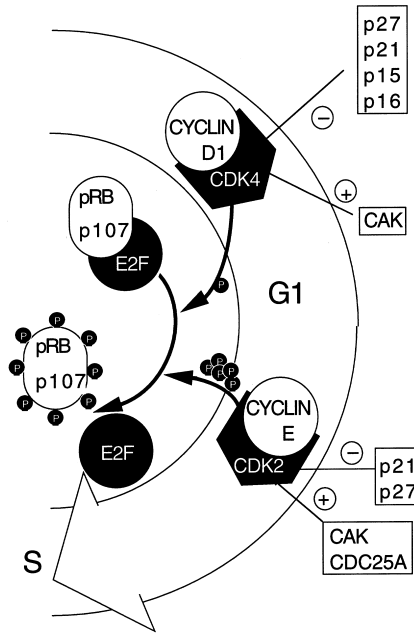


Fig. 3. Cell-cycle regulation during G₁ phase. Progress from G₁ into S phase is regulated by the actions of the molecular pathways illustrated schematically. The major G₁ cyclin complexes in breast-cancer cells, cyclin D1-Cdk4, and cyclin E-Cdk2 are illustrated. The activity of these complexes is regulated at several levels including cyclin abundance, consequent assembly of the cyclin/CDK complex, and activation by both kinases (CAK) and phosphatases (CDC25). Once active the CDKs phosphorylate substrates including pRB and the related “pocket protein,” p107, leading to the release of molecules including the transcription factor E2F and consequent transcription of genes necessary for entry into S phase. The CDK inhibitors p21 and p27 not only interfere with the phosphorylation steps leading to the activation of the CDK but inhibit active CDK complexes. While the p16 CDK inhibitor may also inhibit holoenzyme complexes, a major function is to inhibit the assembly of cyclin D1-Cdk4 complexes. Redrawn from ref. (92).

A further level of control results from the actions of two families of specific CDK inhibitory proteins (CKIs). One family, of which the prototypic member is p16^{INK4}, specifically targets the kinases which associate with the D-type cyclins, Cdk4 and Cdk6. The inhibitory activity of this family appears to result largely from competition with the cyclin for CDK binding although there is also evidence that p16 family members bind to and inhibit cyclin D-Cdk4 and cyclin D-Cdk6 complexes (52,53). The other family, of which p21 (WAF1, Cip1, sdi1) and p27 (Kip1) are the best-studied, interact with cyclin/CDK complexes containing Cdk2 as well as Cdk4 and Cdk6. Recent structural studies of p27 bound to cyclin A-Cdk2 indicate that p27 interacts with both cyclin A and Cdk2, occluding the cat-

alytic cleft of Cdk2, causing multiple structural changes within the complex (54). Despite these multiple modes of inhibition of CDK activity, cyclin/CDK complexes containing p21 or p27 can retain activity in in vitro kinase assays (55–57). More recent data indicate that p21 and p27 as well as a related inhibitor, p57^{Kip2}, stabilize cyclin D-Cdk4 and cyclin D-Cdk6 complexes in vitro (55). Thus low stoichiometry p21 binding promotes assembly of active complexes while at higher stoichiometry kinase activity is inhibited (55). Consequently, these molecules appear to have functions in addition to CDK inhibition, perhaps as adaptors which not only promote assembly of the cyclin-CDK complexes but also target these complexes to specific intracellular compartments or substrates.

In addition to the G₁ cyclins, the proto-oncogene product c-Myc is one of only a limited number of proteins that are known to be rate-limiting for progression through G₁ phase (58). c-Myc-induced stimulation of DNA synthesis is preceded by modulation of the expression or activation of cyclins, CDKs, and CDK inhibitors, although it appears that there are differences in the specific responses to c-Myc activation, perhaps related to cell type or the presence of functional pRB (58). While some data suggest close links between c-Myc and cyclin D1, other data argue that they may be involved in alternative pathways for progression through G₁ phase (58). There is, however, increasing evidence of a role for c-Myc in the activation of cyclin E-Cdk2 (59–61). This activation appears to be indirect, rather than by direct transcriptional regulation of components of the cyclin-CDK complex.

The complexity of control of cyclin-CDK activity provides multiple targets through which physiological regulators of cell proliferation might mediate their effects. However, only a restricted range of these potential targets appear to be utilized. Thus, regulation of cyclin or CDK inhibitor expression is a frequent response to mitogens including steroid hormones, peptide-growth factors and cytokines, and to growth arrest following induction of differentiation or treatment with inhibitory factors, e.g., TGF- β (38–40). In contrast, regulation of the expression or activity of the kinases and phosphatases controlling CDK phosphorylation and hence activation appears to be rare. Consequently, examination of the effects of antiestrogens and estrogens on cell-cycle regulatory molecules has focused on regulation of c-Myc, cyclins/CDKs, and CDK inhibitors.

4. EFFECTS OF ANTIESTROGENS ON CELL-CYCLE REGULATORY MOLECULES

4.1. Antiestrogen Increases Hypophosphorylated Retinoblastoma Protein

Because of the central role of pRB as a regulator of cell-cycle progression in late G₁ phase, we examined whether pRB phosphorylation is altered by antiestrogen treatment, in particular whether this occurs at times compatible with a

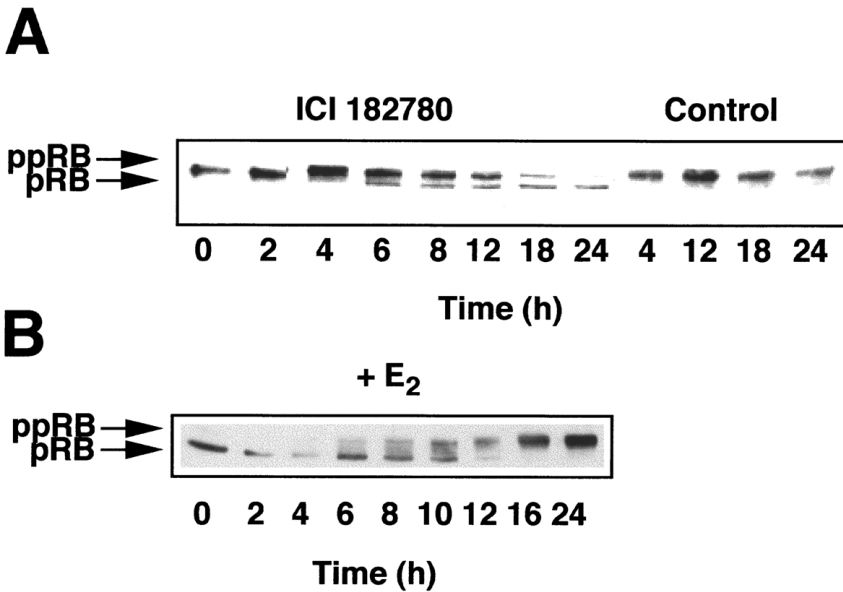


Fig. 4. Effects of estrogen and antiestrogen on phosphorylation of the RB protein. **(A)** Whole-cell lysates from MCF-7 cells treated with 10 nM ICI 182780 or with 0.1% ethanol vehicle (control) were Western blotted with an anti-RB antibody. The upper band, ppRB, represents the hyperphosphorylated form of RB and the lower band, pRB, the hypophosphorylated form. From ref. (62). **(B)** Antiestrogen-treated MCF-7 cells were rescued with estradiol as described in Fig. 1. At intervals thereafter, whole-cell lysates were prepared and Western blotted for pRB as above. From ref. (17).

role for pRB in mediating the cell-cycle effects of antiestrogens, and whether changes in phosphorylation are consistent with antiestrogen regulation of G₁ cyclin/CDK activities (62,63). Western blotting of MCF-7 cell lysates from untreated exponentially growing control cells demonstrated that almost all pRB exists in the more highly phosphorylated, slowly migrating form (Fig. 4A). Treatment with ICI 182780 resulted in a time-dependent decrease in pRB phosphorylation, with a corresponding increase in the growth-inhibitory, hypophosphorylated form of pRB from 4–6 h (62). These early changes in pRB phosphorylation preceded decreases in % S phase cells by several hours, indicating that they are likely to be a cause, rather than a consequence, of antiestrogen-induced inhibition of cell-cycle progression. At 12 h both forms of pRB were still present but at 18 h and 24 h additional hypophosphorylation and a decrease in total pRB protein were observed, such that little or none of the hyperphosphorylated pRB remained. These later changes in phosphorylation occur when major effects on inhibition of entry into S phase are already

apparent. Similar results were seen in another ER-positive breast-cancer cell line, T-47D (63), and with both steroidal and nonsteroidal antiestrogens. Thus given the known function of pRB in controlling progression through G₁ to S phase, early decreases in the degree of pRB phosphorylation may be central to the inhibition of entry into S phase that is the ultimate consequence of anti-estrogen action.

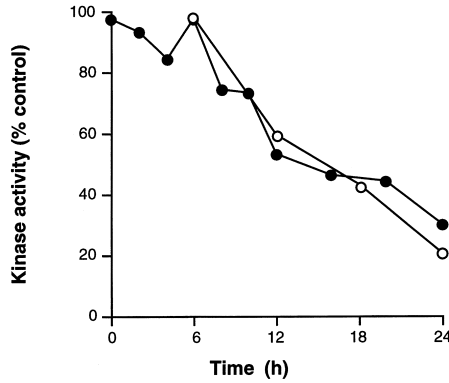
Further support for this conclusion is provided by experiments where cells growth arrested with ICI 182780 for 48 h were “rescued” by addition of E₂ (16,17). This resulted in the synchronous entry of cells into S phase commencing at 12 h, the proportion of cells in S phase reaching a maximum of 60% at 21–24 h (Fig. 1C). After 48 h of ICI 182780 pretreatment, almost all pRB is hypophosphorylated (time 0, Fig. 4B). Following estradiol treatment an increase in more slowly migrating, phosphorylated forms of pRB is first apparent at 6 h. The proportion of phosphorylated pRB increases at subsequent time points such that after 12 h, when cells commence their synchronous entry into S phase, little or no hypophosphorylated pRB remains. Similar results are obtained in estrogen rescue of tamoxifen arrested MCF-7 cells (15). Estrogen treatment also increased the total cellular concentration of pRB (Fig. 4B). These observations, then, are essentially the reverse of those seen when cells are treated with antiestrogen supporting a central role for pRB in mediating the opposing effects of estrogens and antiestrogens on G₁ to S-phase progression in target cells.

Recently we have shown that ICI 182780 not only influences the phosphorylation state of pRB, but also results in hypophosphorylation of p107 and p130 (two related pRB family members) (63a). p107 total protein levels also decrease, but p130 levels accumulate, which is characteristic of growth arrest. Coupled with this, we have detected the association of p130 with its preferred transcription factor (E2F4), suggesting that antiestrogens arrest cells in quiescence (G₀ phase) as opposed to the G₁ phase.

4.2. Antiestrogen Inhibition of Cdk4 and Cdk2 Activities

While the mechanisms responsible for the antiestrogen regulation of pRB phosphorylation have yet to be fully defined, reductions in CDK activity are the most likely explanation, although an alternative explanation that requires further investigation is the possible action of protein phosphatases suggested to control pRB reactivation (44). To investigate which of the CDKs that act during G₁ phase might be responsible, cyclin D1-associated kinase activity (principally Cdk4 activity in MCF-7 cells [(64)]) following ICI 182780 treatment was measured in immunoprecipitates of cyclin D1. Kinase activity towards a recombinant, truncated pRB substrate fell by 40% at 12 h and by 80% at 24 h (Fig. 5A), indicating that initial alterations in kinase activity precede the cell-cycle effects of antiestrogens: only small effects on inhibition of entry of cells

A



B

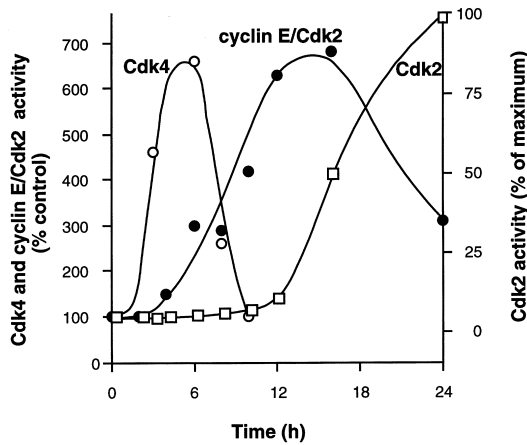


Fig. 5. Effect of estrogen and antiestrogen on cyclin D1- and cyclin E-associated kinase activities. (A) Immunoprecipitates were prepared from whole-cell lysates of MCF-7 cells treated with 10 nM ICI 182780 using anti-cyclin E or -cyclin D1 antibodies. Cyclin D1-associated Cdk4 activity (○) was determined using recombinant pRB(379–928) as a substrate. Cyclin E-associated Cdk2 activity (●) was assayed using a histone H1 substrate. From ref. (62) and unpublished data. (B) MCF-7 cells were rescued with estradiol as described in Fig. 1. At intervals thereafter, whole-cell lysates were prepared, immunoprecipitated with antibodies to either Cdk4, cyclin E, or Cdk2, and kinase activity determined. Cdk4 activity (○), cyclin E/Cdk2 activity (●) and total Cdk2 activity (□). From ref. (17).

into S phase were apparent at 12 h (Fig. 1B). This inhibition is rapidly, though transiently reversed in the estrogen-rescue model, where Cdk4 activity (determined in Cdk4 immunoprecipitates) was elevated several fold by 3 h after estradiol treatment, maximally elevated at 6 h, and thereafter declined (Fig. 5B). Given that the cyclin D1/Cdk4 complex is active in mid-G₁ phase (65), a decrease in cyclin D1/Cdk4 activity is consistent with involvement of this complex in mediating the early- to mid-G₁ phase point of action of antiestrogens on pRB phosphorylation.

Cdk2 is the second major CDK acting in the G₁ phase and its total cellular activity, as measured in Cdk2-immunoprecipitates, appeared to be unaffected by antiestrogen treatment between 2 and 6 h but decreased starting at 8 h (62,63). This profound inhibition of Cdk2 activity might result in pRB hypophosphorylation at late times and contribute to the sustained antiestrogen blockade of cell-cycle progression. However, cyclin A/Cdk2 is the predominant form of this complex and when the subcomponent of Cdk2 associated with cyclin E was examined, a more complex picture emerged as significant decreases in kinase activity were seen prior to 12 h (Fig. 5A). This inhibition increased to 24 h and beyond and suggests that inhibition of cyclin E/Cdk2 complexes contributes to the early effects of antiestrogens on pRB phosphorylation.

Like antiestrogens, estradiol had little effect on total Cdk2 activity prior to changes in S phase and pRB phosphorylation (17) (Fig. 5B). After antiestrogen pretreatment, cyclin E-associated kinase activity is low and estradiol restores this activity by threefold at 6 h (Fig. 5B), coinciding with the time when both the increase in Cdk4 activity and the shift in pRB phosphorylation are first apparent. The substantial and early changes in both Cdk4 activity and cyclin E-associated kinase activity between 4 and 6 h indicate that both kinases were likely to contribute to the initial changes in pRB phosphorylation following estradiol treatment. These results suggest that estrogens and antiestrogens have early specific effects on the activities of both cyclin D1/Cdk4 and cyclin E/Cdk2, which in turn are responsible for the observed changes in pRB phosphorylation associated with their opposing effects on G₁ to S-phase progression.

4.3. Mechanisms of Antiestrogen Regulation of G₁ Phase CDK Activity

CDKs are regulated at multiple levels, each of which, potentially, could be influenced by antiestrogens to inhibit kinase activity. Protein levels of Cdk4, Cdk2, and their partners cyclin D3 and cyclin E are unaltered by antiestrogen treatment over 12 h (62,63). Consistent with their known expression in late G₁/S and S phase, respectively, cyclin D3 and cyclin A protein levels declined significantly at late times probably as a consequence of the decreasing S-phase population. The latter decrease probably results in the observed decreases in total Cdk2 activity. In contrast, decreases in cyclin D1 mRNA expression are

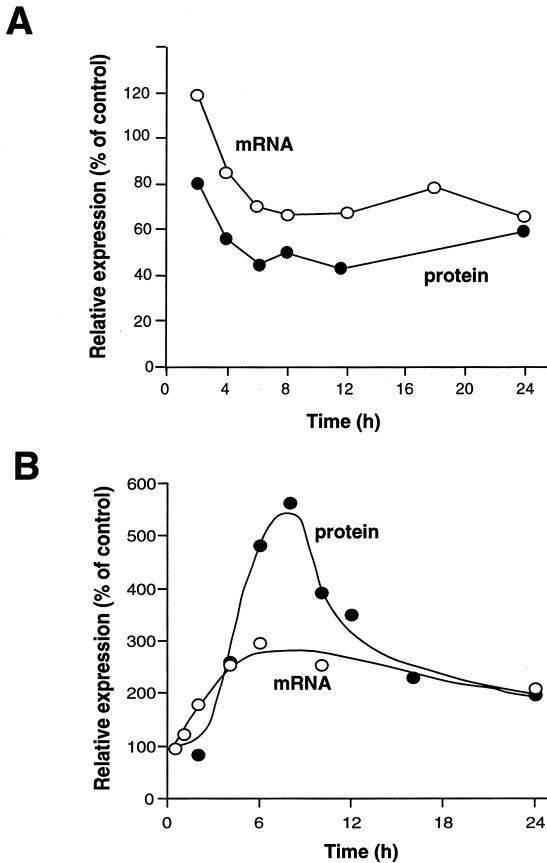


Fig. 6. Effects of estrogen and antiestrogen on cyclin D1 mRNA and protein levels. **(A)** MCF-7 cells were treated with 10 nM ICI 182780 and harvested at intervals thereafter for RNA extraction or preparation of whole-cell lysates. Northern blots were probed for cyclin D1, and mRNA levels were determined by phosphorimager analysis and expressed relative to the ethanol controls. Western blots were probed with an antibody to cyclin D1 and protein levels were determined by phosphorimager analysis and are expressed relative to the ethanol controls. From ref. (62). **(B)** MCF-7 cells were rescued with estradiol as described in Fig. 1 and harvested and analyzed for cyclin D1 mRNA and protein expression as described earlier. From ref. (17).

detected 2–4 h after a variety of antiestrogen treatments, with maximal decreases of approx 50% occurring by 6 h (Fig. 6A), before any major changes in the proportion of cells in S phase (19,21,62,63). Cyclin D1 protein also falls to a minimum level of 50% or less at 6 h (Fig. 6A) in MCF-7 cells and ER-positive MDA-MB-134 cells, a change of similar magnitude to decreases in % S

phase, but occurring several hours earlier. Although mRNA levels fall significantly, no detectable changes in cyclin D1 levels were observed in T-47D cells treated with ICI 164384, perhaps because of Western-blot-sensitivity limitations (63). Close correspondence between the timing of the disappearance of mRNA and protein is in agreement with the known short half-life of cyclin D1 protein in the MCF-7 cell line (less than 1 h) and other cell types (66).

In confirmation of a specific antiestrogen effect on cyclin D1 expression, early and pronounced changes in mRNA and protein expression are also seen in response to estradiol prior to any change in % S phase (15,17,67,68) (Fig. 6B). Although cyclin D1 levels are rapidly altered by antiestrogens and estradiol, it remains to be determined whether these are directly transcriptionally mediated effects or require prior activity of other gene products. Experiments with actinomycin D suggest that the effects of estrogen on cyclin D1 mRNA levels are transcriptionally mediated but the ability of cycloheximide to abolish mRNA induction shows that this is not a direct effect on the cyclin D1 gene and implies a requirement for *de novo* synthesis of intermediary proteins, which mediate either cyclin D1 gene transcription or mRNA stabilization (17). Studies on the cyclin D1 gene promoter have identified several regulatory regions including an AP-1 site (69) providing a link between estrogen-induced AP-1 activity (27) and cyclin D1 induction. A more recent study confirms that this AP-1 site is within the promoter region responsible for estrogen regulation of this gene (67).

Several studies provide evidence for a pivotal role of cyclin D1 in G₁ progression in breast-cancer cells. Ectopic expression of cyclin D1 is sufficient and rate-limiting for G₁-S phase progression in pRB-positive breast cancer cells, and results in increases in cyclin D1-Cdk4 and Cdk2 kinase activities (16,70–72). Furthermore, microinjection of either cyclin D1 antibodies or recombinant dominant negative Cdk4 or p16^{INK4} (protein or cDNA) prevents estradiol-induced G₁-S phase progression in MCF-7 cells (73). Therefore it is possible that the inhibition of cell-cycle progression following antiestrogen treatment may be a consequence of reduced cyclin D1 expression. To examine this further, we generated stable transfectants of T-47D and MCF-7 cells that contained cyclin D1 cDNA downstream of a metal-responsive metallothionein promoter. Cells were treated with the steroidal antiestrogens ICI 164384 or ICI 182780 or the nonsteroidal antiestrogens tamoxifen and 4-hydroxytamoxifen, arresting cells in G₁ phase as described earlier. Subsequent treatment with Zn-induced cyclin D1 protein expression and this was accompanied by cyclin D1-Cdk4 complex formation, activation of cyclin D1-Cdk4 and cyclin E-Cdk2 activities, pRB phosphorylation, and entry into S phase (Fig. 7). Treatment of control cell lines with Zn was without significant effect. Therefore expression of cyclin D1 alone was sufficient to overcome antiestrogen-induced G₁ arrest, suggesting a role for cyclin D1 in antiestrogen arrest. However, a critical role

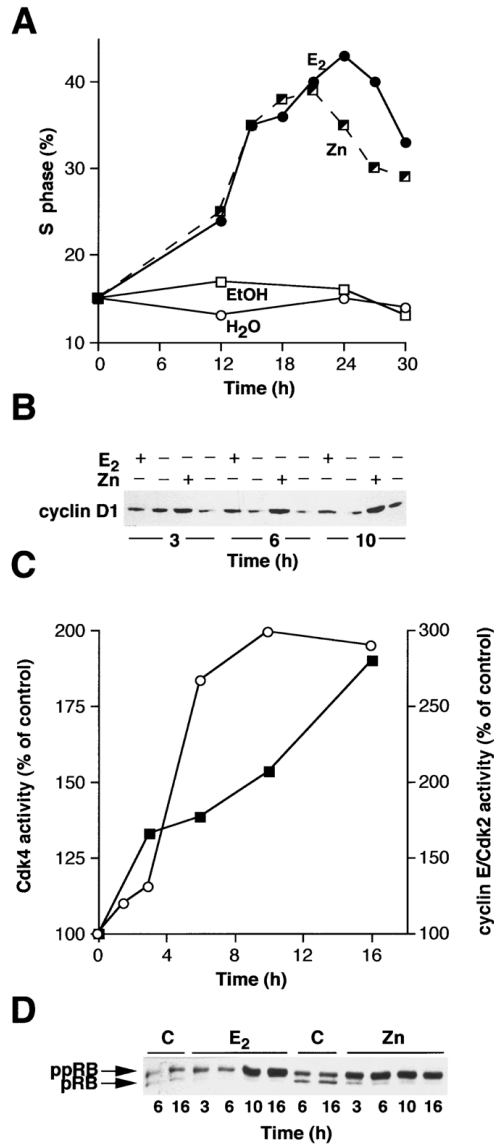


Fig. 7. Inducible expression of cyclin D1 can reverse antiestrogen-induced growth arrest. (A) An MCF-7 cell line stably transfected with the Zn-inducible p Δ MT vector containing cyclin D1 cDNA was growth-arrested for 48 h with 10 nM of the antiestrogen ICI 182780. Cells were treated at time 0 with either 50 μ M Zn (■), vehicle (H₂O, ○), 100 nM estradiol (E₂) (●), or vehicle (EtOH). At intervals thereafter, the proportion of cells in S phase was determined by flow cytometry. (B) Whole-cell lysates were prepared at intervals following treatment (shown in hours) and immunoblotted with antibodies against cyclin D1. (C) Zinc-treated whole-cell lysates were prepared and assessed for Cdk4 (■) and cyclin E/Cdk2 (○) activity as described in Fig. 5. Autoradiographs were quantitated by densitometry and expressed relative to time-matched controls. (D) Cell lysates from the same experiment were immunoblotted with a pRB antibody. From ref. (16).

of cyclin D1 in estrogen-dependent proliferation in other target tissues is less certain since mice carrying null mutations of both cyclin D1 alleles exhibit mammary-gland ductal development and pregnancy-related uterine hyperplasia, known classical estrogen-mediated biological responses (47,48).

4.4. Antiestrogen Effects on the CDK Inhibitors p21^{WAF1/CIP1} and p27^{KIP1}

Although decreases in Cdk2 and cyclin D1-associated kinase activities are predicted in response to antiestrogen treatment as the consequence of corresponding changes in the levels of cyclin D1 and cyclin A proteins, cyclin levels only fall by approx 50% (62). This suggests that antiestrogen action necessitates the activation of additional factors that are responsible for the quantitatively greater inhibition of kinase activities, particularly beyond 12 h. We have therefore examined whether antiestrogens might regulate the levels of expression of the specific inhibitors of CDK activity, p21^{WAF1/CIP1} and p27^{KIP1}. In MCF-7 cells, Western-blot analysis of p21 expression shows little or no change in the first 12 h and an approximate threefold induction by ICI 182780 at 18–24 h (62), coinciding with the timing of inhibition of total Cdk2 activity but later than the first changes in inhibition of cyclin D1-associated pRB kinase and cyclin E/Cdk2 histone H1 kinase activity (Fig. 5A). Similarly, p27 protein levels increased approx 50% by 12 h and attained an approximate threefold maximal increase between 18 and 24 h. Neither inhibitor is markedly altered prior to changes in % S phase indicating that the late changes are more likely a consequence, than a cause, of inhibition of cell-cycle progression. p27^{KIP1} could play a role in the decrease in Cdk4 activity over this period. Similarly, increased expression of p21^{WAF1/CIP1}, also an inhibitor of both Cdk4 and Cdk2 (74,75), may well contribute to inactivation of Cdk4 at these times but neither is likely to be responsible for the earlier inhibition of Cdk4 or cyclin E/Cdk2. The dramatic downregulation of total Cdk2 activity at 18–24 h that occurs without corresponding large decreases in cyclin E or cyclin A levels also suggests the possible inhibitory action of p21 and p27. It will be interesting to determine whether raised levels of these CDKIs contribute to continued growth arrest by antiestrogens upon longer term treatment by maintaining pRB hypophosphorylation.

Further investigation into the inhibition of cyclin D1/Cdk4 and cyclin E/Cdk2 at early timepoints has revealed that the loss of cyclin D1-containing complexes as the result of repressed cyclin D1 transcription resulted in release of free p21 and p27, which was subsequently recruited by and inhibited cyclin E/Cdk2. This shift in inhibitors between cyclin D1/Cdk4 and cyclin E/Cdk2 occurred prior to the increase in total protein levels of p21 and p27, and highlights the general importance to cell-cycle control of redistribution of CDKIs between different cyclin/CDK complexes.

4.5. CDK Complex Formation in Antiestrogen and Estrogen Action

The full interpretation of the previous results will depend on analysis of the components that make up antiestrogen-inhibited cyclin D1/Cdk4 and cyclin E/Cdk2 complexes. Such studies are underway but clues to the possible mode of antiestrogen action come from our most recent studies on the activation of these complexes in the estrogen-rescue model. It is necessary to bear in mind, however, that antiestrogens may not simply act in a way that is the mirror image of estrogen action. The most likely explanation for Cdk4 activation following estrogen rescue is that it is the direct consequence of increased cyclin D1/Cdk4 complex formation resulting from estrogen-induced expression of cyclin D1 protein, a conclusion reached by several recent studies (15,67,68). This is illustrated in Fig. 8A, which shows the alterations in composition of immunoprecipitated cyclin D1 complexes in MCF-7 cells treated with estradiol. This is also a property shared with a number of other mitogens. In T-47D breast cancer-cells progesterins, IGF-1, insulin, serum, and bFGF induce cyclin D1 mRNA, protein, and cyclin D1/CDK complex formation (*see ref. 21* and unpublished observations) as do many other mitogens in a variety of other cell types (66,76). However, the presence of elevated levels of cyclin D1 is not always sufficient for increased kinase activity in quiescent cells stimulated by growth factors, leading to the postulation that an “assembly factor” governs formation of active complexes (65); other authors have suggested that this factor might be p21 (55,57). The increased relative content of p21 in cyclin D1/CDK complexes concurrent with increased activity of the complexes following estrogen rescue (Fig. 8A) is consistent with this possibility. At present the mechanism that allows enrichment of p21 in the cyclin D1/Cdk4 complex is unknown.

As noted earlier (Fig. 5B), estrogen rescue also results in activation of cyclin E-associated Cdk2 at early time points, i.e., 4–6 h (16,17). In contrast to the action of most other mitogens, where activation of cyclin E/Cdk2 occurs through increases in total or Cdk2-associated cyclin E (77,78), we detected no change in either cyclin E mRNA or protein or Cdk2 protein at early times. Furthermore, examination of cyclin E complexes immunoprecipitated from lysates from estrogen-treated cells revealed that the levels of cyclin E, Cdk2, p21, and p27 remained unchanged in cyclin E complexes until 10 h after estradiol treatment (17). While activation of cyclin E/Cdk2 at 16 h was likely to involve loss of p21 and p27 from the complex due to a decline in their total intracellular levels (17), these experiments did not identify a mechanism for the activation of cyclin E/Cdk2 prior to 16 h.

However, using gel-filtration chromatography we demonstrated that following estrogen treatment there was a small but consistent increase in cyclin E migrating in higher molecular-weight complexes, i.e., >250 kDa, and these complexes contained the majority of cyclin E/Cdk2 kinase activity (Fig. 8B). Consequently, the specific activity of these higher molecular-weight complexes

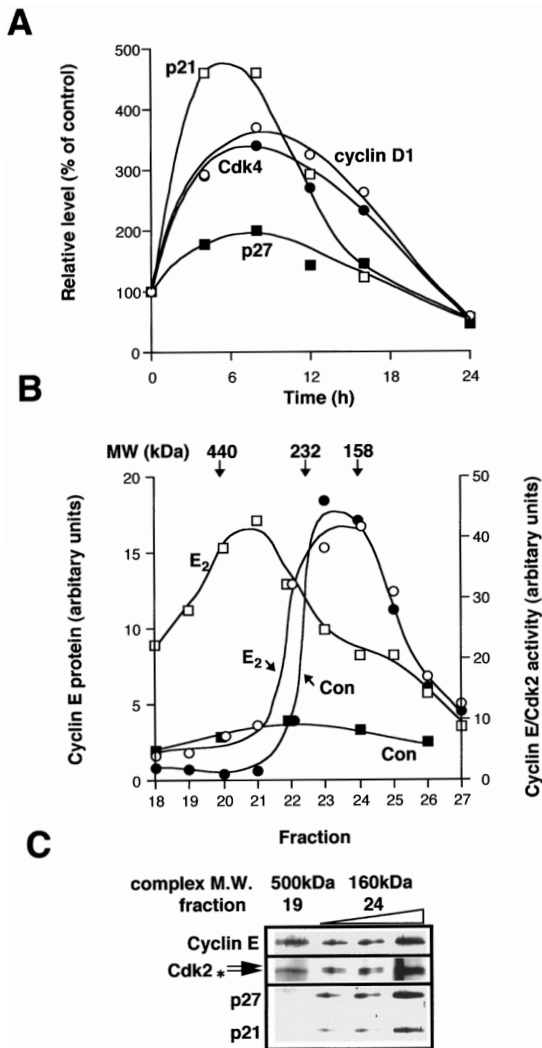


Fig. 8. Effects of estradiol on cyclin D1- and cyclin E-complex formation. (A) MCF-7 cells were rescued with estradiol as described in Fig. 1 and at intervals thereafter whole-cell lysates were prepared and immunoprecipitated with anti-cyclin D1 antiserum. Relative levels of cyclin D1 (○), Cdk4 (●), p21 (□), and p27 (■) were determined by densitometry of Western blots and are expressed relative to the vehicle-treated controls. (B) Cell lysates were prepared 8 h after estrogen (E₂) or vehicle (Con) treatment and fractionated on a Superose 12 gel-filtration column. Fractions were precipitated with acetone and Western blotted for cyclin E or assayed for cyclin E-Cdk2 kinase activity. The elution of known markers (ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa) are indicated at the top of the graph. (C) Cyclin E immunoprecipitates from fractions 19 and 24 of the E₂-treated lysate were analysed by Western blot for cyclin E, Cdk2, p21, and p27. Various quantities of the cyclin E immunoprecipitate from fraction 24 were analyzed to permit comparison of equivalent levels of cyclin E complexes with fraction 19. The asterisk marks the more mobile, active form of Cdk2 that is phosphorylated on Thr-160. From ref. (17).

was 10-fold greater than the bulk of the cyclin E eluting as lower molecular-weight forms. Comparison of the composition of cyclin E immunoprecipitates eluting at these different molecular weights revealed that the larger complexes were markedly depleted of both p21 and p27 (Fig. 8C) in contrast to previous results for cyclin E immunoprecipitates from whole-cell lysates. In different experiments, we and others have demonstrated that estrogen relieves a cyclin E-Cdk2 inhibitory activity that is present in antiestrogen-treated cells, which is attributable to p21 (15,17). Therefore, in contrast to estrogen-induced activation of cyclin D1-Cdk4 by increasing cyclin D1 expression, estradiol-mediated activation of cyclin E-Cdk2 appears to result from decreased association with p21. A potential mechanism for the loss of p21 from these complexes is its sequestration by cyclin D/Cdk4-6 induced by estradiol as suggested by Planas-Silva and Weinberg (15). However, more recent data from this laboratory demonstrate a similar mechanism of activation of cyclin E/Cdk2 following induction of c-Myc but in the absence of increased cyclin D1 gene expression and cyclin D1/Cdk4 complex formation (16). These data point to a more direct effect of estrogen/antiestrogen on p21 perhaps via a c-Myc-mediated mechanism.

4.6. Involvement of c-Myc in Antiestrogen Action

Among the first candidate genes to be investigated as potential targets of estrogen-induced mitogenesis was the immediate early gene *c-myc* which encodes a nuclear phosphoprotein (c-Myc). Regulation of this gene is among the earliest detectable responses to estrogens and has been identified in a number of target tissues including rat uteri in vivo (79,80) and both normal breast-epithelial cells and breast-cancer cells in vitro (17,81). Increased expression of *c-myc* was attributed to estrogen-induced transcriptional regulation, but not necessarily via a classical estrogen response element (ERE), and demonstrated kinetics similar to those following growth factor stimulation of serum-starved cells (81,82). Furthermore, inhibition of *c-myc* expression by antisense oligonucleotides was accompanied by inhibition of estrogen-induced cell proliferation identifying a critical role for *c-myc* in estrogen action (83). In fibroblasts, c-Myc is both necessary and sufficient for G₁-S phase progression (58) and activation of conditional alleles of *c-myc* is followed by the activation of both cyclin D1-Cdk4 and cyclin E-Cdk2 (59–61).

Rapid decreases in *c-myc* mRNA and protein levels are observed in response to a variety of antiestrogens in both in vivo and in vitro models (21,63,84,85), being apparent within 30 min (Fig. 9A). Therefore in addition to cyclin D1 and p21, c-Myc may be a major target molecule through which antiestrogen mediates cell-cycle control. In order to test whether c-Myc expression was critical to antiestrogen arrest, we constructed MCF-7 cell lines stably transfected with c-Myc cDNA under the control of a metal-responsive metallothionein promoter (16). Cells were treated with ICI 182780 resulting in

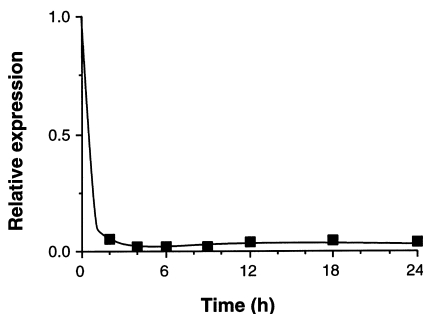
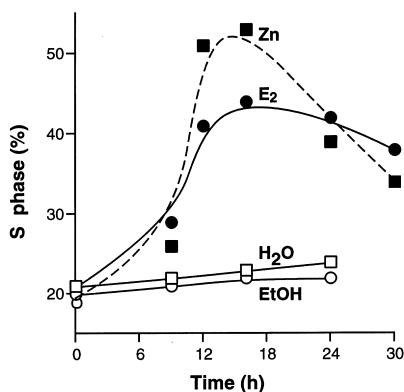
A**B**

Fig. 9. Effects of estrogen and antiestrogen on c-myc expression in breast-cancer cells. **(A)** T-47D cells proliferating in insulin-supplemented serum-free medium were treated with 500 nM ICI 164384 and harvested at intervals for Northern analysis. Densitometric analysis of data for mRNA expression is presented relative to exponentially growing control cells. From ref. (21). **(B)** An MCF-7 cell line stably transfected with the zinc-inducible p_{MT} vector containing c-myc cDNA was growth-arrested for 48 h with 10 nM ICI 182780. Cells were treated at time 0 with either 65 μ M Zn (■) or vehicle (H₂O), or 100 nM estradiol (E₂, ●) or vehicle (EtOH, ○). At intervals thereafter, cells were harvested, stained for DNA content and the proportion of cells in S phase determined by flow cytometry. From ref.

G₁-phase arrest. c-Myc expression was induced by Zn treatment and this was sufficient for subsequent S-phase entry (Fig. 9B). This gives strong support to a role for downregulation of c-Myc in antiestrogen action. An analysis of the molecular events preceding S-phase entry demonstrated that c-Myc induction resulted in the formation of high molecular-weight, high specific activity cyclin E/Cdk2 complexes devoid of p21, apparently identical to those induced

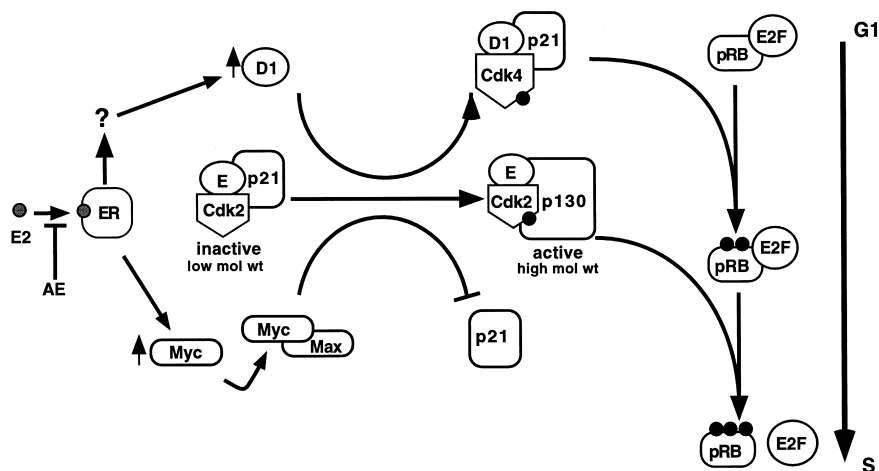


Fig. 10. A model of estrogen and antiestrogen effects on molecules regulating G₁-phase progression. Estrogen (E₂) binding to ER initiates a cascade of events including transcriptional activation of c-Myc and cyclin D1 gene expression, the latter occurring indirectly through the induction of an intermediary factor (?), which in turn regulates cyclin D1 gene expression. The increased expression of cyclin D1 stimulates the formation of active cyclin D1-Cdk4 complexes containing p21, here acting as an assembly factor rather than an inhibitor of the kinase. Activation of cyclin E-Cdk2 involves conversion to a high molecular-weight form lacking p21 and containing p130. Neither induction of cyclin D1 nor activation of the cyclin D1-Cdk4 complex appear to be c-Myc dependent in this model system, but both cyclin D1-Cdk4 (via sequestration of p21) and c-Myc (via an unknown mechanism) appear to contribute to cyclin E-Cdk2 activation. Antiestrogens inhibit binding of estrogen to the ER and act by opposing the downstream effects of estrogen on gene transcription and activation. E₂, estrogen; AE, antiestrogen; D1, cyclin D1; E, cyclin E; ●, phosphorylation sites.

by estradiol. This occurred in the absence of any detectable changes in cyclin D1/Cdk4 complexes and activity (16).

Together these data identify c-Myc and cyclin D1 as major downstream targets of estrogen/antiestrogen action; these pathways are initially separate but converge at or before the formation of active cyclin E/Cdk2 complexes devoid of p21. Thus the movement of p21 into and out of cyclin E/Cdk2 complexes appears to be a critical event in antiestrogen/estrogen regulated G₁- to S-phase progression. The mechanism responsible for this effect is a major unanswered question in estrogen/antiestrogen action.

5. CONCLUSION

Recent research in this and other laboratories has given us a much clearer understanding of the molecular events that mediate the antiproliferative effects

of antiestrogens in ER-positive breast cancer cells. In summary, current evidence suggests that antiestrogens achieve their acute effects on inhibition of breast-cancer cell-cycle progression in G₁ phase via a sequence of events including decreased cyclin D1 and *c-myc* expression, decreased cyclin D1-Cdk4 and cyclin E-Cdk2 activities, at least partially via a redistribution of p21, and finally decreased RB protein phosphorylation. Ectopic expression of either *c-myc* or cyclin D1 is sufficient to overcome antiestrogen arrest in these cells, confirming the critical role of these genes in antiestrogen action. The development of an in vitro model system, where breast-cancer cells are growth-arrested with a pure antiestrogen and cell-cycle progression re-initiated with estrogen, has also contributed to understanding antiestrogen action by allowing much better definition of early molecular events in estrogen action. Current knowledge developed from this model and the results of others is presented in Fig. 10. In summary, mitogenic effects of estrogen appear to be mediated by at least two apparently distinct pathways, involving transcriptional activation of *c-myc* and cyclin D1, the latter requiring *de novo* protein synthesis and leading to formation of active complexes with Cdk4. Both pathways then lead to early activation of cyclin E-Cdk2 by the formation of cyclin E-Cdk2 complexes deficient in the CDK inhibitor p21, and of high molecular weight, presumably due to association with other proteins including p130 (16). Phosphorylation of pRB is a primary action of these active cyclin D1-Cdk4 and cyclin E-Cdk2 complexes, resulting in release of E2F transcription factors necessary for DNA synthesis, and progression from G₁ to S phase of the cell cycle. Antiestrogens act as competitive antagonists of the binding of estrogen to its receptor and appear able to reverse the downstream effects of estrogen at each step along these pathways.

Major questions remain unanswered, however. Further studies on cyclin/CDK complex formation are required to establish the precise mechanisms involved in antiestrogen inhibition of CDK activity, particularly the factors involved in movement of CDKs in and out of these complexes. In addition, the pathway linking alterations in *c-Myc* expression to cyclin E-Cdk2 activation needs definition as does the indirect transcriptional regulation of cyclin D1. The latter studies should lead us to the earliest and primary events in antiestrogen/estrogen action.

Growth inhibition by a number of other agents appears to occur by mechanisms different from those responsible for antiestrogen action. Like antiestrogens, the antiprogesterin RU 486 and retinoic acid are both potent inhibitors of breast cancer cell proliferation. However, we found that neither appears to downregulate cyclin D1 prior to effects on S phase, despite changes in pRB phosphorylation (70,86). Instead, increased p21 abundance appears to be an important mechanism mediating the antiproliferative effects of antiprogesterins (86). CDKs appear to play a central role for several other growth inhibitors, suppressing CDK function and consequently pRB phosphorylation. TGF- β , for example, which arrests cells in mid- to late- G₁, promotes association of p27

with Cdk2 (87) and can also transcriptionally upregulate expression of p15^{INK4}, a Cdk4/Cdk6 inhibitor. p27 is also implicated in the G₁ arrest of murine macrophages by cAMP, where it prevents cyclin D1/Cdk4 activation (88) and in progestin-mediated, long-term growth inhibition of breast cancer cells (89). p21 induction is involved in growth inhibition by diverse stimuli, including serum deprivation (90) and DNA damage (74).

The evidence presented here for the key roles of c-Myc and cyclin D1 suggest potential roles for overexpression of these molecules in constitutive activation of estrogen-regulated growth pathways and in the important problem of clinical antiestrogen resistance. The common amplification and overexpression at the mRNA and protein levels of these genes in breast tumors (*see refs. 50,91 and references therein*) suggest that these might confer a growth advantage to breast epithelial cells and contribute to the development and progression of breast cancer. In support of this concept, we have demonstrated increments in cyclin D1 protein levels with progression from normal epithelium through hyperplasias to intraductal and invasive carcinomas (49). Thus cyclin D1 overexpression is an early event in the evolution of breast cancer and may play a causative role. Our demonstration that ectopic expression of c-Myc (16) or cyclin D1 (16,70) can overcome the growth inhibitory effects of antiestrogens in vitro, suggests a mechanism for antiestrogen resistance in clinical breast cancer that needs further investigation. Further research into the mechanisms of cell-cycle control in breast cancer should aid in the refinement of current procedures for the management of this disease. It is hoped that such knowledge will ultimately also contribute to a better understanding of tumorigenesis and progression in breast cancer, providing useful markers of prognosis and therapeutic response and leading to new molecular targets for therapeutic and preventative intervention. The knowledge gained from in vitro models of antiestrogen and estrogen action in breast cancer should also facilitate the exploration of mechanisms underlying hormone action in the normal breast and other estrogen-target tissues.

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