Mechanisms of Growth Arrest by c-*myc* Antisense Oligonucleotides in MCF-7 Breast Cancer Cells: Implications for the Antiproliferative Effects of Antiestrogens[1]

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ABSTRACT

The proto-oncogene c-*myc* is up-regulated by estrogen stimulation of hormone-dependent breast cancer cells and is frequently overexpressed in breast and other cancers. Therapeutic interventions that inhibit c-Myc expression have been extensively investigated, including antisense oligonucleotides that have high specificity and potential clinical application. This investigation compared antiestrogen-mediated growth arrest with the molecular events after repression of c-Myc expression in MCF-7 breast cancer cells using an antisense oligonucleotide. We show that the decreased cellular proliferation of MCF-7 cells after direct inhibition of c-Myc is a consequence of inhibition of cyclin D1 expression, subsequent redistribution of p21$^{WAF1/CIP1}$ from cyclin D1-Cdk4 to cyclin E-Cdk2 complexes, and a decline in cyclin E-Cdk2 enzymatic activity. Simultaneous repression of p21$^{WAF1/CIP1}$ can attenuate the growth-inhibitory effects of reduced c-Myc expression emphasizing the importance of this cyclin-dependent kinase (CDK) inhibitor in growth arrest. These molecular events are similar to the initial changes in cyclin gene expression, CDK complex formation and CDK activity seen after antiestrogen (ICI 182780)-mediated growth inhibition of MCF-7 cells, which suggests that the down-regulation of c-Myc by ICI 182780 is a primary event that culminates in cell cycle arrest.

INTRODUCTION

Antiestrogens have been successfully used in the treatment of breast cancer, with tamoxifen being the current endocrine therapy of choice in the treatment of ER[3]-positive breast cancers (1). The frequent development of resistance to tamoxifen (2) and its potential agonist activity (3) has led to the development of alternative, potentially more efficacious, endocrine therapies including "pure" steroidal antiestrogens that display no agonist activity (4). One such antiestrogen is ICI 182780, which showed efficacy in early clinical trials (5).

Recent work has identified the molecular mechanisms by which ICI 182780 and other antiestrogens induce cell cycle arrest in MCF-7 breast cancer cells (6, 7). A key initial event is the down-regulation of the G$_1$-specific regulatory protein cyclin D1 (6, 8), which in association with its CDK partners, Cdk4 and Cdk6, phosphorylates and partially inactivates the pocket proteins (pRb, p107, and p130) during normal cellular proliferation (reviewed in Ref. 9). These pocket proteins are responsible for negative regulation of the E2F transcription factors, which are required for a cell to circumvent the G$_1$-S-phase block imposed by underphosphorylated pocket proteins. A conse-

quence of the antiestrogen-mediated decrease in cyclin D1 is the accumulation of free" p21$^{WAF1/CIP1}$ (6). The latter protein, a member of the Cip/Kip family of CDK inhibitors, facilitates the assembly of cyclin D1-Cdk4/6 complexes at low stoichiometry but inhibits cyclin D1-Cdk4/6 at higher concentrations (10). The p21$^{WAF1/CIP1}$ that is liberated from cyclin D1-Cdk4 complexes after the antiestrogen-induced decrease in cyclin D1 binds another G$_1$-specific cyclin-CDK complex, namely, cyclin E-Cdk2, inhibiting its enzymatic activity (6). As growth arrest continues, both p21$^{WAF1/CIP1}$ and the related CDK inhibitor p27^{Kip1} contribute to the inhibition of CDK activity (7). As a result, the pocket protein phosphorylation events catalyzed by the G$_1$ cyclin-CDK complexes do not occur, and cells eventually arrest in a state of quiescence (6). Studies on estrogen-induced mitogenesis have revealed multiple cell cycle-regulatory pathways activated by estrogen (11–13). These include regulation of cyclin D1 protein levels and increased accumulation of the oncoprotein c-Myc, which converge on p21$^{WAF1/CIP1}$ redistribution and modulation of cyclin E-Cdk2 activity (14). This, therefore, suggested a role for c-Myc in antiestrogen action, but little is known of its role during antiestrogen-mediated growth arrest.

The c-Myc protein has been implicated in many physiological events including cellular proliferation, apoptosis, and differentiation (15). We previously investigated the role of c-Myc in estrogen-induced cell cycle progression and demonstrated that induction of c-Myc alone is sufficient to induce cell cycle progression in antiestrogen-arrested cells (11, 14). Because of the importance of c-Myc in normal development and of its high level of expression in many cancers, it has been well studied as a potential therapeutic target. Dominant-negative forms of c-Myc (16) and both full-length antisense mRNA (17) and oligonucleotides to the c-*myc* mRNA (18, 19) inhibit proliferation of cancer cell lines *in vitro*. Watson *et al.* (18) showed that both ER-positive and ER-negative breast cancer cells could be growth arrested with antisense oligonucleotides to the c-*myc* mRNA. However, the molecular mechanisms responsible for cell cycle arrest after the loss of c-Myc have not been clearly defined in these cells.

The role of c-Myc in the control of cell cycle progression has been difficult to elucidate (20). Critical c-Myc transcriptional targets identified from c-Myc overexpression studies differ from those obtained by targeted disruption of c-Myc. For example, the expression of many of the putative downstream *trans*-activated or *trans*-repressed targets of c-Myc do not change in c-*myc* null cells (21). This disparity between systems adds an extra degree of complexity in defining the role of c-Myc. This investigation aimed to clarify the molecular mechanisms of growth arrest after down-regulation of c-Myc in breast cancer cells, because this had not previously been studied in detail. Because antiestrogens elicit an acute decrease in c-Myc levels (22, 23), we also compared the specific down-regulation of c-Myc with antiestrogen action. We show that the specific regulation of c-Myc after antisense treatment of breast cancer cells is sufficient to elicit the downstream cell cycle events that have previously been implicated in antiestrogen-mediated cell cycle arrest (6).

MATERIALS AND METHODS

Cell Culture. Stock solutions of 7α-[9-(4,4,5,5,5-pentafluropentylsulfinyl)nonyl]estra-1,3,5,(10)-triene-3,17β-diol (ICI 182780) and treatment of MCF-7 cells were as described previously (6).

c-*myc* Antisense Oligonucleotides. A 20mer c-*myc* antisense oligonucleotide (AACGTTGAGGGGCATCGTCG) was designed based on a previously published sequence (18) but with a five-nucleotide extension at the 3′ terminus, and synthesized (Geneworks, Adelaide, SA, Australia) with phosphorothioate residues at the 5′-and 3′-terminal nucleotides to minimize exonucleolytic cleavage (24). As controls, complementary (sense) and scrambled (GTACACATGGGGAGTTTCGA) oligonucleotides were manufactured. The scrambled oligonucleotide contained four contiguous guanosine residues, which have been implicated in nonspecific effects when the original 15-mer c-myc antisense oligonucleotide was used (25). MCF-7 cells were harvested and gently syringed four times to minimize clumps, and 5×10^5 cells were grown in 50-cm^2 dishes overnight. Ten μl of Cellfectin (Life Technologies, Inc., Grand Island, NY) and oligonucleotide (1 μM final concentration) were incubated in 1 ml of serum-free RPMI 1640 for 30 min and subsequently added to the monolayer with 1 ml of RPMI 1640 supplemented with 10% FCS. All of the control oligonucleotides were included at 1 μM final concentration. The dishes were placed in a 37°C incubator with 5% CO_2 for 2–3 h with intermittent mixing. The oligonucleotide/Cellfectin solution was then decanted and the monolayer washed once with RPMI 1640 (5% FCS). Ten ml of RPMI 1640 (5% FCS) was then added to each dish. Cells were harvested for protein analysis as described previously (6).

p21$^{WAF1/CIP1}$ and p27^{Kip1} Antisense Oligonucleotides. A 20-mer p21$^{WAF1/CIP1}$ antisense oligonucleotide (TCCCCAGCCGGTTCTGACAT; Ref. 26) and a 15-mer p27^{Kip1} antisense oligonucleotide (27) were synthesized (Geneworks) with phosphorothioate residues at the 5′ and 3′ termini. Complementary (sense) oligonucleotides were also manufactured. For double antisense experiments, cells were set up and treated as described above, except 20 μl of Cellfectin were added to the 1 ml of serum-free medium containing 400 nM p21$^{WAF1/CIP1}$ antisense oligonucleotide (or the appropriate sense control) or 800 nM p27^{Kip1} antisense oligonucleotide (or the appropriate sense control).

Cell Cycle Analysis. Two methods of cell cycle analysis were performed. Initially, MCF-7 cells were seeded at 2×10^3 cells/well in a 96-well plate and grown overnight. BrdUrd (Sigma-Aldrich, Castle Hill, NSW, Australia) was added to a final concentration of 10 μM for 2 h. The incorporation of BrdUrd was measured using the cell proliferation ELISA, BrdUrd (colorimetric) kit (Roche Diagnostics, Castle Hill, NSW, Australia) according to the manufacturer's instructions. The colorimetric result was measured at 370 nm using a Molecular Devices Spectra Max 250 microtiter plate reader.

In other experiments, a FITC-conjugated anti-BrdUrd monoclonal antibody was used. Briefly, MCF-7 cells were seeded into 10-cm^2 dishes at 5×10^5 and pulsed with 10 μM BrdUrd for 2 h. Cells were trypsinized and fixed in three changes of methanol:acetic acid (3:1). The pelleted cells were resuspended in PBS/1% Tween followed by denaturation in 1.5 M HCl. Three washes with PBS/1% Tween followed, after which the cells were resuspended in PBS/1% Tween containing 1 μg of FITC-conjugated BrdUrd monoclonal antibody (Chemicon International, Termecula, CA) and incubated in the dark for 30 min at 37°C. Finally, the cells were pelleted, washed twice with PBS/1% Tween, and made up to 500 μl in 1 $\times$ PBS/1% Tween. Propidium iodide (PI) was added to a final concentration of 10 μg/ml and left at room temperature for 30 min, after which the cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) exciting at 488 nm, and measuring the BrdUrd-FITC on the green fluorescence (514 nm) and the DNA-linked red fluorescence (PI) through a 600-nm wavelength filter after compensation to reduce overlap between red and green fluorescence.

Immunoblot Analysis. Immunoblotting was performed as described previously (6). Primary antibodies used were: c-Myc (C-19) and cyclin E (C-19), from Santa Cruz Biotechnology Inc., Santa Cruz, CA; β-actin (AC-15) from Sigma-Aldrich; pRb from PharMingen, San Diego, CA; cyclin D1 (DCS-6) from Novacastra Laboratories Ltd, Newcastle-upon-Tyne, United Kingdom; p21$^{WAF1/CIP1}$ and p27^{Kip1} from Transduction Laboratories, Lexington, KY.

Kinase Assay and Detection of Cyclin E and Cyclin D1-associated Proteins. Kinase assays and immunoprecipitations were performed as described previously (6).

RNase Protection Assay. MCF-7 cells were set up as described previously and treated with either the c-*myc* antisense or sense oligonucleotide, and RNA was collected at 6 and 16 h using the Ambion (Austin, TX) RNAqueous kit according to the manufacturer's instructions. The RNA was subjected to an RNase protection assay using the Riboquant multiprobe system from PharMingen according to the manufacturer's instruction, using the hCC-2 human cell cycle regulator multiprobe.

RESULTS

The Pure Antiestrogen ICI 182780 Induces a Rapid Decrease in the c-Myc Protein. Previously published work from this laboratory (22) demonstrated that the progestin ORG 2058 and the pure antiestrogen ICI 164384 decreased c-*myc* expression in T-47D cells and that these changes preceded changes in the other cell cycle components. To assess the effects of ICI 182780 on c-Myc in MCF-7 cells, we treated proliferating cells with 10 nM of ICI 182780, harvested protein over a 6-h time course and immunoblotted the lysate for c-Myc (Fig. 1*A*). As seen in Fig. 1*B*, the level of c-Myc protein decreased by 50% within the 1st hour of treatment and declined to 10% of control levels between 2 and 4 h. These changes are consistent with previous data suggesting that repression of c-Myc is one of the first detectable changes in expression of known cell cycle regulatory genes after growth inhibition by antiestrogens (22).

c-*myc* Antisense Oligonucleotides Inhibit Proliferation of MCF-7 Breast Cancer Cells. To assess whether we could mimic changes in proliferation seen after antiestrogen treatment (6) by specifically decreasing c-Myc protein, we treated proliferating MCF-7 cells with an antisense c-*myc* oligonucleotide and measured both changes in c-Myc levels (Fig. 2*A*) and BrdUrd incorporation into DNA. As seen in Fig. 2*A*, c-Myc protein was inhibited to 30% of control by 6-h treatment with antisense c-*myc* oligonucleotide. The rate of proliferation was also decreased to 30% of that in exponentially proliferating cells when the antisense oligonucleotide was administered for 16 h (Fig. 2*B*). These changes in both the levels of c-Myc and the rate of proliferation were not seen when either the sense or the scrambled oligonucleotide was added (Fig. 2).
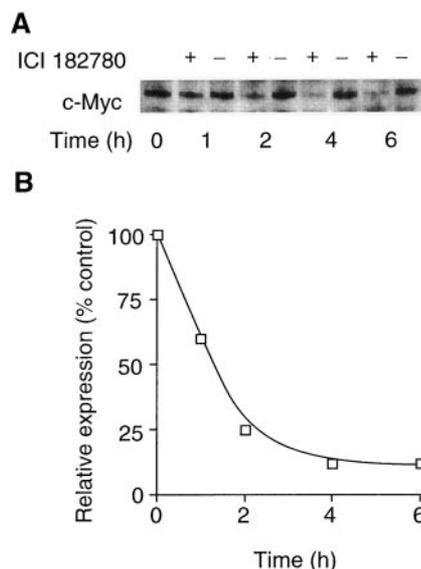

Fig. 1. ICI 182780 rapidly down-regulates c-Myc protein. Exponentially proliferating MCF-7 cells were treated with ICI 182780 (10 nM) or ethanol vehicle, and whole cell lysates were prepared at the time points indicated. *A*, protein level of c-Myc during the first 6 h of ICI 182780 treatment. Total cell lysates were separated by SDS-PAGE and immunoblotted with an antibody to c-Myc. *B*, protein levels were determined using densitometry, as described in "Materials and Methods."
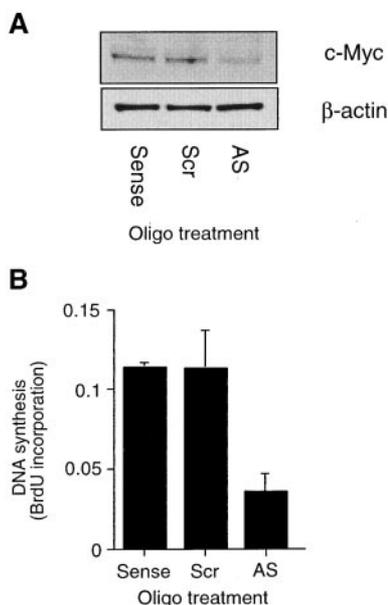
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Fig. 2. Antisense oligonucleotides to c-*myc* decrease c-Myc protein and DNA synthesis in MCF-7 cells. MCF-7 cells were treated with 1 μM of either control [sense or scrambled (*Scr*)] or antisense (*AS*) oligonucleotides and Cellfectin lipid vector for 2 h, after which the medium was replaced with 5% FCS in RPMI for an additional 4 h. *A*, total cell lysates were collected, separated by SDS-PAGE, and immunoblotted for c-Myc. The nitrocellulose was reprobed for β-actin as a loading control. *B*, DNA synthesis after 16 h of control or antisense oligonucleotide treatment was measured by BrdUrd (*BrdU*) incorporation, as described in "Materials and Methods." Data presented are the mean $\pm$ SE of three experiments.

Loss of c-Myc Is Accompanied by a Decrease in Cyclin D1 and Loss of Cyclin E-Cdk2 Activity. We next measured changes in the G$_1$-specific cyclins, cyclin D1 and cyclin E. The level of cyclin D1 decreased by 50% after antisense c-*myc*-oligonucleotide treatment, but there was little change in cyclin E protein levels (Fig. 3*A*), as was observed in antiestrogen-treated MCF-7 cells. There were no changes in either protein in the sense or the scrambled oligonucleotide controls. Our previous investigation showed that a reduction in cyclin D1 protein of this magnitude is sufficient to inhibit cyclin E-Cdk2 activity, which likely contributes to subsequent cell cycle arrest (6). Therefore, we assessed whether a specific reduction in c-Myc alone could decrease the levels of cyclin E-Cdk2 activity. As seen in Fig. 3*B*, cyclin E-Cdk2 activity decreased to less than 40% of the sense or scrambled controls after 8 h of antisense c-*myc* oligonucleotide treatment, which suggests that the presence of the c-Myc oncoprotein is required to maintain active cyclin E-Cdk2. An analysis of changes in the phosphorylation status of pRb (a major downstream target of both cyclin D1-Cdk4 and cyclin E-Cdk2) showed that the hyperphosphorylated, inactive forms of pRb decreased after the targeted repression of c-Myc (Fig. 3*C*).

Loss of c-Myc Does Not Induce Changes in p21$^{WAF1/CIP1}$ and p27^{Kip1} Protein or mRNA Levels, but Causes a Shift of p21$^{WAF1/CIP1}$ from Cyclin D1-Cdk4 to Cyclin E-Cdk2 Complexes. We previously demonstrated that the cell cycle arrest of MCF-7 cells by ICI 182780 induced the accumulation of p21$^{WAF1/CIP1}$ and p27^{Kip1} protein, but not until a majority of the cyclin E-Cdk2 activity was lost (6). To assess whether the same situation occurred in cell cycle arrest after antisense c-*myc* treatment, we immunoblotted lysate from 8-h-antisense-c-*myc*-oligonucleotide-exposed MCF-7 cells for p21$^{WAF1/CIP1}$ and p27^{Kip1}. As seen in Fig. 4*A*, the total levels of p27^{Kip1} and p21$^{WAF1/CIP1}$ did not change. Recent work on the downstream targets of c-Myc suggest that c-Myc negatively regulates the transcription of the *p21$^{WAF1/CIP1}$* gene (28, 29). This being the case,

it would be anticipated that the loss of c-Myc would result in a substantial increase in p21$^{WAF1/CIP1}$ levels. However, RNase protection assays of antisense c-*myc*-targeted cells showed that the transcription of the *p21$^{WAF1/CIP1}$* gene did not change significantly after either 8 or 16 h of antisense-c-*myc*-oligonucleotide treatment (Fig. 4*B*), which is consistent with the protein data.

We and others concluded from previous investigations on ICI 182780 action that the elevated levels of p21$^{WAF1/CIP1}$ and p27^{Kip1} at later time points were the primary factors that maintained long-term cell cycle arrest (6, 7). However, the initial inhibition of cyclin E-Cdk2 occurred, not because of an increase in inhibitor levels but rather as a result of p21$^{WAF1/CIP1}$ redistribution between cyclin D1-Cdk4 and cyclin E-Cdk2. Thus, we analyzed changes in the relative number of cyclin D1-Cdk4- and cyclin E-Cdk2-bound p21$^{WAF1/CIP1}$ complexes. The abundance of cyclin D1-Cdk4-p21$^{WAF1/CIP1}$ complexes decreased by 35% after 16 h of treatment with antisense c-*myc* oligonucleotide (Fig. 4*C*), whereas cyclin E-Cdk2-p21$^{WAF1/CIP1}$ complexes increased by 85%, which suggested that the loss of cyclin D1 resulted in a decrease in cyclin D1-Cdk4-associated p21$^{WAF1/CIP1}$ and a subsequent increase in cyclin E-Cdk2-associated p21$^{WAF1/CIP1}$, coincident with the loss of cyclin E-Cdk2 activity. There was no significant change in the abundance of cyclin E-Cdk2-p27^{Kip1} complexes after the decrease in c-Myc levels (Fig. 4*C*).

p21$^{WAF1/CIP1}$ and p27^{Kip1} Are Essential for Inhibition by c-*myc* Antisense. To assess whether the inhibitors p21$^{WAF1/CIP1}$ and p27^{Kip1} played an essential role in the inhibition of cyclin E-Cdk2
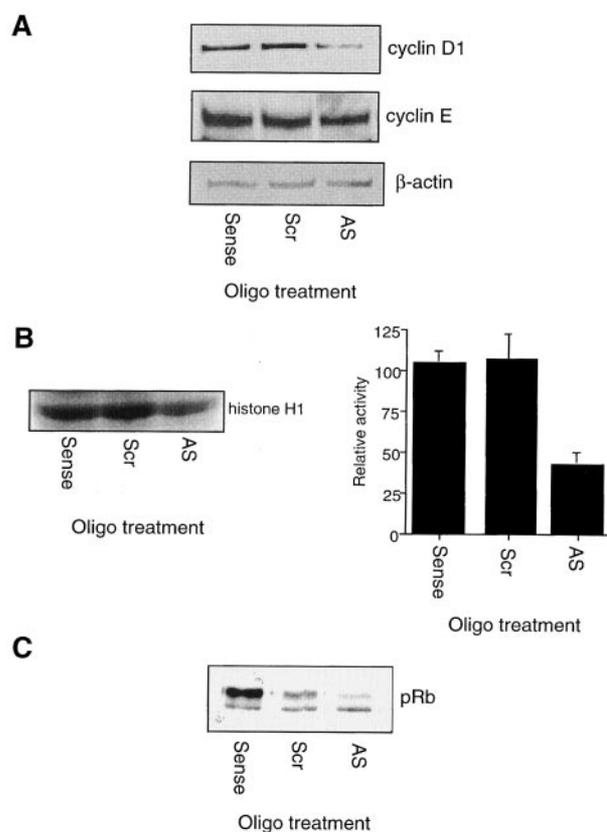

Fig. 3. Reduction in c-Myc causes a decrease in cyclin D1 protein and a decline in cyclin E-Cdk2 activity. MCF-7 cells were treated with either control [sense or scrambled (*Scr*)] or antisense (*AS*) c-*myc* oligonucleotides and Cellfectin lipid vector for 2 h, after which the medium was replaced with 5% RPMI for an additional 6 h. *A*, total cell lysates were collected, separated by SDS-PAGE, and immunoblotted for cyclin D1 followed by cyclin E. The blot was reprobed with an antibody to β-actin as a loading control. *B*, total protein was harvested, cyclin E-immunoprecipitated, and subjected to an *in vitro* kinase assay using histone H1 as substrate, as described in the "Materials and Methods." *C*, changes in pRb phosphorylation state were assessed by Western blotting.
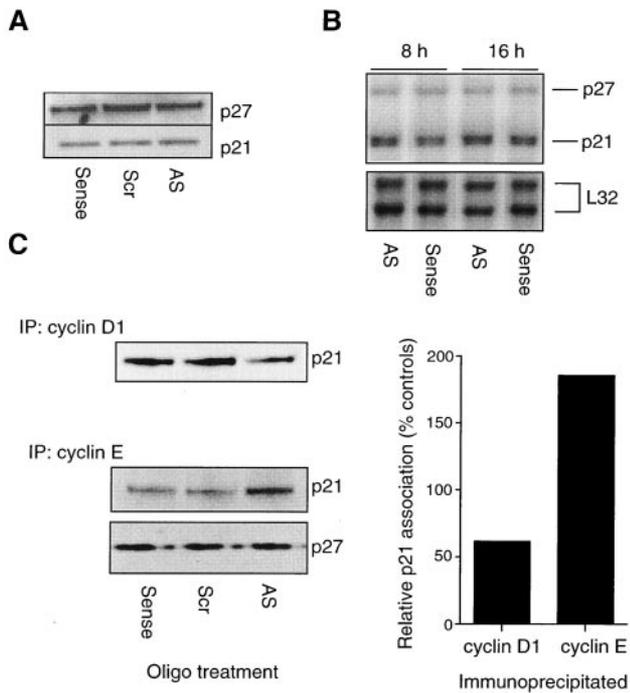
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Fig. 4. Reduction in c-Myc does not change p21$^{WAF1/CIP1}$ or p27^{Kip1} mRNA or protein levels but causes a shift of p21$^{WAF1/CIP1}$ from cyclin D1-Cdk4 to cyclin E-Cdk2 complexes. The experimental design is as described in Fig. 3. *A*, protein levels of p21$^{WAF1/CIP1}$ and p27^{Kip1} in c-*myc*-antisense-treated MCF-7 cells after 8 h. Total cell lysates were prepared as described previously, separated by SDS-PAGE, and immunoblotted for p21$^{WAF1/CIP1}$ and p27^{Kip1}. *B*, RNA was collected from MCF-7 cells treated with either the sense or antisense c-*myc* oligonucleotide after 8 h or 16 h of treatment and subjected to RNase protection as described in the "Materials and Methods." The 'housekeeping' gene *L32* was used as a loading control. *C*, cyclin D1 was immunoprecipitated from MCF-7 cell lysates that had been treated with either the sense or scrambled control or with the c-*myc* antisense oligonucleotide for 16 h, separated by SDS-PAGE, and immunoblotted for p21$^{WAF1/CIP1}$. Cyclin E was also immunoprecipitated from a similar set of lysates and subsequently immunoblotted for p21$^{WAF1/CIP1}$ and p27^{Kip1}. Densitometry was performed as described in "Materials and Methods." *AS*, antisense; *Scr*, scrambled; *IP*, immunoprecipitated.

complexes and subsequent cell cycle arrest after the loss of c-Myc, MCF-7 cells were treated with the antisense c-*myc* oligonucleotide or the sense control, and at the same time, p21$^{WAF1/CIP1}$ or p27^{Kip1} antisense oligonucleotides (or the appropriate sense control). These antisense p21$^{WAF1/CIP1}$ and p27^{Kip1} oligonucleotides are effective in reversing the growth-inhibitory effects of ICI 182780 at these concentrations (6, 7). As seen in Fig. 5*A*, the levels of c-Myc, p21$^{WAF1/CIP1}$, and p27^{Kip1} protein were differentially regulated by the addition of specific antisense oligonucleotides. The presence of any two antisense oligonucleotides in combination could inhibit the production of the specific proteins without any deleterious effects on cell proliferation or morphology (data not shown). The protein changes were not seen in any of the various controls. To assess whether the loss of p21$^{WAF1/CIP1}$ or p27^{Kip1} could influence the inhibition in cyclin E-Cdk2 activity that was observed after the targeted repression of c-Myc, we used the same cell lysates and performed an *in vitro* kinase assay. As shown in Fig. 5*B*, the decrease in cyclin E-Cdk2 activity observed after antisense c-Myc treatment could be reversed when p21$^{WAF1/CIP1}$ was also down-regulated. However, the simultaneous down-regulation of p27^{Kip1} did not appear to attenuate the enzymatic inhibition of cyclin E-Cdk2. To investigate whether the loss of p21$^{WAF1/CIP1}$ or p27^{Kip1} could influence the decrease in DNA synthesis and cellular proliferation after the loss of c-Myc, we used BrdUrd incorporation into DNA as a measure of cell proliferation. As shown in Fig. 5*C*, when c-Myc levels were down-regulated in the presence of p21$^{WAF1/CIP1}$ or p27^{Kip1} sense, control oligonucleotides,

BrdUrd incorporation decreased to ~50% of control levels. However, when p21$^{WAF1/CIP1}$ was simultaneously down-regulated, the S-phase fraction remained at c-Myc sense control levels. When p27^{Kip1} and c-Myc were simultaneously down-regulated, proliferation rates remained only marginally higher than without the p27^{Kip1} antisense oligonucleotide. This confirmed that p21$^{WAF1/CIP1}$ plays a pivotal role in mediating growth arrest after the loss of c-Myc, whereas p27^{Kip1} may play a relatively minor role in the initial growth arrest.

DISCUSSION

c-*Myc* is a potent oncogene, the regulation of which is directly correlated with cellular growth status (30). Expression of c-*Myc* is rapidly lost after mitogenic starvation (31, 32) and ectopic expression
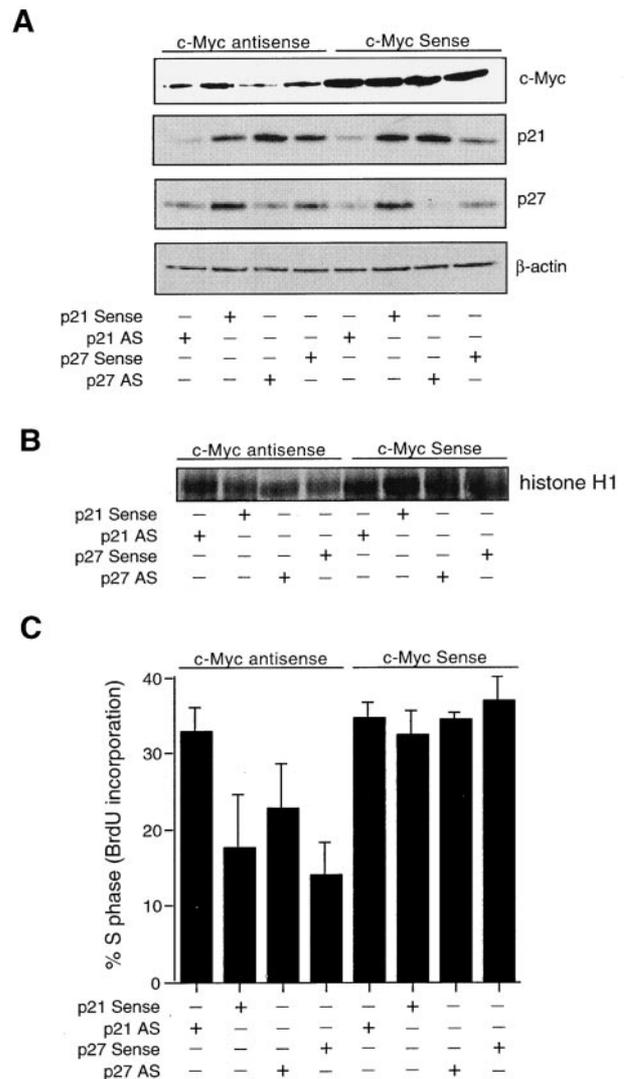

Fig. 5. p21$^{WAF1/CIP1}$ is essential for growth arrest after down-regulation of c-Myc. Exponentially growing cells were treated with 1 μM of antisense c-*myc* oligonucleotide or sense control and simultaneously treated with 400 nM p21$^{WAF1/CIP1}$ antisense (*AS*) oligonucleotide, or 800 nM p27^{Kip1} antisense oligonucleotide, or the appropriate sense control. *A*, total protein levels of p21$^{WAF1/CIP1}$, p27^{Kip1}, and c-Myc after 16 h of antisense treatment. Total cell lysates were prepared as described previously, separated by SDS-PAGE, and immunoblotted for p21$^{WAF1/CIP1}$, p27^{Kip1}, and c-Myc. The nitrocellulose was subsequently blotted with an antibody to β-actin as a loading control. *B*, cyclin E-Cdk2 activity was assessed from total protein by immunoprecipitating cyclin E and subjecting it to an *in vitro* kinase assay using histone H1 as a substrate, as described in the "Materials and Methods." *C*, BrdUrd (*BrdU*) incorporation into control or antisense-treated MCF-7 cells was conducted as described in "Materials and Methods." Data presented are the mean ± SD of three experiments.

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is capable of inducing expression of numerous proliferation-specific genes (33). This direct link between c-Myc expression and cell cycle progression, together with the significant correlation between c-Myc amplification and disease progression or early relapse in several cancers (34), has led to the development of strategies to target this molecule for therapeutic intervention. Specific regulation of the c-*Myc* oncogene has been the focus of several investigations, in which antisense c-*myc* oligonucleotides have been successfully used to inhibit the growth of small cell lung carcinoma (35), hepatoma cells (36), and breast cancer cells (18, 19). However, the molecular mechanisms through which antisense c-Myc strategies induce cell cycle arrest have not been fully defined.

Recent literature has emphasized discrepancies in the implied role of c-Myc in cell cycle progression drawn from studies that have used different experimental systems. Potential c-Myc transcription targets were originally identified based on *in vitro* assays using inducible c-Myc-ER constructs (37). These investigations identified several potential c-Myc targets (15). However, development and characterization of c-*myc* null fibroblasts in culture has shown that expression of many of the proposed c-Myc targets is not changed when c-*myc* is deleted. Several more recent investigations have highlighted a link between c-Myc overexpression and decreased $p21^{WAF1/CIP1}$ transcription (28, 38), although work presented in the present study suggests that $p21^{WAF1/CIP1}$ transcription and protein levels are not influenced by the down-regulation of c-Myc. Deletion of MYC from B cells resulted in a decrease in cyclin E-Cdk2 activity but not in an increase in $p21^{WAF1/CIP1}$ levels (39), a finding in agreement with the data presented here. The divergent conclusions drawn from studies of overexpression of c-Myc, and those studies in which c-Myc expression or function was inhibited, have complicated the definition of c-Myc targets with a pivotal role in cell cycle progression.

Our data suggest that the loss of cyclin D1 protein after the inhibition of c-Myc expression may be a central component of cell cycle arrest. This loss of cyclin D1 results in a redistribution of $p21^{WAF1/CIP1}$ from cyclin D1-Cdk4 to cyclin E-Cdk2, and in a subsequent inhibition of cyclin E-Cdk2 activity. Despite stable levels of $p21^{WAF1/CIP1}$ after the inhibition of c-Myc expression, $p21^{WAF1/CIP1}$ is important in the growth arrest because a simultaneous loss of $p21^{WAF1/CIP1}$ can reverse the growth arrest that typically follows the reduction of c-Myc. This is the first investigation to show that $p21^{WAF1/CIP1}$ is essential for growth arrest after a reduction of c-Myc. At later time points, $p27^{Kip1}$, also, partially contributes to the growth arrest that results from decreased c-Myc production, because a parallel loss of $p27^{Kip1}$ could marginally attenuate the growth arrest.

The mechanistic link between decreased c-Myc expression and consequent decreases in cyclin D1, as presented here, is unclear. Cyclin D1 protein levels can be directly up-regulated by c-Myc (40), not regulated at all (14), and even negatively regulated (41). One investigation suggested that c-Myc can associate with, and sequester, Miz-1, a transcriptional activator of cyclin D1 (42), thereby negatively regulating cyclin D1 transcription. Although overexpression of c-Myc in MCF-7 cells does not influence cyclin D1 protein levels (14), it is clear that studies of the reduction of c-Myc and of c-Myc overexpression do not necessarily identify the same targets. Further support for the data generated here comes from studies of c-*myc* null fibroblasts, in which a loss of c-Myc results in a decrease in cyclin D1 levels (21). Gartel *et al.* suggest that the regulation of the *p21^{WAF1/CIP1}* gene by c-Myc is via the association with Sp1/Sp3 binding sites in the $p21^{WAF1/CIP1}$ promoter (38). The promoter of cyclin D1 possesses several Sp1 sites (43) and may provide opportunity for a direct physical link between the c-Myc protein and cyclin D1 regulation. Despite the controversy surrounding possible regulation of cyclin D1 by c-Myc, cyclin D1 antibodies can prevent c-Myc-induced S-phase
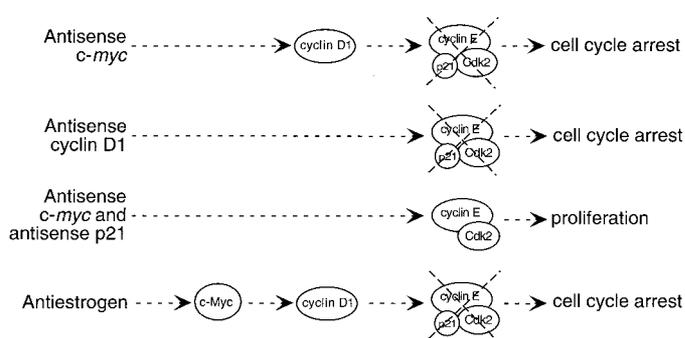

Fig. 6. Model of ICI 182780-mediated growth arrest. ICI 182780 causes a rapid decrease in c-Myc and cyclin D1 protein levels, cyclin E-Cdk2 inhibition, and cell cycle arrest. Antisense c-*myc* oligonucleotides are sufficient to decrease cyclin D1 levels, inhibit cyclin E-Cdk2, and induce cell cycle arrest. Similarly, the direct inhibition of cyclin D1 is sufficient to inhibit cyclin E-Cdk2 activity and induce cell cycle arrest, all of which is dependent on $p21^{WAF1/CIP1}$. Therefore, the current model of ICI 182780 action is an initial decline in c-Myc levels, a consequent decrease in cyclin D1 protein, a redistribution of $p21^{WAF1/CIP1}$ to cyclin E-Cdk2, an inhibition of cyclin E-Cdk2, and cell cycle arrest.

entry (44), and p16 (a specific inhibitor of cyclin D1-Cdk4/6) can block c-Myc-mediated transformation (45), confirming a vital role for cyclin D1 in the oncogenic and proliferative effects of c-Myc.

Growth arrest by ICI 182780 and other antiestrogens involves a rapid decline in c-Myc levels, which, when emulated using antisense strategies, is sufficient to cause cell cycle arrest of MCF-7 breast cancer cells (Fig. 6). This implicates the loss of c-Myc as one of the crucial early events in antiestrogen-mediated growth arrest. c-Myc is commonly overexpressed in many hormone-insensitive breast cancer cells (46, 47), which suggests that, in hormone-independent cells, the constitutive expression of c-Myc may be sufficient to induce changes in the abundance and activity of cyclin/CDK complexes, modify levels of the inhibitors, and regulate pocket protein phosphorylation as documented in the estrogen-induced proliferation of ER-positive cells (11–13). Interestingly, the hyperplastic effects of inducible c-Myc overexpression or c-Myc-ER activation in skin and hematopoietic cells can be reversed by the inactivation of the transgene or its product, which suggests that the continued presence of c-Myc is required for cancer development and not just for initiation (48, 49). As such, specific targeted regulation of c-Myc with antisense oligonucleotides or other therapeutic strategies may be a potential mechanism of regulating established breast cancers. Of particular interest will be determining whether this strategy may be effective in circumventing antiestrogen resistance in breast cancers in which c-Myc down-regulation does not follow antiestrogen treatment.

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