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Antiestrogen ICI 182,780 Decreases Proliferation of Insulin-like Growth Factor I (IGF-I)-treated MCF-7 Cells without Inhibiting IGF-I Signaling[1]

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ABSTRACT

Previous studies have suggested that antiestrogens inhibit MCF-7 cell proliferation by altering the expression or activity of components of the insulin-like growth factor I (IGF-I) signaling pathway, including IGF-I receptor, insulin receptor substrate 1, and phosphatidylinositol 3-kinase. In this report, we examine the effects of the pure antiestrogen ICI 182,780 (ICI) on various targets of IGF-I signaling in MCF-7 cells. ICI treatment led to decreases in the absolute levels of cyclin D1 and cyclin A expression, retinoblastoma protein phosphorylation, and DNA synthesis in IGF-I-treated cells. However, IGF-I retained the ability to induce these events in the presence of ICI, suggesting that ICI treatment did not completely block IGF-I signaling. Consistent with this suggestion, IGF-I-induced phosphorylation of extracellular signal-regulated kinase, AKT, and insulin receptor substrate 1 was unaffected by ICI treatment. Finally, transient expression of either constitutively active phosphatidylinositol 3-kinase or AKT was unable to induce proliferation in ICI-treated MCF-7 cells. Together, these results indicate that ICI can inhibit proliferation without blocking IGF-I signaling and suggest a model in which both estrogen receptor and IGF-I signaling regulate cell cycle components and are required for MCF-7 cell proliferation.

INTRODUCTION

Both growth factors and estrogen regulate proliferation of many breast cancer cells, including the MCF-7 human cell line. Growth factors bind to membrane-bound receptor tyrosine kinases that activate signaling cascades affecting cell cycle progression. IGF-I[3] stimulates proliferation of MCF-7 cells through the IGF-IR (1, 2). Activated IGF-IR recruits and phosphorylates the IRS-1, which in turn activates several downstream signaling pathways including the MAPK and PI3K pathways (3, 4). PI3K activity is essential for proliferation of MCF-7 cells, and blocking its activity with either a chemical inhibitor (5) or by constitutive expression of PTEN (phosphatase and tensin homologue deleted on chromosome 10), a phosphatidylinositol 3-phosphatase (6), causes a G_1 arrest in these cells. PI3K activates downstream kinases including AKT, p70S6 kinase, and glycogen synthetase 3-kinase (7). One of the key downstream targets of these kinases is cyclin D1, an important regulator of cell cycle progression (8).

Estrogens activate ERα and ERβ, which are ligand-activated transcription factors (9). MCF-7 cells are ERα positive (10), and their proliferation is regulated by estrogens and antiestrogens (11). One mechanism by which estrogens such as E induce proliferation in MCF-7 cells is by activating genes that promote cell cycle progression, including *cyclin D1* and *c-myc* (12). Antiestrogens reverse the cell cycle changes induced by estrogen and inhibit proliferation of MCF-7 cells.

In addition to directly regulating cell cycle progression, there is evidence that both E and antiestrogens influence growth factor signaling pathways. For example, antiestrogens are reported to inhibit IGF-I-induced proliferation in MCF-7 cells (13, 14). Under some experimental conditions, this inhibition coincides with a down-regulation of intermediates in the IGF-I signaling pathway, including IGF-IR (15, 16) and IRS-1 protein levels (17), and IRS-1 phosphorylation (18), suggesting that antiestrogens directly inhibit IGF-I signaling. The current study was undertaken to test this model. The results presented here demonstrate that in addition to decreasing proliferation in the presence of IGF-I, ICI treatment of IGF-I-stimulated cells decreases the absolute levels of several important cell cycle regulators, including cyclin D1, cyclin A, and phosphorylated pRb. However, ICI also lowered the basal levels of cyclin D1 expression and proliferation in SFM, and the fold induction of cyclin D1 and proliferation by IGF-I was similar in the absence and presence of ICI. In addition, ICI treatment did not inhibit the ability of IGF-I to induce phosphorylation of ERK or AKT, indicating that this antiestrogen does not completely block IGF-I signaling in MCF-7 cells. Finally, expression of constitutively active PI3K or AKT did not overcome an antiestrogen-mediated growth arrest of MCF-7 cells, indicating that deregulation of the PI3K pathway is insufficient to convert cells to antiestrogen resistance. Together, these results suggest a model in which ER acts downstream of or parallel to the PI3K/AKT pathway to regulate MCF-7 cell proliferation.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were obtained from Dr. Michael Johnson at the Lombardi Cancer Center and maintained in Improved Modified Eagle's Medium (Biofluids, Inc.) containing 5% FBS (HyClone), penicillin (100 units/ml), and streptomycin (100 μg/ml). For serum-free experiments, cell pellets were washed twice in PBS, and the washed cells were plated at 10^6 cells/100-mm plate or 50,000 cells/well on 24-well plates in phenol red-free, serum-free, DMEM F-12 (Sigma) on collagen 1 (4 $\mu g/cm^2$)-coated tissue culture plates as described previously (19). After overnight incubation, cells were treated with ICI (100 nM), E (1 nM), IGF-I (10 ng/ml), ICI + E, IGF-I + E, or IGF-I + ICI. For ICI pretreatment, ICI was added 24 h before the addition of IGF-I.

Plasmids. The HA-tagged AKT encoded by pCEFL-myr-HA-AKT contains a myristylation signal that causes membrane localization and has been described previously (20). The plasmid was a gift of Dr. Yi Li and J. S. Gutkind. The constitutively active Myc-tagged PI3K (p110*) and the kinase dead PI3K (ΔKIN) constructs have been described previously (21) and were provided by Dr. Anke Klippel. The SRE-luc and the CMV β-galactosidase plasmids were obtained from Clontech. The cyclin D1 cDNA plasmid was provided by Dr. Steven Reed. A plasmid containing the 18S rRNA gene was obtained from Dr. Laura McCabe.

Chemicals, Growth Factors, and Antibodies. ICI was a gift of A. Wakeling (Zeneca Pharmaceuticals), and E was purchased from Sigma. IGF-I was from GroPep Pty. Ltd. (Adelaide, Australia), and LY294002 was from Calbiochem. Rat tail collagen 1 was from Collaborative Biomedical Products (Bedford, MA). [^{3}H]Thymidine (50 Ci/mmol) was from ICN Biomedicals. The cyclin D1, IRS-1, and tyrosine phosphate (4G10) antibodies were from Upstate Biotechnology. Antibodies to cyclin E, cyclin A, and pRb were from Phar-

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[3] The abbreviations used are: IGF-I, insulin-like growth factor I, IGF-IR, insulin-like growth factor I receptor; IRS-1, insulin receptor substrate 1; ER, estrogen receptor; E, 17β-estradiol; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; pRb, retinoblastoma protein; Cdk, cyclin-dependent kinase; SFM, serum-free media; HA, hemagglutinin; BrdUrd, bromodeoxyuridine; ICI, ICI 182,780; FBS, fetal bovine serum; SRE, serum response element; CMV, cytomegalovirus; luc, luciferase.

Mingen. The ERα antibody was a monoclonal antibody (Mab17; Ref. 22) and was a gift of Dr. Richard Miksicek. Both the total and phosphoserine 473 AKT antibodies were rabbit polyclonal antibodies from New England Biolabs. Phospho-ERK and total ERK antibodies were from Santa Cruz Biotechnology. Rat anti-HA and mouse anti-Myc (clone 9E10.3) antibodies were from Boehringer Mannheim and Neomarkers, respectively. Sheep anti-BrdUrd and mouse anti-BrdUrd were from Fitzgerald Industries and Boehringer Mannheim, respectively. FITC-conjugated donkey antisheep and goat antirat antibodies were from Sigma, whereas the rhodamine-conjugated goat antimouse antibody was from Boehringer Mannheim. The β-actin antibody was from Sigma.

Transfections and Luc Assays. MCF-7 cells were plated at 5×10^5 cells/60-mm dish and transfected using Superfect reagent (Qiagen). One μg of SRE-luc plasmid was cotransfected with 1 μg of either vector, p110*, or ΔKIN along with 0.2 μg of pCMV β-galactosidase as a control for transfection efficiency. Cells were kept in 5% charcoal dextran-stripped serum containing medium for 24 h and then treated with various media for an additional 24 h. Cells were harvested at 48 h posttransfection, and both luc and β-galactosidase activities were measured using the protocol provided by the manufacturer (Clontech) on a Turner TD 20E luminometer (Turner Designs). Each transfection was done in triplicate, and the luc activity was normalized to β-galactosidase activity in each sample.

[^{3}H]Thymidine Incorporation. Cells were plated at 50,000 cells/well on 24-well plates and treated as described for the respective experiments. One μCi of tritiated thymidine was added to each well, and the cells were harvested in 0.5% NaOH, 0.1% Triton X-100 after 24 h of labeling. Cell lysates were precipitated with trichloroacetic acid (10%), and acid-precipitable counts were measured using liquid scintillation counting. Six wells were harvested for each treatment; three wells were used for thymidine incorporation, and the remaining three wells were used to determine the DNA content by a fluorometric assay (23). [^{3}H]Thymidine incorporation/well was normalized to DNA content.

Immunoblotting. MCF-7 cells were lysed as described previously (24), and protein was quantitated using the Bradford protein assay (Bio-Rad). Protein (20–50 μg) was subjected to SDS-PAGE (12% or 7.5%), transferred to polyvinylidene difluoride membranes, and probed using the appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using the enhanced chemiluminescence reagent (Pierce). Cyclin D1 levels in the experiment shown in Fig. 2*A* were quantitated by densitometric scanning of the resulting films.

Northern Blotting. Cells were lysed, and total RNA was purified using Trizol reagent (Life Technologies, Inc.). Twenty μg of RNA were electrophoresed on 1% agarose-formaldehyde gels, transferred to nitrocellulose membranes, and hybridized with a ^{32}P-labeled cDNA probe for cyclin D1. The membrane was stripped and reprobed for 18S rRNA as a loading control. Bands were quantitated by PhosphorImager scanning (Molecular Dynamics), and the cyclin D1 mRNA levels were normalized to 18S rRNA.

Indirect Immunofluorescence. Cells were plated on coverslips and transfected with either the HA-tagged AKT or the Myc-tagged PI3K (p110*) plasmid using Superfect reagent (Qiagen). After transfection, cells were incubated in medium containing 5% FBS and ICI and labeled with 25 μM BrdUrd for 5 h before fixation at 48 h posttransfection. The double immunofluorescence procedure for detecting proteins and BrdUrd has been described previously (25). BrdUrd incorporation was detected using mouse anti-BrdUrd for HA-AKT and sheep anti-BrdUrd for Myc-PI3K. Cells were viewed under a fluorescence microscope, and the percentage of HA- (or Myc-)positive and -negative cells that were also positive for BrdUrd was determined. At least 100 HA/Myc-positive and -negative cells were counted in each experiment. The images shown in Fig. 5*A* were taken on an Olympus microscope, scanned, and converted to grayscale using Adobe Photoshop.

RESULTS

ICI Treatment Decreases Proliferation of IGF-I-treated MCF-7 Cells. To assess the effects of E, ICI, and IGF-I on proliferation, MCF-7 cells were cultured in SFM and treated with these agents alone or in combination. Proliferation was assayed by measuring [^{3}H]thymidine incorporation, and results from one representative experiment are shown in Fig. 1*A*. MCF-7 cells in SFM incorporated basal levels of [^{3}H]thymidine, which decreased 4–5-fold upon treatment with ICI. This result suggested that a ligand-independent activity of ER was inhibited by ICI. E treatment induced a 2-fold increase in [^{3}H]thymidine incorporation that was completely reversed by ICI. Treatment with IGF-I alone increased proliferation 3–4-fold over control levels, and E had an additive effect with IGF-I. In agreement with previously published results (13, 14), ICI treatment reduced proliferation in the presence of IGF-I, but not down to the level seen in cells treated with ICI alone or in combination with E. This suggested that the ability of IGF-I to stimulate proliferation was not completely inhibited by ICI treatment. To determine the extent to which IGF-I stimulated proliferation in the presence and absence of ICI, [^{3}H]thymidine incorporation was compared in three independent experiments. The results of these experiments confirmed those shown in Fig. 1*A*; IGF-I treatment induced proliferation 3–4-fold in both the presence and absence of ICI, but the absolute levels of proliferation obtained were 2–3-fold higher in the absence of ICI (data not shown).

Both ICI and IGF-I Regulate Cyclin Protein Levels. To investigate the mechanism(s) by which ICI limits proliferation, we examined its effects on the expression of cyclins, key regulators of the G_1-S-phase transition. MCF-7 cells were treated as described above, and protein extracts were prepared and subjected to Western blot analysis for cyclin D1, cyclin E, and cyclin A. Results from one representative experiment are shown in Fig. 1*B*. Cells in SFM expressed basal levels of cyclin D1 that decreased upon treatment with ICI. Treatment of cells with IGF-I or with IGF-I + E increased the levels of cyclin D1 compared with controls, and cells cotreated with both ICI and IGF-I expressed lower levels of cyclin D1 than those treated with IGF-I alone. Cyclin A levels increased in cells treated with either IGF-I or E and were decreased by ICI treatment. Cyclin A is induced at the G_1-S transition (26), and its regulation is consistent with the proliferation data shown in Fig. 1*A*. Cyclin E levels did not

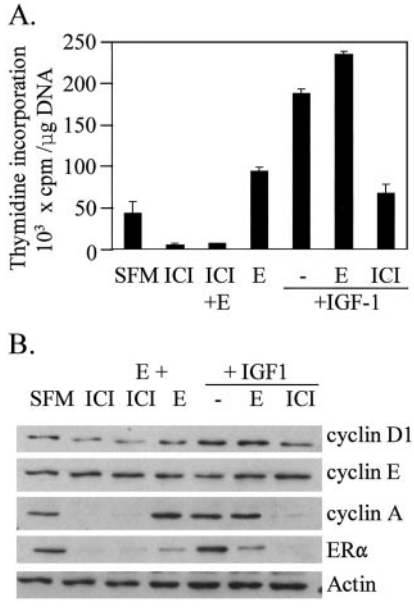

Fig. 1. ICI treatment decreases proliferation in IGF-I-treated cells. MCF-7 cells were plated in SFM, and after overnight culture, cells were treated with ICI (100 nM), E (1 nM), IGF-I (10 ng/ml), or various combinations thereof as described in "Materials and Methods." Untreated cells in SFM served as controls. *A*, [^{3}H]thymidine (1 μCi) was added to each well after 24 h of treatment, and cells were harvested after 24 h of labeling. Acid-precipitable counts were measured and normalized to DNA content. Results from one experiment are shown as the average ± SE from triplicate wells. *B*, in experiments carried out in parallel to those shown in *A*, cells were harvested after 2 days of treatment, and lysates were subjected to immunoblotting for cyclin D1, cyclin E, cyclin A, and ERα. Actin served as a loading control.

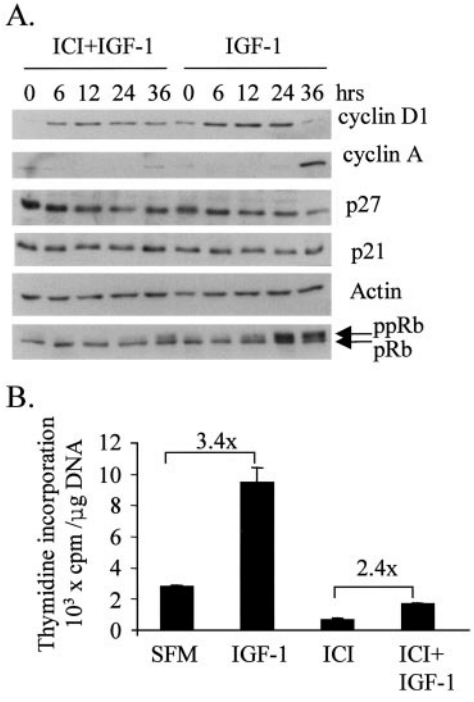

Fig. 2. ICI pretreatment does not inhibit IGF-I-mediated induction of cyclin D1 or proliferation. MCF-7 cells incubated in SFM or pretreated with ICI for 24 h were stimulated with IGF-I. *A,* cell lysates were harvested at the time points indicated and subjected to Western blotting for cyclin D1, cyclin A, $p21^{Waf1}$, $p27^{Kip1}$, pRb, or β-actin. *B,* in a parallel experiment, [^{3}H]thymidine incorporation was determined during the 24-h period after IGF-I treatment. Results represent the average ±SE of triplicate wells normalized to DNA content. Fold induction of [^{3}H]thymidine incorporation in either SFM- or ICI-treated cells is shown *above* the bars. The results shown are representative of three independent experiments.

change with the various treatments. Also shown in Fig. 1*B* is down-regulation of ER levels in the presence of E and ICI. ICI has previously been shown to cause receptor degradation (27), and E down-regulates ERα by decreasing its transcription, mRNA, and protein stability (28, 29). In similar experiments, levels of progesterone receptor, a transcriptional target of ER (30), were increased by E treatment of cells in SFM (data not shown). Together, these results confirm that the SFM was free of E.

ICI Pretreatment Does Not Block IGF-I Induction of Cyclin D1 Expression or Proliferation. The fact that IGF-I retained the ability to induce cyclin D1 and [^{3}H]thymidine incorporation in the presence of ICI suggested that IGF-I signaling was functional in ICI-treated cells. However, because cells were treated simultaneously with IGF-I and ICI, it was possible that ICI had insufficient time to block IGF-I signaling. To investigate the effects of ICI pretreatment on IGF-I signaling, cells were plated in SFM and then either left in SFM or preincubated with ICI for 24 h. They were then stimulated with IGF-I in the presence or absence of ICI, harvested at various times, and analyzed by Western blotting for cyclin D1, cyclin A, the Cdk inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, and pRb. The results of one representative experiment are shown in Fig. 2*A*. Consistent with the results in Fig. 1, ICI treatment decreased the absolute levels of cyclin D1 approximately 2-fold, as determined by densitometric scanning. However, IGF-I treatment induced cyclin D1 expression to similar extents in both the absence and presence of ICI. The levels of cyclin D1 remained constant in ICI + IGF-I-treated cells until 36 h but declined between 24 and 36 h in cells treated with IGF-I alone. This decrease correlated with the entry of cells into S phase, as indicated by cyclin A expression and pRb phosphorylation. Because cyclin D1 levels vary during the cell cycle, the decline in cyclin D1 levels may be a consequence of cell cycle progression (8).

Because both ICI and IGF-I have been reported to regulate $p27^{Kip1}$ and $p21^{Waf1}$ levels in MCF-7 cells (31, 32), we examined the effects of IGF-I and ICI on these proteins. The $p27^{Kip1}$ levels were higher in ICI pretreated cells than in control cells at time 0 (Fig. 2*A*); however, IGF-I treatment decreased $p27^{Kip1}$ levels in both ICI-treated and control cells. In contrast to previous reports (32, 33), the levels of $p21^{Waf1}$ were not reproducibly altered by IGF-I or ICI treatment in these experiments. Although we have not investigated the reason for this difference, it may be due to different cell culture conditions. Under the conditions used in these experiments (cells in SFM plated on collagen), the basal levels of $p21^{Waf1}$ are high, and it is possible that they cannot be further increased by IGF-I or ICI treatment.

The effect of ICI pretreatment on IGF-I-induced proliferation was also assessed. As shown in Fig. 2*A,* ICI pretreatment almost completely blocked cyclin A expression and pRb phosphorylation, although small increases were reproducibly seen at the 36 h time point. These effects were paralleled by changes in DNA synthesis, where ICI treatment decreased [^{3}H]thymidine incorporation approximately 4-fold (Fig. 2*B*). However, even in the presence of ICI, IGF-I increased proliferation 2.4-fold over that seen in untreated cells. The overall decrease in proliferation in ICI-treated cells may be due to the 50% reduction in cyclin D1 levels observed because a threshold level of cyclin D1 may be required to fully activate G_1 Cdks and phosphorylate pRb. Alternatively, additional targets of ICI may be contributing to its effects.

Cyclin D1 mRNA Levels Are Regulated by Both ICI and IGF-I. Mitogens such as IGF-I can lead to the stabilization of cyclin D1 protein (34). It was therefore possible that ICI was regulating cyclin D1 mRNA levels and that IGF-I was regulating cyclin D1 protein stability, in which case cyclin D1 mRNA and protein levels would not be coordinately regulated. To test this possibility, total cellular RNA was extracted at various time points after IGF-I stimulation of ICI-pretreated or control cells, and Northern blotting was carried out as described in "Materials and Methods." Results from one representative experiment are shown in Fig. 3*A,* and the quantitation of results

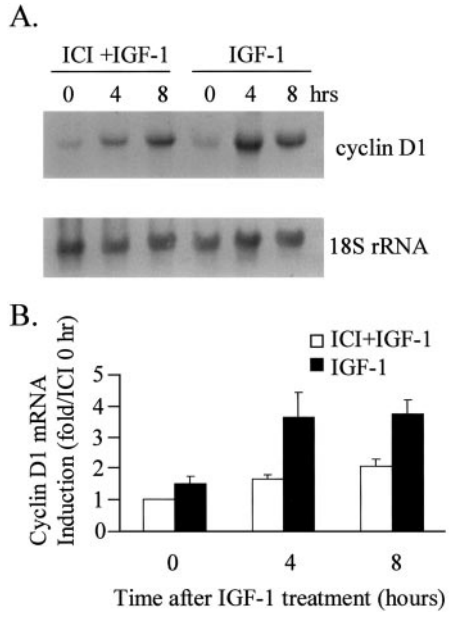

Fig. 3. IGF-I induces cyclin D1 mRNA in the presence and absence of ICI. *A,* MCF-7 cells incubated in SFM or pretreated with ICI for 24 h were stimulated with IGF-I. At the times indicated after IGF-I treatment, total RNA was isolated and analyzed for cyclin D1 mRNA by Northern blotting. *B,* cyclin D1 mRNA levels were normalized to 18S rRNA and are represented as fold induction over the 0 h ICI treatment time point. The results are the mean ± SE of three independent experiments.

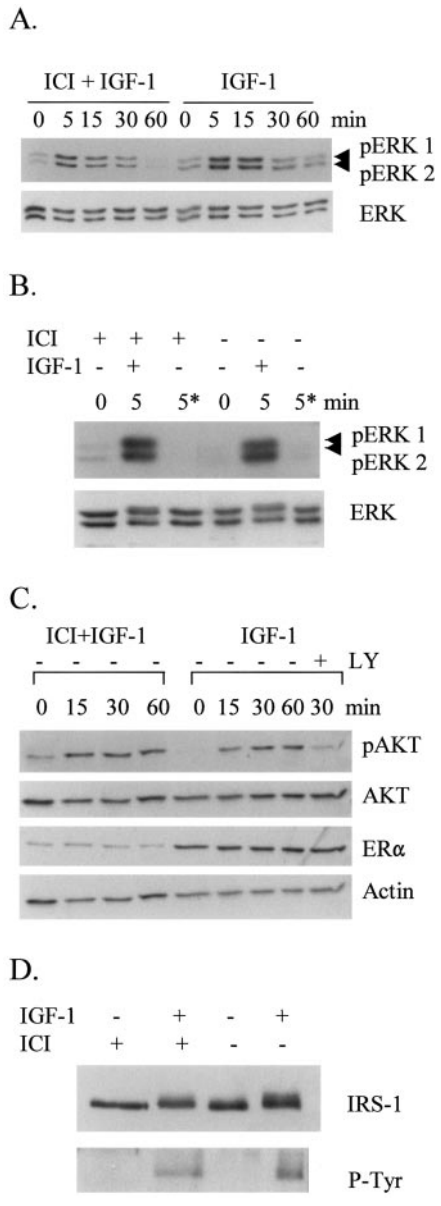

Fig. 4. ICI does not inhibit ERK or AKT activation by IGF-I. MCF-7 cells incubated in SFM or pretreated with ICI for 24 h were stimulated with IGF-I. *A*, cells were harvested at the time points indicated, and MAPK activation was assayed by Western blotting using an antibody specific to phosphorylated ERK1 and ERK2. The blots were then reprobed with an antibody that detects total ERK1 and ERK2. *B*, cells pretreated as described above were incubated with IGF-I or SFM media (*5**) alone and harvested after 5 min. The levels of both phosphorylated and total ERK1 and ERK2 were determined by Western blotting. *C*, cells were pretreated with ICI for 24 h or with LY294002 for 30 min. They were then stimulated with IGF-I, and lysates were prepared at the times indicated and analyzed by Western blotting with an antibody specific for AKT phosphorylated on Ser^{473}. Blots were also probed with antibodies to total AKT, ERα, and actin. *D*, cells in SFM or preincubated with ICI for 24 h were treated with IGF-I or left untreated and harvested after 10 min. Cell lysates were prepared, and 50 μg of total protein were subjected to SDS-PAGE (7.5%) and analyzed for IRS-1 protein levels and phosphorylation by Western blotting with antibodies directed against IRS-1 or phosphotyrosine.

from three independent experiments is shown in Fig. 3*B*. Consistent with the protein data, the absolute levels of cyclin D1 mRNA were approximately 2-fold higher in the absence of ICI than in the presence of ICI. However, when the amount of mRNA at time 0 was defined as 1 for each treatment, the induction by IGF-I was similar (2–2.5-fold) in the presence and absence of ICI. These results demonstrate that cyclin D1 mRNA levels parallel protein levels and suggest that both ICI and IGF-I are regulating cyclin D1 predominantly at the mRNA level under these experimental conditions.

ICI Does Not Inhibit Activation of ERK, AKT, or IRS-1 by IGF-I. To directly examine the effects of ICI on IGF-I signaling, activation of two downstream targets, ERK/MAPK and AKT, was investigated. As shown in Fig. 4*A*, addition of IGF-I to cells in SFM leads to ERK phosphorylation within 5 min of treatment, with the levels of phosphorylation returning to baseline by 60 min. The ability of IGF-I to induce ERK phosphorylation was not significantly inhibited by pretreatment with ICI (Fig. 4, *A* and *B*), indicating that this branch of the IGF-I signaling pathway is independent of ER function.

Activation of PI3K by IGF-I is required to induce cyclin D1 protein and proliferation in MCF-7 cells (5). We confirmed this requirement using LY294002, a specific PI3K inhibitor. Treatment of MCF-7 cells with this inhibitor prevented the induction of cyclin D1 protein, mRNA, and proliferation seen upon IGF-I treatment (data not shown). An important downstream target of PI3K is AKT. The effects of ICI on IGF-I-induced AKT activation were assessed by immunoblotting with an antibody that detects AKT phosphorylated on Ser^{473} (Ref. 35; Fig. 4*C*). IGF-I treatment induced a rapid phosphorylation of Ser^{473}, and the levels of phosphorylated AKT were maintained for 1 h. This induction was not inhibited by ICI pretreatment, which down-regulated ERα protein levels. However, it was inhibited by treatment with the PI3K inhibitor LY294002, indicating that induction of Ser^{473} phosphorylation by IGF-I does not require ER activity but does require PI3K activation.

Because it has been reported previously that ICI treatment decreases IRS-1 protein levels and/or phosphorylation induced by IGF-I, we also examined this aspect of IGF-I signaling under our experimental conditions. As shown in Fig. 4*D*, pretreatment with ICI for 24 h did not decrease IRS-1 protein levels (compare *Lanes 1* and *3*). IGF-I-induced phosphorylation of IRS-1, detected by a change in the mobility of the protein or by probing with a phosphotyrosine-specific antibody, was also unaffected by ICI treatment. Taken together, the results presented in Figs. 2 and 4 establish that ICI can inhibit proliferation of IGF-I-treated MCF-7 cells without directly blocking the IGF-I signaling pathway.

Constitutively Active PI3K or AKT Does Not Confer ICI Resistance. The results presented above indicate that ICI does not directly block the IGF-I/PI3K signaling pathway, although it does limit the total amount of proliferation induced by IGF-I. To determine whether constitutive activation of the PI3K pathway could overcome the antiproliferative effects of ICI, constitutively activated forms of a Myc-tagged PI3K (p110*) or HA-tagged AKT were used. MCF-7 cells transfected with plasmids encoding constitutively active Myc-PI3K or HA-AKT were incubated in ICI-containing medium, labeled with BrdUrd, and then fixed and examined for both AKT/PI3K expression and BrdUrd incorporation as described in "Materials and Methods." The tagged versions of both PI3K and AKT were readily detectable in transfected cells (Fig. 5*A*); however, as shown in Fig. 5*B*, neither protein was able to increase proliferation in the presence of ICI above the level seen in untransfected cells.

Because constitutively active PI3K was unable to overcome an ICI-induced growth arrest, we confirmed the activity of this construct in MCF-7 cells by testing its ability to activate transcription from a SRE promoter-luc reporter construct. The SRE is part of the complex promoters of genes such as c-fos and is activated by IGF-I, and this activation is dependent on PI3K (36, 37). As shown in Fig. 5*C*, the constitutively active PI3K (p110*), but not a kinase dead mutant (ΔKIN), increased luc activity to a similar extent as IGF-I treatment. This confirmed its activity in MCF-7 cells and indicated that its inability to induce proliferation was not due to a quantitative defect in the PI3K pathway. In addition, Fig. 5*C* demonstrates that SRE activation by IGF-I was not inhibited by ICI treatment, further supporting the finding that ICI does not block IGF-I signaling.

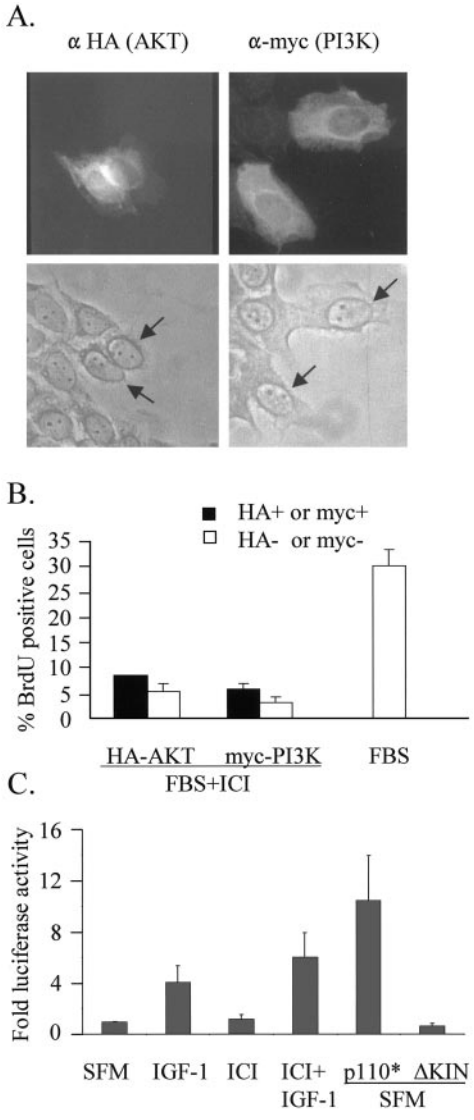

Fig. 5. Constitutively active PI3K and AKT are insufficient to overcome an ICI-induced cell cycle arrest. MCF-7 cells were transfected with constitutively activated AKT (HA tag) or PI3K (Myc tag). After transfection, cells were incubated for 48 h in medium containing 5% FBS + ICI, and BrdUrd was added for the final 5 h before fixation. Cells in medium containing 5% FBS served as a control population of cycling cells. Expression of the transfected proteins and BrdUrd incorporation were detected by double indirect immunofluorescence as described in "Materials and Methods." *A*, representative micrographs showing expression of the tagged proteins. *B*, the percentage of PI3K- or AKT-positive cells that were also BrdUrd positive is represented by *filled bars*. *Open bars* represent the percentage of PI3K- or AKT-negative cells that were BrdUrd positive in the same cultures. The results are the average ± SE of three independent experiments. *C*, MCF-7 cells were cotransfected with a SRE-luc plasmid and either vector alone, constitutively active PI3K (p110*), or a kinase dead version of PI3K (ΔKIN). All transfections also included a CMV promoter-driven β-galactosidase gene as a control for transfection efficiency, and all luc activities were normalized to β-galactosidase activity. The results shown represent fold induction over vector alone control and are shown as the average ± SE of three independent experiments, each done in triplicate.

DISCUSSION

There is considerable evidence for cross-talk between the IGF-I and estrogen signaling pathways in the regulation of breast cancer cell proliferation. Initial work (13, 14) demonstrated that the antiestrogen tamoxifen inhibits insulin/IGF-I-induced proliferation of ER-positive breast cancer cells, including MCF-7 cells. Results from several laboratories have suggested that the decrease in IGF-I-induced proliferation in response to antiestrogens is the result of inhibition of the IGF-I signaling pathway and that estrogens and antiestrogens mediate some of their proliferative effects via this pathway. Both IGF-IR and IRS-1 protein levels have been reported to be down-regulated by ICI (16, 17, 38), as have PI3K (18) and AKT (39) activities. Additional studies have indicated that E can enhance both PI3K and AKT activation by IGF-I (32), and a recent study has reported rapid and transient activation of PI3K by E in MCF-7 cells (40). All of these findings support the idea that estrogens and antiestrogens regulate the IGF-I signaling pathway. However, evidence to the contrary also exists. Several studies have reported that E alone does not affect PI3K or AKT activity (32, 41). In addition, overexpression of IGF-IR (42), IRS-1 (43), or constitutively active AKT (44) in MCF-7 cells did not lead to ICI resistance, indicating that there are additional targets of ICI that mediate its antiproliferative effects. The reason why effects of E and antiestrogens on IGF-I signaling are seen in some experimental systems but not others are not understood but may involve differences in both cell lines and/or cell culture conditions.

In this study, we have investigated the effects of ICI on several targets of IGF-I signaling. As reported previously, ICI treatment lowered the absolute level of proliferation attained in IGF-I-treated cells. However, basal proliferation was also decreased by ICI treatment, and proliferation was induced to similar extents (2–4-fold) by IGF-I in the presence and absence of ICI (Figs. 1*A* and 2*B*). Similar results were obtained with regard to the induction of cyclin D1, an important cell cycle-regulatory protein. Whereas the absolute levels attained were lower in the presence of ICI, the extent of both protein and mRNA induction by IGF-I was similar (Figs. 2*A* and 3). These results are in agreement with a recent report (33) showing that ICI pretreatment of MCF-7 cells did not prevent cyclin D1 induction by insulin, which, at the concentrations used in that study, acts via the IGF-IR (45).

IGF-I activates several different intracellular pathways, including the MAPK and PI3K signaling pathways (3, 4). Under our experimental conditions, MAPK activation by IGF-I was not inhibited by ICI treatment (Fig. 4*A*). To examine the effects of ICI treatment on the PI3K pathway, we tested its ability to inhibit AKT activation. AKT is implicated in both cyclin D1 induction and proliferation, and its phosphorylation and activation are induced by PI3K (7, 46). Our results show that phosphorylation of AKT on Ser^{473} is induced by IGF-I in MCF-7 cells. AKT phosphorylation was inhibited by LY294002 but was unaffected by ICI treatment, indicating that the IGF-I signaling pathway upstream of AKT is intact in ICI-treated cells. This was confirmed by assaying IRS-1 phosphorylation, which was also unaffected by ICI treatment.

Several interesting questions are raised by these results. The first is whether the decreased level of proliferation seen in IGF-I + ICI-treated cells relative to those treated with IGF-I alone is due to lower cyclin D1 levels, or whether additional targets of ICI contribute to its effects. As shown in Fig. 2*A*, although cyclin D1 is induced in the presence of ICI, it accumulates to only 50% of the levels seen without ICI, and there is very little hyperphosphorylated pRb or cyclin A expression. Cyclin D1 has been proposed to activate Cdk4 directly and to contribute to Cdk2 activation by titrating the Cdk inhibitor $p21^{Waf1}$ from cyclin E/Cdk2 complexes into cyclin D1/Cdk4 complexes (47, 48), and a threshold level of expression may be required to fulfill these functions. This possibility is supported by the facts that cyclin D1 overexpression promotes cell cycle progression in the presence of ICI (49) and that a 50% reduction in cyclin D1 levels using antisense oligonucleotides results in a nearly complete inhibition of MCF-7 proliferation and cyclin E/Cdk2 activity (50). However, ICI also alters the expression of other cell cycle regulators including c-Myc, the Cdk inhibitors $p21^{Waf1}$ and $p27^{Kip1}$, and the Cdk-activating phosphatase Cdc25A (31, 47, 51). In particular, the up-regulation of $p21^{Waf1}$ by antiestrogens reportedly contributes to the inhibition of both Cdk2 (47, 48) and Cdk4 (52) complexes.

Although we did not detect changes in $p21^{Waf1}$ expression in these experiments, and ICI did not block the down-regulation of $p27^{Kip1}$, it remains possible that the effects of ICI on molecules other than cyclin D1 may be responsible for the ability of ICI to decrease proliferation in the presence of IGF-I.

A second question is whether ICI inhibits IGF-I-induced proliferation by directly interfering with IGF-I signaling. Under our experimental conditions, IGF-I-induced activation of IRS-1, AKT, ERK, and SRE-luc expression was unaffected by ICI treatment, although proliferation was strongly inhibited. This argues against a direct effect of ICI on IGF-I signaling, although it is possible that ICI blocks elements downstream of or parallel to AKT activation. An alternative explanation, and one that is consistent with our finding that expression of constitutively active PI3K is not sufficient to promote proliferation in the presence of ICI, is that ER and IGF-I signaling pathways converge to regulate the expression of cell cycle components such as cyclin D1 and that both pathways are required to obtain maximal proliferation.

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