The Cyclin-dependent Kinase Inhibitor p21WAF1/Cip1 Is an Antiestrogen-regulated Inhibitor of Cdk4 in Human Breast Cancer Cells*

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The MCF-7 cell line is a model of estrogen-dependent, antiestrogen-sensitive human breast cancer. Antiestrogen treatment of MCF-7 cells causes dramatic decreases in both Cdk4 and Cdk2 activities, which leads to a G1 phase cell cycle arrest. In this report, we investigate the mechanism(s) by which Cdk4 activity is regulated in MCF-7 cells. Through time course analysis, we demonstrate that changes in Cdk4 activity in response to estrogen or antiestrogen treatment do not correlate directly with cyclin D1 protein levels or association. In contrast, Cdk4 activity does correlate with changes in the level of the Cdk inhibitor p21WAF1/Cip1. Furthermore, we show that extracts of antiestrogen-treated cells contain a factor capable of inhibiting the Cdk4 activity present in extracts of estrogen-treated cells, and immunodepletion experiments identify this factor as p21WAF1/Cip1. These results identify p21WAF1/Cip1 as an important physiological regulator of Cdk4 complexes in human breast cancer cells.

Many estrogen receptor (ER)1-positive breast tumors require estrogen for growth and can be successfully treated with antiestrogens (1, 2). The MCF-7 cell line, which was derived from a human breast adenocarcinoma, serves as a model for such estrogen-responsive and antiestrogen-sensitive breast tumors (3). MCF-7 cells require estrogen to proliferate and arrest in the G1 phase of the cell cycle when they are deprived of estrogen or treated with antiestrogens (4–7). Understanding the mechanisms by which antiestrogens arrest the growth of breast cancer cells is an area of active investigation with potential clinical significance.

In normal cells, the transition from G1 to S phase requires the activity of two classes of cyclin-dependent kinases (Cdks), Cdk4/6 and Cdk2. Cdk4/6 phosphorylates the tumor suppressor protein pRb, which leads to transcription of E2F-regulated genes including cyclins E and A. Cdk2 can also phosphorylate pRb, as well as additional substrates necessary for genome duplication (8–11). Cdk activity is regulated by multiple mechanisms, including phosphorylation (12–15) and association with both positive and negative regulatory proteins, and each of these mechanisms is potentially modified by ER signaling. Cdk activation requires association with a cyclin partner as follows: Cdk4/6 associates with D-type cyclins, whereas Cdk2 associates with either cyclin E or cyclin A (16, 17). Cdk activity can be inhibited by two different families of cyclin-dependent kinase inhibitors (CdkIs). Members of the INK4a family, including p16INK4a (p16), bind specifically to monomeric Cdk4/6 and prevent its association with a D-type cyclin (18, 19). Members of the WAF1/Cip1 family, which include p21WAF1/Cip1 (p21) and p27kip1 (p27), bind to G1 cyclin-Cdk complexes and not to monomeric cyclins or Cdkks (19–25). Although both p21 and p27 can inhibit the activity of Cdk2 and Cdk4/6, p21 can also serve as an assembly factor for cyclin D-Cdk4 complexes, increasing the efficiency of complex formation and Cdk4 activity (26–28).

Based on numerous studies, a general model for the regulation of passage from G1 to S phase in normal cells has been proposed, and deregulation at various stages in the model are proposed to be responsible for cellular transformation and tumorigenesis (8, 10, 17, 20, 29, 30). Many mitogens induce cyclin D expression, either through increased mRNA synthesis and/or stabilization of the protein. Cyclin D binds to Cdk4/6, resulting in an increase in Cdk4/6 kinase activity, pRb phosphorylation, and synthesis of cyclins E and A. Increasing the cellular concentration of cyclin D-Cdk4/6 complexes also provides additional binding targets for p21 and/or p27, thereby titrating these CdkIs from their inhibitory association with Cdk2 complexes (19, 31). Together, the increased levels of cyclins and the decreased association of CdkIs lead to Cdk2 activation and progression to S phase (8). Although this model is widely accepted, mitogens and anti-mitogens can also affect progression through G1 in other ways, for example by regulating the levels of CdkIs (32, 33), the assembly and nuclear import of cyclin-Cdk complexes (34), or the levels and activity of Cdk-activating kinases or phosphatases (13–15).

The specific mechanism(s) by which estrogen and antiestrogens regulate MCF-7 cell proliferation are not completely understood. Others have reported (31, 35–38) that antiestrogen treatment decreases cyclin D1 and cyclin A protein levels, Cdk4 and Cdk2 activity, and pRb phosphorylation and increases Cdk1 levels. All of these effects are reversed upon removal of antiestrogen and replacement with estrogen (38–40). Although both total Cdk2 and cyclin E/Cdk2 activities are highly regulated, cyclin E protein is expressed at constant levels in estrogen- and antiestrogen-treated MCF-7 cells. The lack of activity of cyclin E-Cdk2 complexes in the presence of antiestrogen is believed to be due to inhibition by p21 and/or p27 (31, 40). Cyclin E/Cdk2 activation after estrogen treatment may therefore be a result of decreased levels of these inhibitors and/or sequestration of p21 or p27 into newly formed cyclin D1-Cdk4 complexes (31, 40).

Previous reports (31, 40) suggest that Cdk4 activity in estro-
gen- or antiestrogen-treated MCF-7 cells is regulated primarily by the levels of cyclin D1 protein. Although p21 and p27 are found in association with Cdk4, evidence for a physiological role in inhibiting Cdk4 is lacking. The CdkI p21 is reported to be an activator and an inhibitor of Cdk4, so its function in the regulation of Cdk4 activity by antiestrogen is unclear. In the current report, we investigate the mechanisms by which 17β-estradiol (E2) and the pure antiestrogen ICI 182,780 (ICI) regulate Cdk4 activity in MCF-7 cells. We find that Cdk4 activity is not directly correlated with cyclin D1 protein levels or association. By utilizing an in vitro mixing assay, we demonstrate that extracts of ICI-treated cells contain a factor that inhibits the Cdk4 activity present in extracts of estrogen-treated cells and that this inhibitory factor is specifically removed by immunodepletion of p21 but not p27. These studies identify p21 as an important physiological target of antiestrogen action that inhibits Cdk4 in addition to Cdk2 in human breast cancer cells.

MATERIALS AND METHODS

Cell Lines and Culture Media—MCF-7 cells were obtained from Dr. Michelle R. Bregni, Memorial Sloan-Kettering Cancer Center. Cells were grown in suspension. They were routinely passaged in improved Eagle’s medium (BioFluids), supplemented with 5% fetal bovine serum (HyClone) and 100 µg/ml streptomycin (Invitrogen). For experiments, cells were cultured in improved modified Eagle’s medium without phenol red (BioFluids) containing 5% charcoal-stripped serum (HyClone) and penicillin/streptomycin, with either 10% Eagle’s Medium (Gibco) bound to 7.5 µg/ml aprotinin (American Qualex) or 10 µl/tube ICI (Astra Zeneca). Cells were cultured at 37°C with 5% CO2.

Cell Cycle Analysis—Cells were trypsinized, washed in phosphate-buffered saline (PBS), suspended in PBS + 1% fetal bovine serum, fixed with 80% cold ethanol, and stored at −20°C. Prior to analysis, cells were washed twice with PBS and then suspended in PBS + 1 mg/ml RNase A, 0.2 mg/ml propidium iodide, 0.5 mM EDTA, and 0.1% Triton X-100. Cells were then analyzed for red fluorescence on a FACS-4 flow cytometer; cell cycle distribution was determined using ModFit software. Three 60-mm plates were analyzed for each time point.

Immunoprecipitations and Cdk4 Kinase Assays—Immunoprecipitations (IP) and Cdk4 activity measurements were performed using a modification of a published method (41). Unless indicated otherwise, all manipulations were carried out on ice. Cells were washed twice in ice-cold PBS and harvested by scraping in PBS and pelleted; the pellets were frozen and stored in liquid nitrogen until the day of analysis. Cells were re-suspended and lysed by sonication in IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM dithiorethiol, 10 µM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, 1 mg/ml phospho-molybdenum, 10 µg/ml aprotinin, and 2 µg/ml aprotinin). The lysates were cleared by centrifugation, and protein concentrations were quantitated using the protein assay reagent from Bio-Rad. For IPs, 75 µg of total protein was diluted to 500 µl with IP buffer and then incubated for 60 min by rocking at 4°C with 1.5 µg of antibody (anti-Cdk4 H-22-G or normal goat IgG, Santa Cruz Biotechnology) bound to 7.5 µl of protein G-agarose beads (Roche Molecular Biochemicals). Beads were pelleted and washed four times with 100 µl of IP buffer and twice with 100 µl of kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM Na3VO4, and 1 mM NaF). Pellets were then suspended in 40 µl of kinase buffer containing 10 µCi of [γ-32P]ATP, 20 µM cold ATP, and 1.0 µl of glutathione-Sepharose 4B (Amersham Biosciences) to which −2.0 µg of a fusion protein between glutathione S-transferase (GST) and human pRB were bound (GST-Rb bacterial expression vector was kindly provided by Dr. William Kaelin of the Dana Farber Cancer Institute). The reactions were incubated at 30°C for 30 min with occasional mixing, after which they were boiled in SDS loading buffer containing mercaptoethanol. Beads were pelleted, and the supernatants were transferred to clean tubes. Aliquots of the reaction products were resolved on 10% SDS-polyacrylamide gels, which were then dried and exposed to autoradiography film. Phosphorylated GST-Rb bands were quantitated by PhosphorImaging with a Storm Phosphorlmager (Molecular Dynamics) using ImageQuant software.

Western Blotting—Either total cell lysates (20 µg), IP supernatants, or IP pellets were resolved on 12% SDS-polyacrylamide gels, transferred to membranes, and probed with antibodies for cyclin D1 (Upstate Biotechnology, Inc., catalog number 06 137), p21[wp12] (p21-c-19, Santa Cruz Biotechnology), p27Kip1 (C-19, Santa Cruz Biotechnology), Cdk4 (H22, Santa Cruz Biotechnology), or actin (Sigma clone AC-40). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit (Bio-Rad) or goat anti-mouse (American Qualex) secondary antibodies, and immunoreactive proteins were detected using Super Signal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

ICI and E2 Regulate Cdk4 Activity Independent of Cyclin D1 Levels or Association in MCF-7 Cells—Cyclin D-Cdk4 complexes are key integrators of positive and negative growth signals (8, 17, 20, 30, 42), and both cyclin D1 levels and Cdk4 activity are regulated by E2 and ICI in MCF-7 cells (31, 38, 40). To determine whether changes in cyclin D1 protein levels and/or association correlate directly with Cdk4 activity and cell cycle progression in these cells, we investigated the effects of ICI and E2 on cell cycle distribution, Cdk4 activity, and cyclin D-Cdk4 complex formation. The experiments were performed in the presence of charcoal-stripped serum, which is free of steroid hormones.

To study the inhibition of proliferation by ICI, MCF-7 cells were plated at low density and incubated in the presence of E2 for 48 h to generate an asynchronous, proliferating population. The E2-containing medium was then removed, and fresh medium containing ICI was added to block ER signaling, and cells were harvested at 6-h intervals. As shown in Fig. 1A, ICI caused a gradual decrease in the percentage of cells in S phase beginning by 18 h after treatment and reaching a minimum of 5% by 48 h. Under these conditions, Cdk4 activity was reduced by 50% by 12 h and 90% by 24 h (Fig. 1B). The decrease in Cdk4 kinase activity preceded the observed changes in cell cycle phase distribution, consistent with the concept that the loss of Cdk4 activity was responsible for the cell cycle arrest observed.

To determine whether the decrease in Cdk4 activity shown in Fig. 1B correlated with changes in cyclin D1 and/or Cdk4 protein levels, Western blot analyses were performed (Fig. 1C). No changes in Cdk4 protein levels were detected, confirming that the decrease in Cdk4 activity was not due to a decrease in the level of the catalytic subunit. These analyses also indicate that Cdk4 activity did not correlate directly with cyclin D1 protein levels. At 24 and 30 h after antiestrogen treatment, when there was a virtual complete inhibition of Cdk4 activity, the cyclin D1 levels in the treated cells were similar to those observed in asynchronous MCF-7 cells prior to antiestrogen treatment (0 h). More significantly, the amount of cyclin D1 co-immunoprecipitating with Cdk4 did not change or decrease detectably over the course of treatment. Whereas the levels of p27 remained relatively constant during this experiment, the levels of p21 increased after ICI treatment, and this increase correlated with Cdk4 inactivation (Fig. 1C). These data indicate that
the inhibition of Cdk4 activity following antiestrogen treatment is independent of changes in cyclin D1 levels and suggest that increased p21 levels may inhibit Cdk4.

To examine further the effects of ICI and E2 on Cdk4 activity, the above experiment was performed in reverse. Cells were pre-arrested with ICI, released by the removal of ICI and the addition of E2, and harvested at 6-h intervals. At each time point, samples were analyzed for cell cycle distribution, for Cdk4 levels and activity, and for cyclin D1, p21, and p27 protein levels and Cdk4 association. As shown in Fig. 2A, the percentage of cells in S phase remained low until 18 h and then increased to 40–50% at the 24- and 30-h time points. Cdk4 activity was undetectable at 0 h and increased by 24 and 30 h (Fig. 2B). In agreement with the results presented in Fig. 1, activation of Cdk4 did not correlate with changes in the levels of cyclin D1 or in its association with Cdk4. A transient increase in cyclin D1 was reproducibly observed at 6 and 12 h after treatment in both the total lysate and the Cdk4 IP, but this increase was not coincident with Cdk4 activation. When Cdk4 activity was highest, at 24 and 30 h after E2 treatment, cyclin D1 protein levels were similar to before treatment (Fig. 2C). In this experiment, both p21 and p27 levels decreased after E2 treatment in both the total cellular lysate and Cdk4 IP. These data, taken together with the results of Fig. 1, suggest that Cdk4 activity in E2- or ICI-treated cells is not directly determined by the total amount of cyclin D1 in the cell or by the amount of cyclin D1 in complex with Cdk4. Both experiments provide correlative data suggesting that p21 and/or p27 might be responsible for the inhibition of Cdk4 activity in response to ICI treatment.

ICI Regulates Cdk4 Activity through an Inhibitory Factor—

The results presented above provide correlative data that accumulation of p21 and/or p27 might be responsible for the inhibition of Cdk4 activity in ICI-treated cells. We therefore designed in vitro mixing experiments to test this possibility directly. MCF-7 cells were pre-arrested in ICI for 48 h and then treated with medium containing either E2 or fresh ICI for an additional 24 h. Lysates were prepared, and increasing amounts of lysate from ICI-treated cells were mixed with a constant 75 μg of lysate from E2-treated cells. The single and mixed lysates were diluted to a constant volume, incubated for 30 min, and then subjected to a Cdk4 activity assay as described under “Materials and Methods.”

This experiment was designed to distinguish between three potential mechanisms for regulating Cdk4. If the lack of Cdk4 activity in ICI-treated cells was due to a post-translational modification of the complex, such as a change in phosphorylation state, we would expect the Cdk4 activity in the lysate of E2-treated cells to be unaffected by the addition of the lysate from ICI-treated cells. If the lack of Cdk4 activity was due to the absence of a Cdk4 activator (such as a D-type cyclin), we might expect to see an increase in total activity in the mixed lysates. Finally, if the lack of Cdk4 activity was due to the presence an inhibitory factor, we would expect the addition of the extract of ICI-treated cells to lower the activity present in the E2-treated lysates.

The results of one representative mixing experiment are shown in Fig. 3. As predicted from the time course experiments described above, only background levels of Cdk4 activity were recovered from the ICI-treated cells, and a 10-fold increase in Cdk4 activity was observed after E2 treatment (Fig. 3A, 1st and 2nd lanes). Under these experimental conditions, the amount of immunoprecipitating antibody was limiting, so that the Cdk4 activity recovered was not the total activity in the lysate but rather a representative fraction thereof. This is demonstrated by the fact that equal activity was detected in a second sequential Cdk4 IP of the same lysate (Fig. 3A, 5th to 7th lanes, marked 2nd IP).

Because the IPs were performed in “lysate excess,” we predicted that the mixed lysates would exhibit decreased Cdk4 activity in the absence of any activating or inhibitory activities. Because Cdk4 is present at equal levels in active and inactive extracts and IPs (see Western blots in Fig. 2C; note the constant level of Cdk4 detected in Cdk4 IPs despite differences in kinase activity), and because the mixed reactions contained a greater amount of total cellular protein, the predicted decrease would be proportional to the ratio of inactive to active lysate in the mixture. For example, at the 1:2 mixing ratio, 37.5 μg of ICI-treated lysate was mixed with 75 μg of E2-treated lysate, and the predicted Cdk4 activity in the mixture would be 75/75 + 37.5 = 0.67 = 67% of the activity of the E2-treated lysate; this fraction is represented by black bars in Fig. 3B.

Three identical mixing experiments were performed, and the activity recovered in mixed lysates was expressed as a fraction of the activity recovered from the E2-treated lysate. As shown in Fig. 3B, at each mixing ratio the amount of activity recovered was significantly less than the fractional representation of the E2-treated lysate in the mixtures. These results provide the first direct evidence that ICI treatment induces a factor capa-
Table of inhibiting Cdk4 activity and that this factor is present in excess of cyclin D-Cdk4 complexes in ICI-treated lysates. Aliquots of the lysates and Cdk4 IP pellets used in Fig. 3A were analyzed for cyclin D1 and p21 protein levels by Western blotting (Fig. 3C). Consistent with our previous result, similar amounts of cyclin D1 were present in the lysates from ICI- and E2-treated cells, despite the differences in kinase activity. The amount of p21 in both the total lysate and in complex with Cdk4 was slightly higher in the ICI-treated than in the E2-treated lysates.

Depletion of p21, but Not p27, Removes the Cdk4 Inhibitory Activity from ICI-treated Lysates—To determine whether a known protein accounted for the inhibition of Cdk4 activity in extracts of ICI-treated cells, we modified the lysate mixing experiment described above. Prior to mixing, aliquots of the ICI-treated lysate were subjected to two rounds of immunodepletion with antibodies against p21, p27, Cdk4, or with a normal goat IgG control (mock). The immunodepleted ICI-treated lysates were then incubated with E2-treated lysate at a 1:1 mixing ratio and subjected to a Cdk4 kinase assay as described above. Aliquots of the untreated and depleted extracts were also analyzed by Western blotting for p21, p27, Cdk4, cyclin D1, and actin levels.

As shown in Fig. 4A, two rounds of immunodepletion were sufficient to reduce significantly the amount of the target proteins in the ICI-treated lysate, although all were detectable by Western blotting with very long exposures. Two rounds of treatment with a control antibody (goat IgG, “mock”) had no effect on the levels of the proteins examined. Immunodepletion of either Cdk4 or p21 removed the majority of cyclin D1 from the lysate of ICI-treated cells, suggesting that virtually all the cyclin D1 in ICI-treated cells is in complex with both Cdk4 and p21. Immunodepletion of Cdk4 reduced the amount of p21, whereas immunodepletion of p21 resulted in only a slight decrease in the amount of Cdk4 present in lysates from ICI-treated cells. Depletion of p27 did not change the levels of p21, Cdk4, or cyclin D1, although we have consistently observed p27 co-precipitating with Cdk4 (see Fig. 6), as reported by others (21, 27).

Fig. 4B shows the autoradiogram of the in vitro kinase assay for this experiment; the quantitation of phosphorylated GST-Rb band intensities is shown in Fig. 4C. Immunodepletion of p21 or p27 had no effect on the Cdk4 activity recovered from the ICI-treated lysate, indicating that the removal of inhibitors alone is not sufficient to activate Cdk4. Consistent with the results described in Fig. 3, mixing the non-immunodepleted ICI-treated lysate with the E2-treated lysate resulted in inhibition of Cdk4 activity to less than the 50% predicted by the fractional representation of the E2-treated lysate in the mixture. When the ICI-treated lysate was immunodepleted of p27, Cdk4, or treated with control antibodies, a similar inhibition of the Cdk4 activity in the E2-treated lysate was observed. However, immunodepletion of p21 from the ICI-treated lysate resulted in a loss of inhibition; the amount of Cdk4 activity...
recovered in the mixture was ~60% of the amount in unmixed E2-treated lysate (Fig. 4B), which is greater than the 50% predicted if no inhibitor was present. We therefore conclude that the Cdk4 inhibitory activity present in ICI-treated MCF-7 cells is the CdkI p21.

To confirm the role of p21 in regulating Cdk4 activity, additional immunodepletion/mixing experiments were performed. MCF-7 cells were pre-arrested with ICI, treated with ICI or E2, and lysates were then prepared as described above. The ICI-treated lysate was immunodepleted of p21 and mixed with the E2-treated lysate at three different mixing ratios. The mixed lysates were then subjected to Cdk4 in vitro kinase assays. Three independent experiments were performed, and the average inhibition observed in the mixed lysates was compared with the predicted value based on the proportional representation of the E2-treated lysate in the mixtures. The dashed line represents 50% of the activity in the lysate from E2-treated cells, which is the fractional representation of the E2-treated lysate in the mixtures. This experiment was repeated once with similar results (not shown).
A.  

- supernatants
- Cdk4 IP

ID:  - p21 mock -  - p21 mock -  - p21 ID -  - mock ID - 

E2  1:5  1:1  1:5  1:1

p21  Actin

B.  

ID:  - p21 mock -  - mock IP -

No ID  1:5  1:1

p21 ID  1:5  1:1

mock ID  1:5  1:1

GST-Rb

C.  

Fraction of total activity

E2  6  1.2  1.0  1.0

ICI  1.2  1.0  1.0  1.0

FIG. 5. Effects of p21 immunodepletion on the Cdk4 inhibitory activity in extracts of ICI-treated cells. Mixing assays were carried out as described in Fig. 4. Prior to mixing, aliquots of ICI-treated extract were subjected to two rounds of immunodepletion with anti-p21 or control antibodies (mock). A, aliquots of IP supernatants and IP pellets were immunoblotted for p21 and actin. B, representative autoradiogram of a Cdk4 kinase assay. Lanes marked − were undepleted. C, three replicate experiments were quantitated by PhosphorImaging, and the fractional activity recovered from the mixed lysates relative to the activity in the E2-treated lysate was plotted as described in Fig. 4. Gray bars, Cdk4 activity from undepleted extracts from ICI-treated cells and their mixtures. Striped bars, Cdk4 activity in mock-depleted ICI-treated extracts and mixtures. White bars, Cdk4 activity from p21-immunodepleted extracts and mixtures. Black bars, fractional representation of the E2-treated lysate in the mixed lysates. Error bars represent ± 1 S.D.

The experiments are shown in Fig. 5C. Consistent with the results in Fig. 4, removal of p21 from the ICI-treated lysate abolished the Cdk4 inhibitory activity. At each mixing ratio, the Cdk4 activity was significantly higher when p21-depleted ICI-treated lysate was mixed with E2-treated lysate than when non-depleted or IgG-depleted lysates were used. A one-tailed t test was performed to compare the Cdk4 activities recovered from mixtures of undepleted lysates with mixtures of IgG-depleted lysates (43). The p values were 0.31, 0.14, and 0.19 for the 1:5, 1:2, and 1:1 mixing ratios, respectively. When undepleted mixtures were compared with p21-depleted mixtures, p values were 0.015, 0.023, and 0.020. When IgG-treated mixtures were compared with p21-depleted mixtures, p values were 0.017, 0.011, and 0.0013. These data confirm an important role for p21 in inhibiting Cdk4 kinase activity in estrogen-treated human breast cancer cells.

The Majority of Cyclin D1 in E2-treated MCF-7 Cells Is in Complex with Both p21 and Cdk4—Because cyclin D1 was co-immunodepleted with both Cdk4 and p21 antibodies in extracts of ICI-treated cells, we conclude that virtually all of the cyclin D1 was present in complexes containing both Cdk4 and p21 under these conditions (Fig. 4A). To investigate whether a pool of p21-free cyclin D1-Cdk4 complexes accounted for the Cdk4 activity in extracts of E2-treated cells, additional immunodepletion experiments were performed on extracts of both E2- and ICI-treated cells using antibodies directed against p21, p27, and Cdk4.

As shown in Fig. 6A, cyclin D1 was almost completely removed by immunodepletion of either Cdk4 or p21 in extracts of both E2- and ICI-treated cells. This confirms that the majority of cyclin D1 in ICI-treated MCF-7 cells is in complex with both Cdk4 and p21, and demonstrates that this is also true in E2-treated cells. Thus, even though p21 levels are decreased by E2 treatment, the p21 remaining is sufficient to co-immunoprecipitate the majority of the cyclin D1. Immunodepletion of p27 partially removed cyclin D1 from both lysates (Fig. 6A), and immunodepletion of Cdk4 lowered p27 levels. Because most of the cyclin D1 is removed by immunodepletion of p21, this suggests that at least a subset of cyclin D1-Cdk4 complexes must contain both p21 and p27.

Others have suggested (31, 40) that there is a redistribution of p21 and/or p27 from Cdk2 to Cdk4 complexes after E2 treatment. As shown in Fig. 6A, there appears to be less p27 remaining in the supernatant following Cdk4 immunodepletion in the lysate from E2-treated cells than in lysate from ICI-treated cells, which is consistent with this proposal. To test whether there is an increase in Cdk1 association with Cdk4 following E2 treatment, the pellets from the sequential immunodepletions were analyzed by Western blotting (Fig. 6B). In lysates of both ICI- and E2-treated cells, equal amounts of p21 co-immunoprecipitated with Cdk4, and equal amounts of Cdk4 and cyclin D1 co-immunoprecipitated with p21, even though the overall level of p21 was decreased in the E2-treated cells. These results differ somewhat from those shown in Figs. 3C and 5A, in which lower amounts of p21 co-precipitated with Cdk4 in extracts of E2-treated cells than ICI-treated cells. Despite this inconsistency, all of these experiments indicate that the amount of p21 bound to Cdk4 does not increase in response to E2, at least by 24 h after treatment.

An increase in the amount of p27 co-immunoprecipitating with Cdk4 was detected in lysates from E2-treated cells, although a high background was observed. We investigated the binding of p27 to control antibodies and found that p27 binds nonspecifically to pre-immune goat IgG-coated beads (Fig. 6B, panel labeled mock ID, p27 WB). No such nonspecific binding was observed for cyclin D1, Cdk4, or p21 (not shown). The increase in p27 association with Cdk4 in E2-treated cells was confirmed in a second independent experiment which is shown in the panel marked with an asterisk in Fig. 6B. Because the total amount of p27 in the lysate was not significantly different in lysates prepared from E2- and ICI-treated cells, the reduction of p27 in lysates from E2-treated cells following immunodepletion of Cdk4 and the increase in p27 co-immunoprecipitating with Cdk4 in E2-treated cells suggests that, in response to E2, p27 is redistributed to Cdk4 containing complexes from Cdk2 or some other cellular pool.

**DISCUSSION**

The experiments described herein were designed to investigate the mechanisms by which E2 and ICI regulate Cdk4 activity in MCF-7 cells. As reported by others (31, 38–40), we observed transient increases in cyclin D1 protein levels between 6 and 18 h after E2 treatment of ICI pre-arrested cells (Fig. 2C). However, this increase in cyclin D1 protein did not correlate directly with Cdk4 activity, which remained low until
A. 

| ID: | p21 | p27 | Cdk4 | mock |
|-----|-----|-----|------|------|
|     |     |     |      |      |

\[
\text{Cdk4} \\
\text{Cyclin D1} \\
p21 \\
p27 \\
\text{actin}
\]

B. 

\[
\begin{align*}
\text{IP} & \quad \text{WB} \\
\text{Cdk4} & \quad \text{Cdk4} \\
\text{Cyclin D1} & \quad \text{p21} \\
p21 & \quad \text{p21} \\
p27 & \quad \text{p27} \\
\text{mock} & \quad \text{p27} \\
\text{Cdk4} & \quad \text{Cdk4} \\
\text{p21} & \quad \text{p21} \\
p27 & \quad \text{p27} \\
\text{Cyclin D1} & \quad \text{Cdk4} \\
p27 & \quad \text{p27}
\end{align*}
\]

**Fig. 6.** Cdk4 complex composition in E2- and ICI-treated cells. Cells were arrested with ICI for 48 h and then treated for 24 h with either ICI or E2. Extracts were prepared and immunodepleted of p21, p27, or Cdk4 as described in Fig. 4, with the exception that the amount of antibody used in each depletion was doubled. Mock-depleted extracts were treated with preimmune goat IgG. A, cell lysates and supernatants from the second immunoprecipitation were analyzed by Western blotting for levels of Cdk4, cyclin D1, p21, p27, and actin. The co-immunodepletion experiments shown in Fig. 6A demonstrate that the majority of the cyclin D1 is present in complexes containing Cdk4 and p21 in both E2- and ICI-treated cells. This argues against a model in which there is a large pool of active, “p21-free” cyclin D1-Cdk4 complexes in E2-treated cells. However, it remains possible that the activity recovered is due to p21-free complexes that represent a small minority of the total Cdk4 complexes in the lysate and thus have evaded our detection. In fact, a similar situation has been reported for Cdk2 in MCF-7 cells, in which active complexes that lack p21 can only be identified after biochemical fractionation (40).

If both active and inactive Cdk4 complexes contain p21, there are several alternative mechanisms by which it may regulate Cdk4 activity. The stoichiometry of binding has been proposed to account for the regulation of cyclin A-Cdk2 complexes by p21 (26–28), and because we show that high levels of p21 protein are associated with Cdk4 inactivation, it was of interest to investigate the composition of the active Cdk4 complexes in E2-treated cells. The co-immunodepletion experiments shown in Fig. 6A demonstrate that the majority of the cyclin D1 is present in complexes containing Cdk4 and p21 in both E2- and ICI-treated cells. This argues against a model in which there is a large pool of active, “p21-free” cyclin D1-Cdk4 complexes in E2-treated cells. However, it remains possible that the activity recovered is due to p21-free complexes that represent a small minority of the total Cdk4 complexes in the lysate and thus have evaded our detection. In fact, a similar situation has been reported for Cdk2 in MCF-7 cells, in which active complexes that lack p21 can only be identified after biochemical fractionation (40).

Because p21 has been reported to play both activating and inhibitory roles in regulating Cdk4 activity (26–28), and because we show that high levels of p21 protein are associated with Cdk4 inactivation, it was of interest to investigate the composition of the active Cdk4 complexes in E2-treated cells. The co-immunodepletion experiments shown in Fig. 6A demonstrate that the majority of the cyclin D1 is present in complexes containing Cdk4 and p21 in both E2- and ICI-treated cells. This argues against a model in which there is a large pool of active, “p21-free” cyclin D1-Cdk4 complexes in E2-treated cells. However, it remains possible that the activity recovered is due to p21-free complexes that represent a small minority of the total Cdk4 complexes in the lysate and thus have evaded our detection. In fact, a similar situation has been reported for Cdk2 in MCF-7 cells, in which active complexes that lack p21 can only be identified after biochemical fractionation (40).

If both active and inactive Cdk4 complexes contain p21, there are several alternative mechanisms by which it may regulate Cdk4 activity. The stoichiometry of binding has been proposed to account for the regulation of cyclin A-Cdk2 complexes by p21 (26–28), and p21 may also regulate Cdk4 by this mechanism. Consistent with this model, in a majority of our experiments (9 of 14) less p21 co-precipitated with Cdk4 in extracts of E2-treated than ICI-treated cells (see Figs. 3C and 5A). In the remaining five experiments, however, the amount of p21 co-precipitating with Cdk4 was indistinguishable in E2- and ICI-treated cells, as represented by Fig. 6B. We cannot account for this variability, although one possibility is that some or all of the p21 in Cdk4 complexes is loosely bound and is therefore susceptible to removal during washing of the IP pellets. It is interesting to note in this regard that the pellets in Fig. 3C were washed six times to remove any contaminating kinases, whereas those in shown in Fig. 6B, which were not used for kinase assays, were only washed once.

Because p21 is a phosphoprotein (47), it is also possible that p21 may be differently modified in E2- and ICI-treated cells, and that such a modification may modulate its Cdk4 inhibitory activity. Finally, a p21-associated protein might be responsible for the regulation of Cdk4 activity; a p21-associated factor has been reported that specifically modulates its inhibition of cyclin E/Cdk2 activity (48). Future studies will seek to identify how estrogen and antiestrogens regulate p21 levels, and how p21 regulates the activity of Cdk4 in response to ER signaling. Because we have identified p21 as an important mediator of the effects of antiestrogen in human breast cancer cells, it will be of interest to investigate whether a defect in the inhibition of
Cdk4 by p21 could contribute to the development of antiestrogen resistance.

Our results clearly demonstrate that the p21 that accumulates in ICI-treated cells inhibits Cdk4 activity. They also suggest that the decrease in p21 levels after E2 treatment is required for sustained Cdk4 activity. The data described above, together with previous reports, suggest that an antiestrogen-induced cell cycle arrest is due to a shift in the balance between activators (cyclins) and inhibitors (p21 or p27) of G1 Cdns. Consistent with this idea, both overexpression of cyclin D1 (49–51) and ablation of p21 or p27 expression (52) can cause G1 → S progression in the presence of antiestrogen, at least in the short term, in MCF-7 cells. In different model systems and in clinical cases of breast cancer, the relative levels of activators and inhibitors may affect which factor limits Cdk activity and ultimately proliferation. They may also play a role in determining the sensitivity of ER-positive breast tumors to antiestrogen therapy. For example, tumors expressing high levels of cyclin D1 and low levels of p21 may be less sensitive to hormone ablation than tumors expressing low cyclin D1 and high p21.

Finally, changes in the relative expression levels of Cdk activators and inhibitors during treatment may contribute to the development of antiestrogen resistance.

Acknowledgments—We thank Drs. Michele Fluck, Kathy Gallo, and Richard Schwartz for review of the manuscript and helpful comments. Dr. Pam Fraker and Dr. Louis King at the Michigan State University Flow Cytometry Facility provided assistance in cell cycle analysis.

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