ICI182,780 Induces p21Waf1 Gene Transcription through Releasing Histone Deacetylase 1 and Estrogen Receptor α from Sp1 Sites to Induce Cell Cycle Arrest in MCF-7 Breast Cancer Cell Line*

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We used the estrogen-responsive MCF-7 breast cancer cell line as a relevant model to study the anti-proliferative effects of ICI182,780 and identified the negative cell cycle regulator p21Waf1 as a specific target of ICI182,780. Furthermore, silencing of the p21Waf1 expression by small interfering RNA overcame the G0/G1 cell cycle arrest induced by ICI182,780, suggesting that the induction of p21Waf1 expression has a direct role in mediating the ICI182,780-induced G0/G1 arrest. We further demonstrated that the induction of p21Waf1 by ICI182,780 is mediated at transcriptional and gene promoter levels through the proximal Sp1 sites located near the transcription start site. Co-immunoprecipitation, DNA “pull-down,” and chromatin immunoprecipitation experiments together showed that in cycling cells, estrogen receptor α and histone deacetylase 1 (HDAC1) are recruited to the proximal Sp1 sites of the promoter to repress p21Waf1 expression. In the presence of ICI182,780, estrogen receptor α and HDACs are dissociated from Sp1, resulting in increased histone acetylation and de-repression of the p21Waf1 promoter and induction of p21Waf1 expression. The fact that p21Waf1 expression is normally repressed by HDAC activity in cycling cells is further demonstrated by the finding that p21Waf1 transcription can be induced by the silencing of HDACs with small interfering RNA or treatment with HDAC inhibitors.

Estradiol (E2) is the principle steroid mitogen for normal breast epithelial cells and has been shown to be important for the development and progression of de novo breast cancers. E2 exerts its effect on target cells predominantly through binding to and activating the estrogen receptor α (ERα), which is a member of the steroid/thyroid hormone superfamily of ligand-dependent transcription factors. The ligand-bound receptor binds to promoter regions of estrogen-regulated genes, where it recruits co-activators or co-repressors as well as the transcriptional machinery necessary to modulate gene expression. The recruitment of ligand-bound ER to the promoter regions of target genes can occur directly through binding to specific DNA sequences in target genes known as estrogen response elements (EREs) or indirectly through interaction with other DNA-binding transcription factors, such as AP1 and Sp1 (1). In addition to estradiol, the transcription activity of ER can also be modulated by a group of ligands called selective estrogen receptor modulators (SERMs), which have agonist and/or antagonist functions depending on the target promoters and tissues. Some of these SERMs, including tamoxifen, raloxifene, and ICI182,780 (also clinically termed faslodex or fulvestrant), have been employed to antagonize estrogen actions in order to treat breast cancer and protect high risk individuals against breast cancer (2). However, SERMs, such as tamoxifen and raloxifene, also behave as agonists in certain tissues. In fact, the modes of action of SERMs are often promoter context- and cell type-specific. For example, whereas tamoxifen is an effective adjuvant treatment for breast cancer, it can also promote endometrial cancer. Recent gene array data showed that anti-estrogens, such as tamoxifen, raloxifene, and ICI182,780, function by either partially or completely antagonizing the actions of estrogen on most genes in breast cancer cells (3). Although it was initially believed that anti-estrogens function merely by competing with endogenous estrogens for receptor binding, previous studies using mutated ERs and recent x-ray-based structural studies demonstrated that tamoxifen, raloxifene, and ICI182,780 can each induce distinct conformational changes to ER, implying that the ligand-bound ER can recruit specific co-regulators to different gene promoters, thereby selectively regulating target gene expression (4, 5). Interestingly, ICI182,780 is a “pure” steroidal anti-estrogen, and its mode of action appears to be different from that of tamoxifen (6). Unlike tamoxifen and raloxifene, ICI182,780 does not display partial agonist activity. Consistent with this, breast cancer patients who have become tamoxifen-resistant are often responsive to ICI182,780 (7, 8). It has been proposed that the anti-estrogens not only inhibit estradiol-dependent growth through blocking estrogen actions through ER but also have a direct growth-inhibitory effect on ER-positive cells (9).

In mammalian cells, transition through the G1 and S phases of the cell cycle is regulated by cyclin D (cyclin D1, D2, and D3), cyclin E, and cyclin A-dependent kinases, respectively (10). The D-type cyclins bind and activate CDK4 and CDK6 preferentially, whereas cyclins E and A interact predominantly with CDK2 (10). One of the major substrates of these CDKs is the retinoblastoma protein (pRB) family of pocket proteins (pRB, p107, and p130) (11, 12). During the G1 to S transition, phosphorylation of pRB is initiated by cyclin D-dependent kinases and is completed by cyclin E-CDK2 and cyclin A-CDK2.
Hyperphosphorylation of the pocket proteins causes dephosphorylation/activation of genes predominantly required for G1/S progression and DNA synthesis. Two families of cyclin kinase inhibitors function in order to prevent unscheduled proliferation by inhibiting the kinase activity of these complexes. The Cip/Kip family includes p21Waf1(Cip1), p27Kip1, and p57Kip2, and the INK4 family consists of p15INK4a, p16INK4a, p18INK4c, and p19INK4d. The Cip/Kip family functions by binding to cyclin-CDK complexes to sterically inactivate kinase activity, whereas the INK4 family bind to CDK4 and -6 to prevent association with cyclin D family members (10, 13).

Previous studies have shown that estrogens and anti-estrogens modulate cell cycle progression of ER-positive breast epithelial cells at the G1 phase of the cell cycle, but the exact molecular mechanism by which the estrogen signaling pathway modulates the cell cycle machinery has not been elucidated. Although a great deal of effort has been channeled toward understanding the mechanism of action of tamoxifen, relatively little is known about the molecular mechanisms of IC1182,780 action and, in particular, how it regulates the cell cycle machinery. With respect to breast cancer, aberrations in the normal cell cycle machinery are commonplace, such as the increased expression of growth-promoting cyclin D1 and cyclin A as well as the reduced expression of the growth-inhibitory p27Kip1. Consequently, improving our understanding of the mode of action of IC1182,780 will not only be useful for breast cancer treatment but can also lead to better knowledge of ER and nuclear receptor function in other estrogen-dependent tissues such as bone and ovary.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were originally obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin (Sigma, Poole, UK). Cells were grown to 50% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for at least 1 day. Following treatment (described in results) for 16 h, cells were trypsinized and lysed in high salt lysis buffer (1% Triton 100, 0.1% deoxycholate, 0.05 M Tris-HCl, pH 8.1, 5 mM EDTA, pH 8, 0.5 mM NaCl, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Sigma), and 1 × Complete protease inhibitor mixture in 25 ml (Roche Applied Science, Hemel Hempstead, UK). One-tenth of the volume was used as loading control. Samples were diluted in 2 volumes of low salt lysis buffer (same as high salt lysis buffer, omitting NaCl). One volume of a 50% slurry of protein G beads (Sigma) in PBS was added to the samples, and the samples were incubated at room temperature for 30 min before fluorescence-activated cell sorting analysis is performed using a FACSCalibur (Becton Dickinson, Cowley, UK). FACS analysis was performed using the Cell Quest software (Becton Dickinson, Cowley, UK).

**Immunoprecipitation Assay**—Cells were grown to 50% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for at least 1 day. Following treatment (described in results) for 16 h, cells were trypsinized and lysed in high salt lysis buffer (1% Triton 100, 0.1% deoxycholate, 0.05 M Tris-HCl, pH 8.1, 5 mM EDTA, pH 8, 0.5 mM NaCl, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Sigma), and 1 × Complete protease inhibitor mixture in 25 ml (Roche Applied Science, Hemel Hempstead, UK). One-tenth of the volume was used as loading control. Samples were diluted in 2 volumes of low salt lysis buffer (same as high salt lysis buffer, omitting NaCl). One volume of a 50% slurry of protein G beads (Sigma) in PBS was added to the samples, and the samples were incubated at room temperature for 30 min before fluorescence-activated cell sorting analysis is performed using a FACSCalibur (Becton Dickinson, Cowley, UK). FACS analysis was performed using the Cell Quest software (Becton Dickinson, Cowley, UK).

**Induction of p21Waf1 Transcription by ICI1182,780 via Sp1 Sites**

The human p21Waf1 promoter contains mutant and wild-type constructs pGL3 (−2325/+8), pGL3 (−215/+8), pGL3 (−143/+8), pGL3 (−115/+8), pGL3 (−103/+8), pGL3 (−79/+8), pGL3 (−2325/+8 ∆−143/+8), and pGL3 (−2325/+8) plasmids containing mutations in elements 1–6 (mutants 1–6, 2325/H11001/H9004/H11002/H11001/H11002/H11001/H11002). Plasmid transfections were performed using a 50% slurry of protein G beads (Sigma) in PBS before samples were collected for either Western blot analysis or propidium iodide staining.

**Cell Cycle Phase Analysis by Propidium Iodide Staining**—Cells were collected and fixed in 90% ethanol in PBS and stored at 4 °C until cell cycle analysis was carried out. Cells were spun down, washed in PBS and resuspended in a solution of PBS containing 10 μg/ml propidium iodide. Cells were then incubated at room temperature for 30 min before propidium iodide uptake was visualized using the ECL detection system (Amersham Biosciences). The anti-phospho-pRB (Ser807/811) antibody was purchased from New England Biolabs, and the anti-phospho-pRB (Thr821) antibody was from BIOSOURCE. The β-tubulin (556321) antibody was acquired from BD Pharmingen (Cowley, UK), the cyclin D1 (DCS-6) antibody is from Nova-cabra Labs Ltd. (Newcastle-upon-Tyne, UK), and the p21 (C24420) antibody is from Transduction Laboratories (Cowley, UK). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-phospho-pRB (Ser807/811) antibody was purchased from New England Biolabs, and the anti-phospho-pRB (Thr821) antibody was from BIOSOURCE. The β-tubulin (556321) antibody was acquired from BD Pharmingen (Cowley, UK), the cyclin D1 (DCS-6) antibody is from Nova-cabra Labs Ltd. (Newcastle-upon-Tyne, UK), and the p21 (C24420) antibody is from Transduction Laboratories (Cowley, UK). Primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).
with 10% fetal bovine serum for at least 1 day. Following the addition of 10% fetal bovine serum for at least 1 day. Following the addition of ICI182,780 or TSA for 16 h, cells were washed twice with PBS and cross-linked with 1% (v/v) formaldehyde at 37 °C for 10 min and were subjected to the CHIP procedure as described previously (21). Briefly, cells were harvested in 100 mM Tris-HCl (pH 9.4), 10 mM dithiothreitol and incubated for 15 min at 30 °C, and following washes, they were sonicated four times for 15 s in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1× protease inhibitor mixture (Complete Protease Inhibitor Mixture; as instructed by the manufacturer (Roche Applied Science)) at the maximum setting (Sanyo Soniprep MSE, Model 150) followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by immunoclearing with 2 μg of sheared salmon sperm DNA, 20 μl of preimmune serum (DAKO), and protein A-Sepharose (45 μl of 50% slurry in 10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 2 h at 4 °C. Immunoprecipitation was performed overnight at 4 °C with specific antibodies. The antibodies used for ERα were the same as the one used for IP. Antibodies for acetylated H3 (catalog no. 06-599) and H4 (catalog no. 06-866) were purchased from Upstate (Milton Keynes, UK). The protein-DNA complexes were extracted in 1% SDS, 0.1 M NaHCO3, and heated at 65 °C for at least 8 h to reverse the formaldehyde cross-linking. DNA fragments were purified using the QiAquick Spin Kit (Qiagen, Crawley, UK). For PCR, one-twentieth of the extracted DNA was used for 20- to 25-cycle reactions using specific primers (p21-1205) CTTTATTTA-GAGCGCGGGTC-CCGCTCTTGGGAGGGCGGCCGGGCATCAATGATGGCGTGTTATAT-3' or mutant (5'-GGACCTAGTTCAACTTGGGAGGGCGGCCGGGCATCAATGATGGCGTGTTATAT-3') oligonucleotides (Invitrogen) previously coupled to streptavidin-agarose beads (Sigma, Poole, UK). The wild-type oligonucleotide was primed to the region of −92 to −4 of the human p21Waf1 promoter. The biotinylated oligonucleotide-coupled streptavidin beads were washed at least six times with low salt buffer containing 150 mM NaCl and denatured in SDS-sample buffer before running on a SDS-acrylamide gel. The separated proteins were then Western blotted for Sp1, HDAC1, and ERα using specific antibodies. For competition experiments, the extracts were incubated with the oligonucleotide-coupled beads in the absence or presence of 1×, 10×, or 50× molar excess of either a nonbiotinylated double-stranded oligonucleotide containing a high affinity consensus Sp1 binding site (5'-CGATGGGCGGGGCGGA-3') (22) or a mutant oligonucleotide with the Sp1 mutated (5'-GATGGGCGGGGCGGA-3').

RESULTS

Induction of p21Waf1 Expression in Response to ICI182,780 in MCF-7 cells and Not MBA-MD-231 Cells—The ERα-positive breast cancer cell line MCF-7 has been shown to depend on estrogens for proliferation. To study the molecular mechanism by which the "pure" anti-estrogen ICI182,780 modulates cell cycle arrest, proliferating MCF-7 as well as the control ERα-negative MBA-MD-231 cell lines were treated with ICI182,780 for 24 h. ICI182,780 treatment caused the ERα-positive breast cancer cell line MCF-7 to accumulate predominantly in the G0/G1 phase of the cell cycle but had little effect on the ERα-negative MBA-MD-231 cell line (data not shown; also see Fig. 2).

In order to delineate the mechanism whereby ICI182,780 treatment mediates G0/G1 cell cycle arrest, we examined the effect of ICI182,780 on the temporal expression of cell cycle regulatory molecules by Western blot analysis (Fig. 1A). The data shown in Fig. 1A demonstrated that ICI182,780 treatment resulted in an increase in the expression of the cyclin kinase inhibitor, p21Waf1, whereas the related p27Kip1 was unaffected. Expression of both p21Waf1 and p27Kip1 was at low levels in the MBA-MD-231 cell line. There was little or no change in expression levels of cyclin D1 and the cyclin-dependent kinases, CDK2, -4, and -6 in MCF-7 cells, although a slight down-regulation of cyclin E and A expression was detected in response to ICI182,780. Notably, cyclin A and E as well as CDK4 and -6 were expressed at higher levels in MBA-MD-231; nevertheless, the expression levels of the cyclins (D1, E, and A) and CDKs (CDK4, -6, and -2) remained largely unchanged in MBA-MD-231 following ICI182,780 treatment.

Since one of the principal cellular targets of the cyclin-CDK complexes in cell cycle regulation is the retinoblastoma family of pocket proteins, we performed Western blots using antibodies specific for phosphorylated consensus sequences for either CDK2 or CDK4/6 to assess the in vivo CDK4/6- and CDK2-associated kinase activity in these cells. The data shown in Fig. 1A demonstrates that the endogenous CDK2 activity was down-regulated as early as 4 h after ICI182,780 treatment, whereas the CDK4/6 activity only declined at a much later time probably as a result of the initial cell cycle arrest. These results suggested that the G1 cell cycle arrest induced by ICI182,780 treatment in the MCF-7 cells is primarily the result of a decline in CDK2 activity. The expression pattern of p21Waf1 demonstrated a good inverse correlation with CDK2-associated kinase activity, whereas the overall expression level of CDK2 protein remained unchanged. The initial increase in p21Waf1 expression occurred as early as 4 h, coinciding with the drop in CDK2-associated kinase activity. Since the down-regulation of cyclin E and A occurred at later time points than the up-regulation of p21Waf1 and the decrease in CDK2 activity, p21Waf1 may well be responsible for inhibiting the phosphorylation of pRB and related proteins by direct inhibition of CDK2 activity in the ER-positive MCF-7 cells following ICI182,780 treatment. Nevertheless, the involvement of other mechanisms could not be excluded. Although p21Waf1 has previously been shown to be a gene target of p53, there was no evidence that p53 mediates the induction of p21Waf1 expression by ICI182,780, since no change in p53 expression was observed in response to ICI182,780. Interestingly, the accumulation of p21Waf1 was associated with a down-regulation of ERα expression in the ER-positive MCF-7 cells.

To provide further evidence that the ICI182,780-induced accumulation of p21Waf1 is responsible for the down-regulation of CDK2 activity, we performed co-immunoprecipitation experiments to examine the amount of p21Waf1, p27Kip1, cyclin E, and cyclin A binding to CDK2. The results showed that whereas there was little change in the level of p27Kip1 associated with CDK2, the amount of p21Waf1 interacting with CDK2 increased substantially at 4 h and even further at 24 h following ICI182,780 treatment. The CDK2 level remained largely unchanged, whereas the cyclin E and A associated with CDK2 decreased only at 24 h. The initial down-regulation of CDK2-associated kinase activity was first observable at 4 h, and at this time point, there was no change in CDK2 level or the amount of cyclin E, cyclin A, and p27Kip1 binding to it. It therefore appeared that the down-regulation of CDK2 activity in response to ICI182,780 treatment was a result of its increased association with the CDK inhibitor p21Waf1. Whereas these results demonstrated an increase in p21Waf1 expression at the protein level, we performed Northern blot analysis in order to examine whether the increase in p21Waf1 protein was a direct result of an increase in p21Waf1 mRNA. The data in Fig. 1C clearly show that the ICI182,780 also up-regulated the p21Waf1 mRNA level in the MCF-7 cells. In addition, transient transfection of a reporter construct containing 2325 bp of the human p21Waf1 promoter driving expression of the luciferase reporter gene clearly demonstrated that treatment of MCF-7 cells with ICI182,780 resulted in an increase in p21Waf1 promoter activity (Fig. 1D). Furthermore, the kinetics for the induction of p21Waf1 mRNA and promoter activity correlated with the observed increase in p21Waf1 protein (Fig. 1E), sug-
Induction of p21Waf1 Transcription by ICI182,780 via Sp1 Sites

Fig. 1. Expression of p21Waf1 and other cell cycle regulators following ICI182,780 treatment in MCF-7 and MDA-MB-231 cells. A, cycling MCF-7 and MDA-231 were treated with 100 nM ICI182,780 for 24 h. Cell lysates were prepared at the times indicated after ICI182,780 treatment, separated on SDS-polyacrylamide gels, and immunoblotted with specific antibodies. The expression of p21Waf1, p27Kip1, cyclin D1, E,
gesting that ICI182,780 regulates p21<sup>Waf1</sup> expression at mRNA and gene promoter levels.

**Silencing of p21<sup>Waf1</sup> by siRNA Can Overcome the G<sub>1</sub> Cell Cycle Arrest Induced by ICI182,780 in MCF-7**—To investigate whether the induction of p21<sup>Waf1</sup> mRNA and protein levels has a direct role in the G<sub>1</sub> cell cycle arrest induced by ICI182,780 in the MCF-7 cells, gene silencing experiments were performed. To this end, MCF-7 cells were either untransfected, mock-transfected, or transfected with a p21<sup>Waf1</sup>-specific siRNA and then treated with 100 nM ICI182,780 (ICI) for 48 h. The expression of p21<sup>Waf1</sup> and tubulin was analyzed by Western blotting.

The Proximal Sp1 Sites of the p21<sup>Waf1</sup> Promoter Mediate the ICI182,780-dependent Transcriptional Up-regulation of p21<sup>Waf1</sup>—In order to identify those transcriptional elements in the p21<sup>Waf1</sup> promoter responsible for mediating the increase in p21<sup>Waf1</sup> transcription following ICI182,780 treatment, a series of deletion/mutation reporter constructs of the p21<sup>Waf1</sup> promoter were transfected into MCF-7 cells and analyzed for transcriptional activity 24 h after treatment with either vehicle or ICI182,780 (Fig. 3A). The average induction of the longest wild-type human promoter (P1<sub>11002</sub> to P1<sub>11001</sub>) after ICI182,780 treatment was 2.2-fold, and deletion of the 5<sup>'</sup>-distal and A; CDK2, -4, and -6; pRB; p53; and ERα was analyzed by Western blotting. CDK4/6 and CDK2 activities were determined with specific phospho-pRB antibodies. Tubulin was used as a loading control. B, cycling MCF-7 cells were treated with 100 nM ICI182,780 for 0, 4, and 24 h. Cell lysates were prepared and immunoprecipitated (IP) with antibody against CDK2. The precipitated complexes were examined for cyclin A and E, p21<sup>Waf1</sup>, p27<sup>Kip1</sup>, and CDK2 expression. C, total RNA was extracted from MCF-7 cells following ICI182,780 (100 nM) treatment for the times indicated and analyzed for expression of p21<sup>Waf1</sup> mRNA by Northern blotting. D, cycling MCF-7 cells were transfected with a wild-type human p21<sup>Waf1</sup> promoter-luciferase reporter plasmid, pGL3 (−2325/+8s hp21-luc) and were treated with ICI182,780 (100 nM) 16 h after transfection. The transfected cells were harvested at the times indicated after ICI182,780 and assayed for luciferase activity. Values are normalized with the cotransfected Renilla activity. All data shown represent the average of three independent experiments and the error bars show the S.D. E, statistical analysis of the kinetics for p21<sup>Waf1</sup> mRNA and protein expression following ICI182,780 treatment. The error bars show S.D.
A

FIG. 3. Transactivation of p21<sup>Waf1</sup> promoter constructs in MCF-7 cells in response to ICI treatment. A, cycling MCF-7 cells were transfected with different human p21<sup>Waf1</sup> promoter-luciferase reporter constructs as indicated and were treated with ICI182,780 (100 nM) 6 h after transfection. The treated cells were harvested 16 h after ICI182,780 (ICI) and assayed for luciferase activity. Values are normalized with the cotransfected Renilla activity. All data shown represent the average of three independent experiments, and the error bars show the S.D.

B

Induction of p21<sup>Waf1</sup> Transcription by ICI182,780 via Sp1 Sites

Fig. 3. Transactivation of p21<sup>Waf1</sup> promoter constructs in MCF-7 cells in response to ICI treatment. A, cycling MCF-7 cells were transfected with different human p21<sup>Waf1</sup> promoter-luciferase reporter constructs as indicated and were treated with ICI182,780 (100 nM) 16 h after transfection. The treated cells were harvested 16 h after ICI182,780 (ICI) and assayed for luciferase activity. Values are normalized with the cotransfected Renilla activity. All data shown represent the average of three independent experiments, and the error bars show the S.D. B, cycling MCF-7 cells were transfected with various mouse p21<sup>Waf1</sup> promoter-luciferase reporter constructs as indicated, and the transfected cells were analyzed for promoter activity as described in A.
region (−2325 to −143 bp) reduced the basal p21Waf1 promoter activity but did not affect the ability of ICI182,780 to induce the promoter activity. The -fold activation by ICI182,780 reduced significantly when the proximal region (−143/−8) of the promoter containing six putative Sp1 binding sites was deleted. Similarly, a decrease in the level of induction was also observed when any one of the Sp1 sites of the full-length p21Waf1 promoter was mutated, suggesting that the Sp1 sites are responsible for mediating the response to ICI182,780 treatment. The structure of the mouse p21Waf1 promoter is highly homologous to that of the human form and also subject to regulation by ICI182,780 (Fig. 3B). Deletion of the most 5′ region of this mouse promoter (−4542 to −3038) reduced the activation by ICI182,780 from 4.2- to 2.0-fold, but further deletion of the 5′ region (−3038 to −60) had little effect on the induction by ICI182,780. Similarly, mutation of some of the Sp1 sites rendered the mouse promoter insensitive to ICI182,780 induction. Together, these transfection results indicated that the proximal Sp1 sites mediate the promoter’s response to ICI182,780.

p21Waf1 is specifically targeted by ICI182,780 and TSA in MCF-7 cells—Previous studies have shown that the p21Waf1 promoter is negatively regulated by HDAC activity via their Sp1 sites. It is therefore likely that ICI182,780 induces transcription from the p21Waf1 promoter through down-regulating HDAC activity. To examine if the induction of p21Waf1 in MCF-7 is specific to ICI182,780 and also to explore the molecular mechanism involved, we used Northern and Western blot analyses to determine the expression of p21Waf1 protein and mRNA in the MCF-7 cells after treatment with ICI182,780, tamoxifen, estradiol, and the HDAC inhibitor TSA (Fig. 4). The result showed that whereas ICI182,780 induced the expression of p21Waf1 at both protein and mRNA levels, tamoxifen and estradiol had relatively little effect on p21Waf1 expression, indicating that p21Waf1 is a specific target of the anti-estrogen, ICI182,780. Like ICI182,780, TSA induced the expression of p21Waf1 protein and mRNA in the MCF-7 cells. Interestingly, the Northern and Western blot analyses also revealed that ICI182,780 down-
regulates the expression of the ERα protein but not RNA, whereas TSA represses the expression of ERα at mRNA level. We further confirmed that p21Waf1 expression is negatively regulated by HDAC by showing that other HDAC inhibitors, including apicidin and H. carbonum toxin, also induced the expression of p21Waf1 in MCF-7 cells (Fig. 4C). We then performed further gene silencing experiments using an siRNA specific for HDAC1 and -3. The Western blot shown in Fig. 4D clearly demonstrates that in cycling MCF-7 cells, the transfection of an siRNA specific for HDAC1 caused a dramatic increase in the expression of p21Waf1. In the untransfected cells and cells transfected with an HDAC3 or a control nonspecific siRNA, there was no change in p21Waf1 expression. The fact that silencing of HDAC3 expression has no significant effect on p21Waf1 expression indicates that specific HDACs are involved in the repression of the p21Waf1 promoter. Together, these findings suggested that ICI182,780 might activate p21Waf1 expression via the proximal Sp1 sites of the gene promoter through modulating HDAC activity.

The Proximal Sp1 Sites of the p21Waf1 Promoter Mediate the TSA-dependent Transcriptional Activation of p21Waf1—To further test this hypothesis, the panel of p21Waf1 promoter constructs were transfected into MCF-7 cells and analyzed for transactivation activity after treatment with either vehicle or TSA for 24 h (Fig. 5). The transfection result showed that sequential removal of 5′-sequence from the human p21Waf1 promoter reduced the basal promoter activity. However, deletion of the region from position −2325 to −115 significantly increased, whereas further deletion from position −115 to −78 reduced, the -fold induction by TSA. However, when individual Sp1 sites were mutated in the context of the full-length promoter, there was a decrease in the -fold activation by TSA. This result indicated that mutation of any of the Sp1 sites reduces the ability of TSA to activate the full-length human p21Waf1 promoter, suggesting that the Sp1 sites are important for the activation of the p21Waf1 promoter by TSA in MCF-7 cells. The discrepancy between the results from deletion and mutation constructs is likely to be due to the fact that the upstream sequences are missing in the deletion constructs, and they may modulate the effects of TSA on the promoter. Given that ICI182,780 induces the p21Waf1 promoter activity via the Sp1 sites, it is attractive to speculate that ICI182,780 regulates p21Waf1 transcription by modulating HDAC activity via the Sp1 binding sites. ICI182,780 and TSA Inhibit the Interactions between Sp1, ERα, and HDACs in MCF-7 Cells—The findings that both the “pure” anti-estrogen ICI182,780 and the HDAC inhibitor TSA both regulate the p21Waf1 transcription and promoter activity through the proximal Sp1 sites suggested that Sp1 might interact with ERα and HDACs to mediate p21Waf1 expression in...
the ER-positive MCF-7 cells. To determine whether Sp1 binds to ERs and HDACs and to investigate whether these interactions are altered in response to ICI182,780 and TSA, we performed co-immunoprecipitation experiments on MCF-7 cells after treatment for 24 h with either vehicle, ICI182,780, tamoxifen, or estradiol, and TSA (Fig. 6A). Western blot analysis of the input lysates for co-immunoprecipitation assays showed that the ERα expression was down-regulated by ICI182,780, estradiol, and TSA but up-regulated by tamoxifen and ERα, whereas HDAC1 and ERs were recruited by Sp1 via protein-protein interactions to the proximal GC-rich region of the p21Waf1 promoter in the presence of estrogen to repress p21Waf1 expression. The result also showed that both ICI182,780 and TSA treatments decrease the binding of HDAC1 and ER to Sp1. This result confirms the findings from the co-immunoprecipitation experiments, demonstrating that both ICI182,780 and TSA can both reduce the formation of the HDAC1-ER-Sp1 complex, although via distinct mechanisms.

FIG. 6. Analysis of Sp1, ERα, and HDAC complexes in MCF-7 cells following ICI182,780 (ICI), tamoxifen, E2, and TSA treatment. A, cell extracts prepared from cycling MCF-7 cells 16 h after treatment with either ethanol, ICI182,780 (100 nm), tamoxifen (100 nm), estradiol (100 nm), or TSA (1 μM) were immunoprecipitated (IP) with antibodies against Sp1 and a control antibody. B, in parallel, the MCF-7 cell extracts were also immunoprecipitated with antibodies against ERα and HDAC1. The precipitated complexes were examined for Sp1, ERα, and HDAC1 expression.

The results showed that HDAC1 bound to Sp1 and ERα in vivo in proliferating cells, and these complexes were decreased significantly in response to ICI182,780 and TSA treatment but not tamoxifen and estradiol treatment. Similarly, in proliferating MCF-7 cells, ERα complexes with Sp1 and HDAC1, and these complexes were inhibited by ICI182,780 and TSA but not tamoxifen and estradiol. Taken together, these data suggested that Sp1 recruits ERα and HDAC1 to the p21Waf1 promoter to repress its activity and that both ICI182,780 and TSA reduce the levels of ERα and HDAC1 binding to Sp1, thereby decreasing the p21Waf1 promoter, resulting in increased p21Waf1 expression. Notably, it appears that ICI182,780 treatment functions to disrupt the Sp1-ERα-HDAC complex, whereas TSA decreases the amount of this complex primarily through down-regulating the expression levels of both Sp1 and ERα.

HDAC1 and ER Are Recruited to the Prominal Region of the p21Waf1 Promoter through Sp1—We attempted to use gel mobility shift analysis to confirm the formation of these complexes on the proximal Sp1 sites of the p21Waf1 promoter; however, as reported by others, we could only detect Sp1-DNA complex and not other higher order complexes using this assay (data not shown). The failure of the mobility shift assays is likely to be due to the fact that the protein complexes interacting with Sp1 are either unstable or of high molecular weight and cannot enter the electrophoresis gel. In an attempt to circumvent this problem, we used biotinylated double-stranded oligonucleotides coupled to streptavidin-agarose beads to “pull-down” protein complexes interacting with the Sp1 sites of the p21Waf1 promoter. The bound protein complexes were then analyzed by Western blotting. The result showed that in cycling cells in the presence of estrogen, HDAC1, ERα, and Sp1 all bind to the putative proximal Sp1 sites of the p21Waf1 promoter (Fig. 7A) and that these interactions depend on Sp1, since these bindings can be competed out using increasing amounts of an oligonucleotide containing a high affinity Sp1 site but not a similar oligonucleotide with the Sp1 site mutated (Fig. 7B). This confirms that Sp1 protein binds directly to the Sp1 sites, whereas HDAC1 and ERs were recruited by Sp1 via protein-protein interactions to the proximal GC-rich region of the p21Waf1 promoter in the presence of estrogen to repress p21Waf1 expression.

The result also showed that both ICI182,780 and TSA treatments decrease the binding of HDAC1 and ER to Sp1. This result confirms the findings from the co-immunoprecipitation experiments, demonstrating that both ICI182,780 and TSA can both reduce the formation of the HDAC1-ER-Sp1 complex, although via distinct mechanisms.

Sp1, HDAC1, and ERα Interact with the Proximal Region of the p21Waf1 Promoter in Vivo—We next performed ChIP assays to determine the occupancy of the proximal region of the p21Waf1 promoter in vivo in response to ICI182,780 and TSA (Fig. 8, A and B). The results demonstrated that the anti-Sp1 and anti-ERα antibodies, but not the control species-matched antibodies, can precipitate the proximal region of the p21Waf1 promoter in the untreated proliferating MCF-7 cells. However, the amount of DNA precipitated by anti-ERα decreased significantly following treatment with either ICI182,780 or TSA for 16 h. This result suggested reduced binding of these proteins to this p21Waf1 promoter region in vivo in response to ICI182,780 and TSA. Unlike ERα, the binding of Sp1 to the proximal region of the p21Waf1 promoter was not decreased after ICI182,780 and TSA, indicating that the recruitment of Sp1 to the promoter is independent of ERα and HDAC1. In contrast to the DNA pull-down finding, we consistently observed a small but significant increase in vivo binding of Sp1 to the p21Waf1 promoter in response to ICI182,780 treatment. This discrepancy is likely to be due to the fact that the in vivo DNA pull-down analysis is unable to detect changes in Sp1 recruitment as a result of changes in chromatin structure. To ensure proper DNA shearing, we used primers detecting an unrelated upstream region of the p21Waf1 promoter (primers C, about 1 kb upstream of the Sp1 sites). As shown in Fig. 8B, the PCR results showed no specific chromatin precipitation with either the anti-Sp1 or anti-ERα antibodies. We were not able to dem-
Induction of p21<sup>Waf1</sup> Transcription by ICI182,780 via Sp1 Sites

We identified the negative cell cycle regulator p21<sup>Waf1</sup> as a specific target of the "pure" anti-estrogen ICI182,780, since other ER ligands, such as tamoxifen and estrogen, failed to activate p21<sup>Waf1</sup> expression. We further demonstrated that the induction of p21<sup>Waf1</sup> by ICI182,780 is mediated at mRNA and gene promoter levels and is associated with the down-regulation of CDK2 activity and cell cycle arrest at G<sub>1</sub>/G<sub>S</sub>. Our finding that silencing of the p21<sup>Waf1</sup> expression by siRNA can overcome the G<sub>0</sub>/G<sub>S</sub> cell cycle arrest induced by ICI182,780 suggests that the induction of p21<sup>Waf1</sup> expression has a direct role in mediating the ICI182,780-induced G<sub>1</sub>/G<sub>S</sub> arrest. This finding is consistent with previous studies showing that antisense inhibition of p21<sup>Waf1</sup> or p21<sup>Waf1</sup> expression can abrogate the G<sub>0</sub>/G<sub>S</sub> cell cycle arrest induced by ICI182,780, tamoxifen, or estradiol depletion (23) and that overexpression of p21<sup>Waf1</sup> by adenovirus transduction can inhibit the synergism between estradiol and insulin on S-phase entry in MCF-7 cells (24).

Using a series of mutant p21<sup>Waf1</sup> promoter constructs in transient transfection experiments, we localized an essential ICI182,780-responsive element to the proximal Sp1 sites of the p21<sup>Waf1</sup> promoter. In a number of other cell systems, histone deacetylases inhibitors, such as TSA and apicidin, have previously been shown to modulate the p21<sup>Waf1</sup> promoter activity through these proximal Sp1 sites (17, 25–28). Similarly to these studies, we demonstrated that inhibition of HDACs by TSA can also activate p21<sup>Waf1</sup> promoter activity through these proximal Sp1 sites in proliferating MCF-7 cells in the presence of estrogen, indicating that the p21<sup>Waf1</sup> gene expression is repressed by HDACs under these conditions. The fact that the minimal p21<sup>Waf1</sup> promoter region required for ICI182,780 induction is the same as that necessary for TSA to induce p21<sup>Waf1</sup> expression raises the possibility that ICI182,780 activates the endogenous p21<sup>Waf1</sup> gene expression through modulating histone acetylase activity. Although p21<sup>Waf1</sup> has previously been shown to be an important target gene of the tumor suppressor p53 (29, 30), the induction of p21<sup>Waf1</sup> by ICI182,780 is probably not dependent on p53, since the transfection assays showed that deletion of the two p53-responsive sites on both the human and the mouse p21<sup>Waf1</sup> promoters has little effect on the activation by ICI182,780. Furthermore, treatment of MCF-7 cells with ICI182,780 resulted in the induction of p21<sup>Waf1</sup> but not the stabilization of p53 protein. In agreement with this, the induction of p21<sup>Waf1</sup> by HDAC inhibitor TSA has also been demonstrated previously to be independent of p53 (17, 28).

The observation that the Sp1 sites are necessary for the induction of p21<sup>Waf1</sup> promoter by ICI182,780 and TSA suggests that their respective cellular targets ERα and HDACs are involved in the regulation of the p21<sup>Waf1</sup> promoter through these Sp1 binding sites. The fact that HDACs do not bind DNA directly coupled with the fact that there are no detectable whole or half ERE elements present in the proximal region of the p21<sup>Waf1</sup> promoter indicates that ERα and HDACs are recruited indirectly to the p21<sup>Waf1</sup> promoter through complexing with the Sp1 transcription factor. Supporting this idea is the co-immunoprecipitation experiment showing both ERα and HDAC1 bound to Sp1 under normal proliferative conditions (in complete medium). More importantly, the DNA “pull-down” assay demonstrates that the ERα and HDAC1 precipitated with the proximal p21<sup>Waf1</sup> promoter fragment could be competed off with a molar excess of oligonucleotides containing wild-type, but not mutant, consensus Sp1 sites. This confirms that ERα and HDAC1 are recruited to the proximal region of the p21<sup>Waf1</sup> promoter through Sp1. Indeed, besides binding directly to EREs on target genes, ERα can also modulate gene transcription indirectly by interacting with other DNA-binding transcription factors. A number of studies have demonstrated...
that ERα can bind to transcription factors, such as AP-1 complexes as well as Sp1, to confer estrogen responsiveness to target genes, including the ovalbumin (31), c-fos (32), collagenase (33), insulin-like growth factor 1 gene (34), creatine kinase B (35), c-myc (36), and retinoic acid receptor α expression (37). However, unlike 21Waf1, the majority of these estrogen-responsive gene promoters contain half ERE sites in close proximity to either the Sp1 or AP-1 binding sites (38, 39). Previously, the ERα-Sp1 complexes have been demonstrated to activate transcription in response to estrogens and anti-estrogens. The data shown here suggest a repression mechanism by which the ERα-Sp1 complex functions to recruit HDACs in the presence of estrogen to inhibit 21Waf1 transcription. Supporting this hypothesis are a number of recent studies demonstrating that certain gene promoters are also repressed by HDACs through their Sp1 sites (17, 40–43). Although our work focused on HDAC1, it is likely that other HDACs are recruited to the Sp1 sites. In fact, previous studies have demonstrated that other HDACs are recruited to the Sp1 sites and are responsible for the regulation of the 21Waf1 gene (27). The nature of the interaction between HDACs and the Sp1-ERα complex is complicated. Whereas class I and II HDACs have been demonstrated to interact directly with Sp1 (27, 43), HDACs can also be recruited to Sp1 indirectly through ERα. In the latter scenario, it is likely that in the presence of estrogen, HDACs are recruited by co-repressors, such as L-CoR and RIP140, to the estrogen-bound ERα (44). In either event, our co-immunoprecipitation and DNA “pull-down” assays have unequivocally shown that ICI182,780 treatment can effectively disrupt the interactions between HDACs, Sp1, and ERα, resulting in releasing HDACs from the Sp1 complexes, concomitant with increased histone acetylation and 21Waf1 gene transcription. The fact that HDACs have a role in repressing 21Waf1 promoter activity has been shown by others (45–47) and also here in this cell system using the HDAC inhibitors, including TSA and apicidin. Acetylated histones H3 and H4 are often associated with a more open and active chromatin structure, indicative of actively transcribed genes (48). We observed an increase in histone H3 and H4 acetylation in the promoter region encompassing the Sp1 sites following treatment with ICI182,780 and TSA, indicating increased levels of transcription activation. This also shows that the histones associated with the proximal GC-rich region of the 21Waf1 promoter become more acetylated, consistent with the hypothesis that ICI182,780 can increase histone acetylation through releasing HDACs and ERα from Sp1 on the 21Waf1 promoter. Taken together, these data suggest that ICI182,780 disrupts the association between HDACs, ERα, and Sp1 on the 21Waf1 promoter and releases HDACs and ERα from Sp1 sites on the 21Waf1 promoter, leading to increased acetylation of histone and nonhistone proteins and gene transcription. In this study, the ChIP analysis demonstrated that ICI182,780, but not TSA, treatment also increased the recruitment of Sp1 to the 21Waf1 gene promoter, without affecting Sp1 expression levels. The fact that both ICI182,780 and TSA lead to the reduction of the ERα-Sp1-HDAC complex but only ICI182,780 results in increased Sp1 binding to the 21Waf1 suggests that the result is genuine and not due to reduced steric hindrance following the breakdown of the ERα-Sp1-HDAC complexes. Thus, this could represent an additional secondary mechanism by which ICI182,780 activates 21Waf1 expression via Sp1, following derepression, since non-HDAC-bound Sp1 has been shown to be able to recruit the transcription coactivator CREB-binding protein/p300 with intrinsic histone acetyltransferase activity (49).

Previous studies have shown that ICI182,780 binding can induce proteasome-mediated degradation of ERα, and recent data demonstrated that the ubiquitin-like protein NEDD-8 is involved in this process (50). As a result, one possible mechanism that might explain the dissociation of the HDACs, Sp1, and ERα complex by ICI182,780 is that ICI182,780 induces ERα degradation and thereby disrupts the association of HDACs with the ERα-Sp1. In our experiment, although we could detect the down-regulation of ERα expression by ICI182,780, the level of reduction in protein expression is in-

![Figure 8](http://www.jbc.org/)

**FIG. 8.** ChIP assay on MCF-7 cells before and after ICI182,780 and TSA treatment. A, schematic diagram of the human 21Waf1 promoter with PCR primers. B, cycling MCF-7 cells were treated with either ethanol (control), ICI182,780 (ICI), or TSA for 16 h. Protein-DNA complexes were formaldehyde-cross-linked in vivo. Chromatin fragments from these cells were subjected to immunoprecipitation (IP) with antibodies against Sp1 or ERα as indicated. After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR using the indicated primers and resolved on 2% agarose gels.
sufficient, on its own, to account for the almost complete abolition of the interactions between ERs, HDAC1, and Sp1 as well as the recruitment of ERα and HDAC1 to the proximal Sp1 sites of the p21Waf1 promoter, as revealed by the co-purification and DNA “pull-down” experiments. Consequently, further stochastic mechanisms have to be invoked in order to explain the ability of ICI182,780 to disassociate the ERα, HDACs, and Sp1 complexes, and this warrants further investigation.

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