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3,3′-Diindolylmethane (DIM) induces a G_1 cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of $p21^{WAF1/CIP1}$ expression

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3,3′-Diindolylmethane (DIM) is a promising cancer chemopreventive agent derived from *Brassica* food plants. To determine whether this natural indole has a direct growth inhibitory effect on human breast cancer cells, we examined the cell cycle regulatory effects of DIM in estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) human breast cancer cell lines. Results of flow cytometry studies showed that DIM treatment produced a marked increase (from 51 to 79%) in the proportion of cells in the G_1 phase of the cell cycle, regardless of estrogen-receptor status. Analyses of G_1-acting cell cycle components indicated that the enzymatic activity of cyclin-dependent kinase (CDK) 2 was also strongly reduced. Western blot analyses showed that, concurrent with the DIM-induced cell cycle arrest, DIM stimulated a rapid and pronounced increase in levels of the CDK inhibitor, $p21^{WAF1/CIP1}$ (p21). Northern blot analysis demonstrated that DIM increased p21 mRNA expression with a maximal 6–7-fold induction, and exposure to cycloheximide did not block the response. Similar increases in expression of p21 protein and mRNA were observed in both MCF-7 and MDA-MB-231 human breast cancer cells, suggesting that DIM induction of p21 expression is independent of estrogen-receptor signaling and p53. Transient transfection of 5′-deletion constructs of the p21 promoter demonstrated that the first 291 bp segment of the proximal promoter, which contains six promoter specific transcription factor 1 (Sp1) elements, maintained DIM responsiveness. Consistent with a role for Sp1 in this response, a reporter construct driven by three consensus Sp1 binding sites was responsive to DIM. In addition, electrophoretic mobility shift assays showed that DIM induced the binding of Sp1 and Sp3 to the consensus Sp1 responsive element. Thus, our observations have uncovered an antiproliferative pathway for DIM that implicates Sp1/Sp3-induced expression of p21 as a target for cell cycle control in human breast cancer cells.

Introduction

Dietary indoles present in the *Brassica* plants, including turnips, kale, broccoli, cabbage, Brussels sprouts and cauliflower, have been shown to be protective against several cancers (1). Indole-3-carbinol (I3C), produced by the myrosinase-mediated hydrolysis of glucobrassicin present in *Brassica* food plants, exhibits protective activities *in vitro* and *in vivo*, especially associated with mammary neoplasia (2–5). For example, feeding a diet containing I3C to female C3H/OuJ mice resulted in a 50% reduction in both spontaneous mammary tumor incidence and multiplicity compared with the untreated control mice (2). I3C also inhibited the proliferation of estrogen-responsive (3) and estrogen-receptor deficient (4) human breast cancer cells.

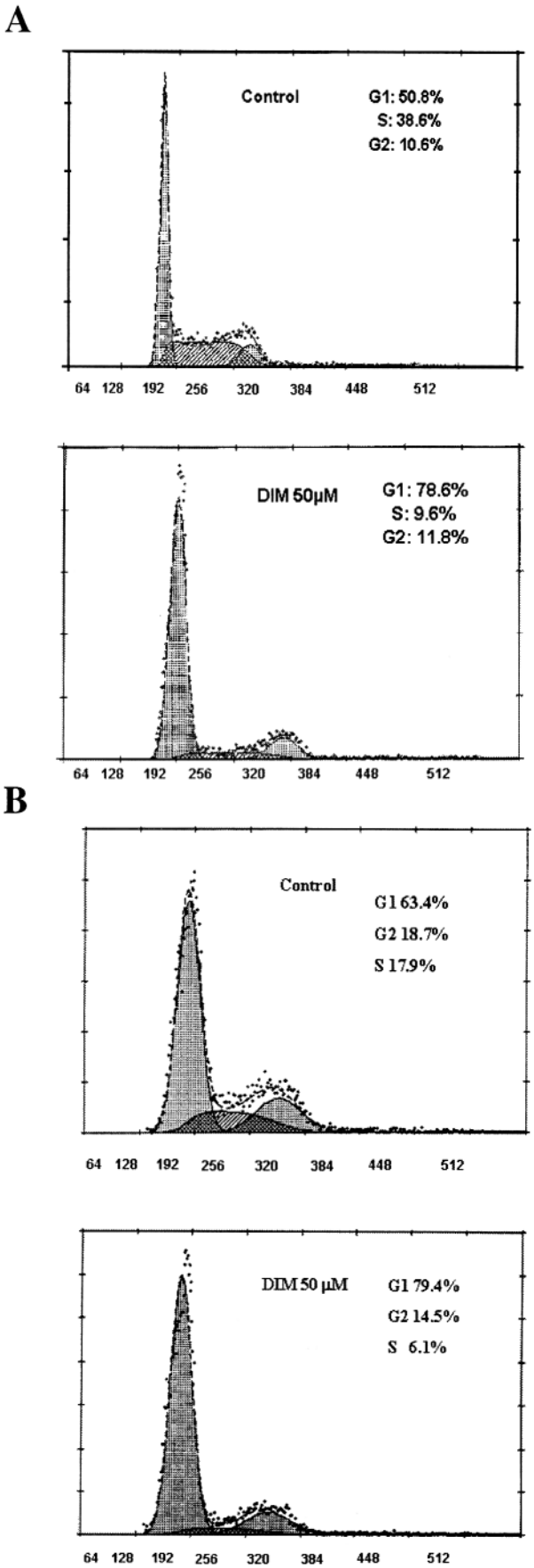
On exposure to gastric acid, I3C is converted to many self-condensation products with distinctive biological activities (6,7), including 3,3′-diindolylmethane (DIM). DIM is a major acid condensation product of I3C (8) that is readily detected in the livers and feces of rodents fed with I3C. The parent I3C compound could not be detected in tissues of I3C treated rodents (9,10), suggesting that DIM, and not I3C, may mediate the observed physiological effects of dietary I3C.

Results of several studies indicate that DIM exhibits promising cancer protective activities. Incorporation of DIM into the feed repressed AFB_1-DNA binding and tumor incidence in trout (10). A single gavage dose of DIM during the initiation stage of tumorigenesis reduced DMBA-induced mammary tumor incidence and multiplicity in rats by 70–80% (11). Repeated oral intubation of DIM during the promotion stage reduced tumor growth by 95% (12). We have shown that DIM inhibited the proliferation of estrogen-dependent and -independent human breast cancer cells and induced apoptosis in both cell types by decreasing cellular Bcl-2 levels and increasing levels of free Bax and the Bax/Bcl-2 ratio (13).

The eukaryotic cell cycle is regulated by activating or deactivating cyclin/cyclin-dependent kinase (CDK) through coordinating internal and external signals at several key checkpoints. CDK activity is modulated positively by various means, most notably through binding of regulatory cyclins, and negatively by interacting with CDK inhibitors (CKIs). The first identified CKI, $p21^{WAF1/CIP1}$ (14), is a direct regulator of CDK2, CDK4 and CDK6 activity (15). p21 binds and inhibits cyclin/CDK complexes resulting in cell cycle arrest (14). Regulation of p21 transcription is controlled by p53-dependent and -independent pathways (16). p53-independent activation of p21 is involved in terminal differentiation and has been observed in several cell types following treatment with chemotherapeutic agents (17,18).

Relatively little is known about the mechanism by which DIM inhibits the growth of human breast cancer cells. In the present study, we observed that DIM induced a G_1 cell cycle arrest that coincided with a strong induction of p21 gene expression and promoter activity in both estrogen-responsive and non-responsive breast cancer cells. Our observations have defined an antiproliferative pathway for DIM that implicates Sp1/Sp3 activation of p21 as a target for cell cycle control in human breast cancer cells.

Abbreviations: CAT, chloramphenicol acetyl transferase; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; DIM, 3,3′-diindolylmethane; DMBA, dimethylbenzanthracene; EMSA, electrophoretic mobility shift assay; I3C, indole-3-carbinol; DMSO, dimethylsulfoxide; Sp1, promoter specific transcription factor 1; Sp3, promoter specific transcription factor 3.



Materials and methods

Materials

Dulbecco's modified Eagles' medium (DMEM), Opti-MEM and Lipofectamine were supplied by Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Sigma Chemical (St Louis, MO). DIM was prepared from I3C as described (8,19,20) and recrystallized in toluene. Actinomycin D and cycloheximide were purchased from Sigma. [α-^{32}P] ATP (3000 Ci/mmol) and [^{3}H]acetyl coenzyme A (200 mCi/mmol) were obtained from NEN Life Science Products (Boston, MA). Salts and other chemicals used were of the highest purity available, and generally purchased from Sigma Chemical.

Cell lines and cell culture

The human breast adenocarcinoma cell lines MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) were obtained from the American Type Culture

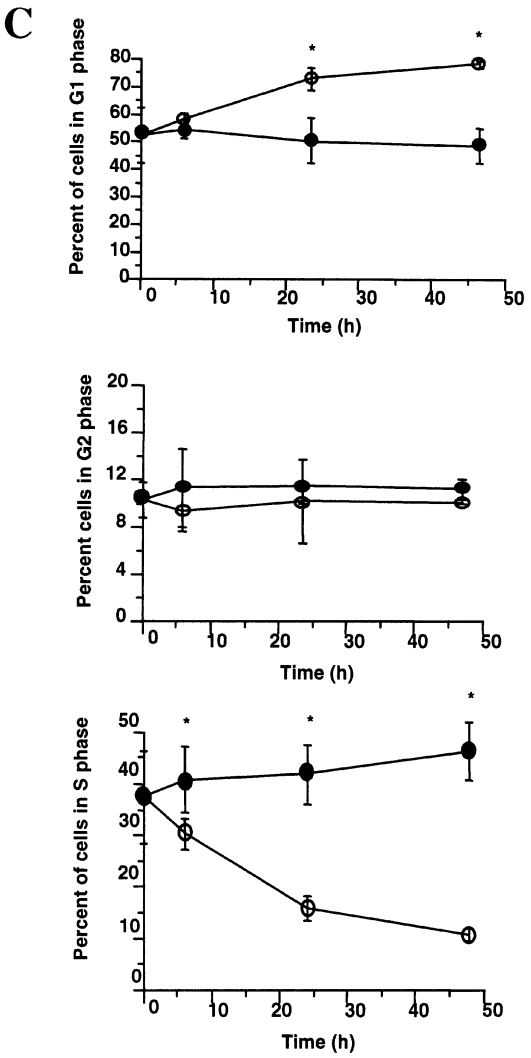


Fig. 1. DIM-mediated G_1 cell cycle arrest in MCF-7 and MDA-MB-231 human breast cancer cells. Histograms of distribution of DNA content with flow cytometry in MCF-7 (**A**) or MDA-MB-231 (**B**) cells. After cells were treated with control vehicle, DMSO or DIM (50 μM) for 48 h, they were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite Laser. A total of 10 000 nuclei were counted from each sample. The percentages of cells within different cell stages were determined as described in the Materials and methods. (**C**). The distribution of cells at different cell cycle stages. MCF-7 cells were treated with vehicle (filled circle) or DIM (50 μM) (open circle) for the indicated times and DNA content of nuclei was analyzed with flow cytometry, as described above. Reported values were average ± SD from two independent experiments with duplicates for each treatment. The asterisks indicate significant difference between the treatments at different time points with ANOVA analysis ($P < 0.05$).

Collection (ATCC, Manassa, VA). MCF-7 and MDA-MB-231 cells were maintained in 10% FBS DMEM, supplemented with 3.0 g/l glucose, 3.7 g/l sodium bicarbonate. The cells were cultured as monolayers in a 95% air, 5% CO_2 water-saturated atmosphere at 37°C and passaged at ~80% confluency. Cultures used in subsequent experiments were at <20 passages.

Flow cytometric analyses of DNA content

Breast cancer cells (5×10^5)/60 mm Corning tissue culture dish were seeded for 24 h. A final concentration of DIM (50 μM) or dimethylsulfoxide (DMSO) control was added for 6–48 h. The medium was changed every 24 h. After cells were incubated at the indicated times, they were hypotonically lysed in 1 ml of DNA staining solution [0.5 mg/ml propidium iodide (PI), 0.1% sodium citrate, 0.05% Triton X-100]. Nuclear emitted fluorescence with wavelength >585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Nuclei (10 000) were analyzed from each sample. The percentage of cells in G_1, S and G_2/M phases of the cell cycle were determined by analysis with the Multicycle computer software provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California at Berkeley.

Western blot analysis

After the indicated treatments, cells were first washed with PBS twice and harvested in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris) containing 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin. Protein concentrations were determined by Bradford-Coomassie dye binding assay. Equal amounts of total protein were mixed with 5× loading buffer (25% glycerol, 7.5% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue, and 156 mM Tris–HCl, pH 6.8) and fractionated by electrophoresis on 4% polyacrylamide/0.1% SDS stacking gels and 15% polyacrylamide/0.1% SDS resolving gels. Biotinylated molecular weight marker (New England Biolabs, Beverly, MA) was used as a standard. Proteins were electrotransferred to nitrocellulose or PVDF Immobilon-P transfer membranes (Millipore, Bedford, MA) using transfer buffer (25 mM glycine, 25 mM ethanolamine and 20% methanol) and then blocked overnight at 4°C with blocking buffer 5% NFDM (1× TBS, 0.1% Tween-20 with 5% w/v non-fat dry milk). Blots were then incubated for 2 h at room temperature with primary mouse anti-human cyclin E, cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. #247 and 450, respectively), at 4 h with rabbit anti-CDK4, CDK6, anti-mouse CDK2, p27 (Santa Cruz Biotechnology, Cat. #749, 177, 6248 and 1641, respectively) or 4 h with mouse anti-p21 and p53 (Oncogen Research Products, Darmstadt, Germany. Cat. #OP64-100UG and OP73-100UG, respectively) and washed with the wash buffer (10 mM Tris–HCl, pH 9.5, 10 mM NaCl and 10 mM $MgCl_2$. Working concentration for all antibodies was 1 mg/ml 5% NFDM. The membranes were incubated for 1 h with secondary anti-mouse or anti-rabbit IgGs conjugated with alkaline phosphatase (1:1000 dilution) (Santa Cruz Biotechnology or Cell Signaling Technology, Beverly, MA, Cat. #7052-1), together with alkaline phosphatase-conjugated anti-Biotin IgG (New England Biolabs) for detection of the biotinylated molecular weight standards. Blots were treated with the CDK-Star reagent (New England Biolabs), and the proteins were detected by autoradiography. Images were scanned and quantified by the densitometer analysis software. Equal protein loading was confirmed by probing β-actin or staining with Ponceau S solution.

Immunoprecipitation and CDK2 activity assay

MCF-7 cells were cultured for 0, 6, 24, 48 or 72 h in growth media with or without 50 μM DIM and rinsed twice with PBS, harvested and stored as dry pellets at –80°C. For the immunoprecipitation (IP), cells were lysed for 15 min in IP buffer (50 μM Tris–HCl, pH 7.4, 200 μM NaCl, 0.1%Triton X-100) containing protease and phosphatase inhibitors (50 μg/ml phenylmethylsufonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, 0.1 μM sodium orthovanadate). Samples were diluted to 800 μg of protein in 1 ml of IP buffer, and samples were pre-cleared for 2 h at 4°C with 30 μl of a 1:1 slurry of GammaBind G–Sepharose (Cat. #17-0885-01) (Amersham Pharmacia Biotech, Uppsala, Sweden) in IP buffer and 1 μg of mouse IgG. After a brief centrifugation to remove pre-cleared beads, 1 μg mouse anti-CDK2 antibody was added to each sample and incubated on a rocking platform at 4°C for 2 h. Then 20 μl of 1:1 slurry of GammaBind G–Sepharose were added to each sample, and the slurries were incubated on the rocking platform at 4°C for 30 min. The beads were then washed five times with IP buffer and twice with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, 0.1 mM sodium orthovanadate). Half of the immunoprecipitated sample was checked by western blot analysis to confirm the IP.

For the kinase assay, the other half of the immunoprecipitated sample was re-suspended in 25 μl of kinase buffer containing 20 mM ATP, 5 mM dithiothreitol, 0.28 μg of Rb C-terminal domain protein substrate (Santa Cruz Biotechnology, Cat. #sc-4112) and 10 mCi of [γ-^{32}P]ATP (3000 Ci/mmol). Reactions were incubated for 30 min at 30°C and stopped by adding an equal volume of 2× loading buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 6.25 mM Tris–HCl, pH 6.8, and bromophenol blue). Reaction products were boiled for 10 min and then electrophoretically fractionated in SDS–10% polyacrylamide gels. Gels were stained with Coomassie Blue and destained overnight with 3% glycerol. Subsequently, gels were dried and visualized by autoradiography.

RNA extraction, mRNA purification and northern hybridization

Cells were lysed by addition of Tri-reagent (Molecular Research Center, Cincinnati, OH) and chloroform was used for phase separation. After centrifugation, the aqueous upper phase was collected and total RNA was precipitated by isopropanol, and washed with 75% ethanol. mRNA was isolated from total RNA with oligo(dT) cellulose columns following Micro-FastTrack 2.0 user manual (Invitrogen, Carlsbad, CA). mRNA (5–10 μg) was electrophoresed on a 1.2% agarose gel containing 3% formaldehyde using MOPS as the running buffer. The gel was then washed gently with 10× SSC and blotted with a Zeta nylon membrane (Bio-Rad, Hercules, CA) overnight to transfer the mRNA onto the membrane. The mRNA was fixed to the membrane by UV cross-linking. cDNA probes were biotinylated using NEBlot Phototope kit (New England Biolabs), purified via precipitation with 3 M sodium acetate (pH 5.2) and washed with 70% ethanol. After hybridization with specific probes, the membrane was incubated with streptavidin and washed twice with solution I (0.5% SDS, 12.5 mM NaCl, 2.5 mM sodium phosphate at pH 7.2). Biotinylated alkaline phosphatase was then incubated with the membrane for 5 min followed by blocking solution. Wash solution II (10 mM Tris–HCl, 10 mM NaCl and 1 mM $MgCl_2$ at pH 9.5) was then used to wash the membrane for 15 min twice, followed by the CDK-Star assay, and X-ray film exposure for mRNA detection. The amount of mRNA was quantified by Gel Densitometery (Bio-Rad) and normalized with β-actin as an internal control.

Lipofectamine transfection

Transfections were performed by the Lipofection method using Lipofectamine (Gibco). The full-length human p21 promoter chloramphenicol acetyl transferase (CAT) reporter construct pWWP and a series of mutant p21 promoter CAT constructs were gifts from B.Vogelstein, and has been described previously (21). Sp1-CAT contains the promoter driven by three consensus Sp1 binding sites from the SV40 promoter. Cells were grown in 10% FBS-DMEM to subconfluence and transferred to 60 mm Petri plates 24 h before transfection. For each 60 mm plate, 8 μl of Lipofectamine was diluted with 92 μl of Opti-MEM serum-free medium (Gibco). Plasmid DNA (1.0 μg) was diluted in 100 μl of serum-free medium. Lipid and plasmid dilutions were combined, mixed gently and incubated at room temperature for 30–45 min. Meanwhile, the cells were washed with 4 ml of serum-free medium, and 2 ml of serum-free medium was added to each plate. Next, 200 μl of the lipid–DNA suspension was added to each plate and mixed gently. The plates were returned to the incubator for 5–6 h, and 2 ml of medium containing 10% FBS serum was added. The next day, the cells were re-fed with fresh medium and the 24 h treatments were started by the addition of 1 μl of 1000× solutions in DMSO/ml of medium. The transfection efficiency was determined using the constitutive galactosidase expression plasmid CMV in an identical set of plates and was found to be unaffected by the DIM treatments.

CAT assay

The CAT assay was performed using a modification of the phase extraction assay described by Seed and Sheen (22). At the end of the 24 h treatment period, the transfected cells were harvested by scraping with a rubber policeman, transferred with the medium to a conical 15 ml tube, centrifuged at 600 *g* for 2 min, re-suspended in 1 ml of cold PBS, transferred to Eppendorf tubes, centrifuged at 600 *g* for 2 min, and washed in PBS a second time. Cell pellets were re-suspended in 200 μl of 0.1 M Tris, pH 8.0, and lysed by three cycles of freeze–thaw treatment (alternating 5 min in a dry-ice/alcohol bath and 5 min in a 37°C bath). Cell lysates were incubated at 65°C for 15 min to inactivate acylases and centrifuged at 14 000 *g* for 8 min. A 165 μl aliquot of the cytosol was transferred to a 7 ml scintillation vial, and a 20 μl aliquot was reserved for determination of protein concentration by the Bradford assay. The substrate mixture (85 μl) was added to the scintillation vial to obtain final concentrations of 100 mM Tris–HCl, pH 8.0, 250 nmol chloramphenicol, 1 μCi [^{3}H]acetyl-CoA (200 mCi/mmol) in a total volume of 250 μl and mixed thoroughly. The organic scintillation fluid (4 ml) was added slowly to avoid mixing with the aqueous phase, and the vials were incubated at 37°C for 1–2 h or until sufficient counts were obtained.

Electrophoretic mobility shift assay

Complementary Sp1 consensus oligonucleotides (5′-GATCATTCGATCGG-GGCGGGGCGAGC-3′ and 5′-GATCGCTCGCCCCGCCCCGATCGAAT-3′) were annealed, and labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The resulting labeled double-stranded DNA probe was purified on a Sephadex

G50 spin-column, precipitated in ethanol, dissolved in TE buffer, and diluted in 25 mM HEPES, 1 mM DTT, 10% glycerol, 1 mM EDTA to contain ~25 000 c.p.m. ^{32}P/µl. Nuclear protein (20 µg), was mixed with 90 ng poly(dI–dC), 25 mM HEPES, 1 mM DTT, 10% glycerol, 1 mM EDTA, 160 mM KCl in a total volume of 21 µl. For antibody super-shift experiments, 1 µg of monoclonal mouse IgG anti-human-Sp1 or rabbit polyclonal Sp1 or Sp3 antibody (Santa Cruz Biotechnology, #sc-420X and #sc-644X) was added to the incubation mixture. After incubation for 15–20 min at room temperature, 4 µl (100 000 c.p.m.) of end-labeled [^{32}P]Sp1 probe was added and incubated for another 15 min at room temperature. In the reaction mixture for competition assays, an excess of unlabeled wild-type oligonucleotides or mutant oligonucleotides with the Sp1 site mutated (5′-GATCATTCGATCGGTTCG-GGGCGAGC-3′ and 5′-GATCGCTCGCCCCGAACCGATCGAAT-3′) were added and incubated at room temperature for 15 min. After the addition of 2.8 ml of 10× Ficoll loading buffer (0.25% bromophenol blue, 25% Ficoll type 400), 22 ml aliquots were loaded onto a pre-run, non-denaturing 6.0% polyacrylamide gel in TAE (67 mM Tris, 33 mM sodium acetate, 10 mM EDTA, pH 8.0) at 120 V for 2 h. Finally, the gel was dried and autoradiographed to an X-ray film.

Statistical analysis

The statistical differences between groups were determined using ANOVA and Tukey's Studentized Range test. The levels of significance are noted at level of $P < 0.05$. The results are expressed as means ± SD for at least three replicate determinations for each assay.

Results

DIM induces a G_1 cell cycle arrest in MCF-7 and MDA-MB-231 cells

In a previous study, we showed that DIM inhibited the proliferation of human breast cancer cells independent of estrogen-receptor status. To assess the cell cycle effects of DIM, MCF-7 cells were treated with vehicle control or 50 µM DIM for 48 h, and then hypotonically lysed with PI DNA staining solution. Flow cytometric analysis of nuclear DNA demonstrated that DIM induced a G_1 cell cycle arrest in these breast cancer cells. Results shown in Figure 1A indicate that by 48 h of treatment of an asynchronous growing cell population, DIM increased the proportion of cells in the G_1 phase of the cell cycle from 50.8 to 78.6% and decreased the proportion of S phase cells from 38.6 to 9.6%, clearly indicating a G_1 block in cell cycle progression. Quantification of the results shown in Figure 1C, revealed that the effects of DIM on cell cycle became evident by 6 h of treatment. Maximal cell cycle arrest was observed by 48 h of DIM treatment. We observed a similar but less pronounced cell cycle blockade in MDA-MB-231 cells (Figure 1B).

DIM increases p21 protein levels and reduces CDK2 activity in MCF-7 cells

Our observation that DIM induced a G_1 block in cell cycle progression in MCF-7 cells suggested that DIM might selectively regulate the expression of G_1 cell cycle components. Therefore, over a time course of DIM treatment, the expression level of G_1 cell cycle components was monitored by Western blot analysis. DIM induced a rapid increase in p21 protein production that peaked and plateaued after 6 h of treatment (Figure 2). The kinetics of DIM-induced p21 protein levels coincided with the DIM-mediated G_1 cell cycle arrest suggesting a causal relationship between p21 protein production and DIM-mediated growth arrest in MCF-7 cells.

To functionally test whether the DIM-mediated induction of p21 protein had an effect on CDK activity as established in other systems (23,24), CDK2-mediated phosphorylation of exogenous retinoblastoma protein (Rb) *in vitro* was examined. As shown in Figure 3A, Rb phosphorylation by immunoprecipitated CDK2 was strongly inhibited with DIM treatment in a concentration-dependent manner; a concentration of 50 µM strongly decreased CDK2 activity, while virtually no CDK2 enzymatic activity was detected at 75 µM DIM and above. This inhibition reached a maximum by 24 h of DIM treatment (Figure 3B). Levels of immunoprecipitated CDK2 were not affected by DIM treatment (data not shown), thus, confirming the results of western blotting shown in Figure 2. The loss in

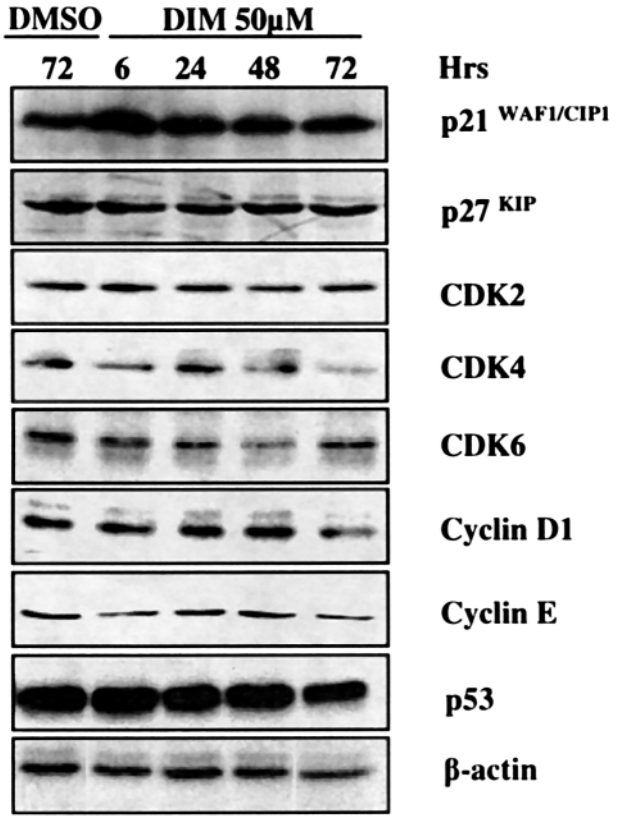


Fig. 2. The effect of DIM on expression of p21 and other cell cycle components in MCF-7 cells. Cells were treated with DIM (50 µM) for up to 72 h, and the levels of the p21, CDKs, cyclins and CKIs were determined by Western blot analysis using specific antibodies. Equal sample loading was determined by β-actin level measurement. Experiments were repeated three times with similar results.

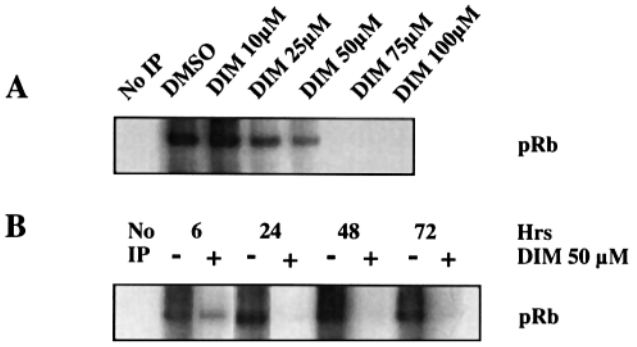


Fig. 3. The reduction of CDK2 activity in MCF-7 cells with DIM in a time- and concentration-dependent manner. MCF-7 cells were cultured with different concentrations of DIM for 24 h (**A**) or with DIM (50 µM) for various time periods (**B**). CDK2 was immunoprecipitated from cell lysates and assayed for *in vitro* kinase activity using the C-terminus of the Rb protein as substrate. The lane marked No IP was the control with rabbit anti-IgG, but no anti-CDK2 antibodies. The kinase reaction mixtures were fractionated and the level of [^{32}P]Rb was analyzed by autoradiography. The kinase assay was repeated three times with similar results.

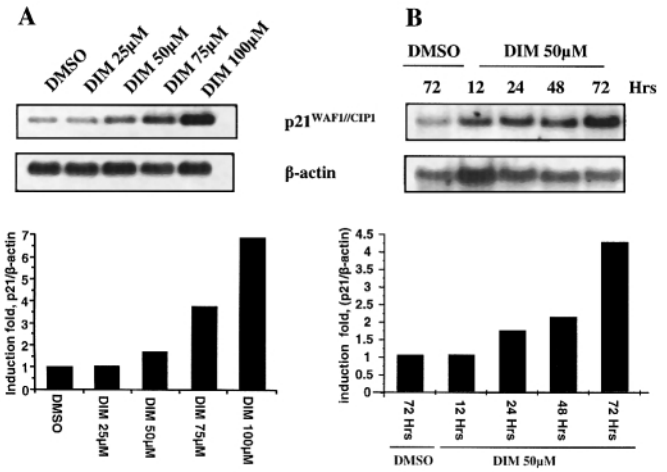


Fig. 4. The induction of p21 mRNA transcription with DIM treatment in MCF-7 cells. Cells were treated with different concentrations of DIM for 24 h (**A**) or with DIM (50 μM) for the times indicated (**B**). The isolated poly(A)$^+$ RNA was electrophoretically fractionated, and Northern blots were probed for p21 transcripts as described in the Materials and methods. As a loading control, the Northern blots were probed for β-actin. The results shown are representative of three independent experiments.

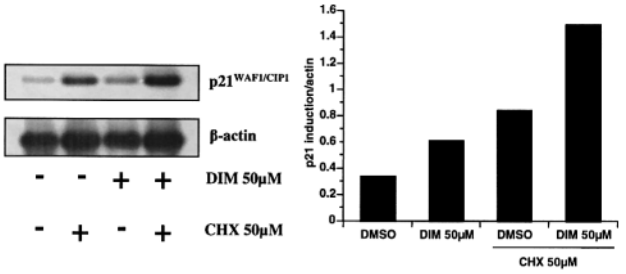


Fig. 5. The induction of p21 transcripts following 24-h co-treatment of MCF-7 cells with DIM and cycloheximide. MCF-7 cells were treated with DMSO or DIM (50 μM) in the presence or absence of 50 μM cycloheximide for 24 h. The isolated poly(A)$^+$ RNA was electrophoretically fractionated and northern blots were hybridized with p21 probe as described in the Materials and methods. The β-actin level was probed for loading control. The blot shown is representative of three independent experiments.

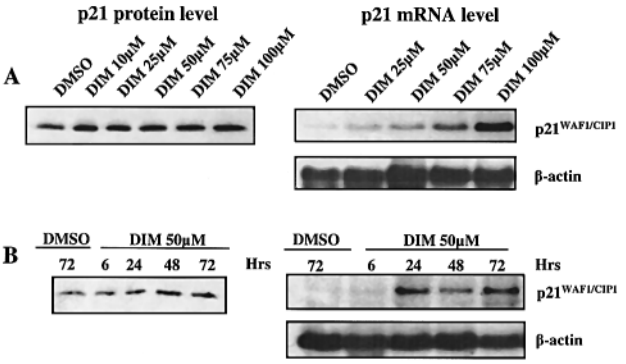


Fig. 6. The inductive effect of DIM on the levels of p21 protein and transcripts in MDA-MB-231 cells. MDA-MB-231 cells were treated with different concentrations of DIM for 24 h (**A**) or with DIM (50 μM) for various periods (**B**). Equal amounts of protein or mRNA were fractionated and probed with p21 antibodies or labeled cDNA as described previously. The blots are typical of three different experiments.

CKD2 enzymatic activity, presumably by the increased p21 levels, probably accounts for the DIM-mediated cell cycle arrest.

DIM increases levels of p21 transcript without significantly changing mRNA stability by a process that is independent of de novo protein synthesis

To determine the potential effect of DIM on p21 transcript levels, poly(A)$^+$ RNA was isolated from MCF-7 cells treated with different concentrations of DIM or with DIM (50 μM) for various time periods. Results of Northern blot analyses showed (Figure 4A) that DIM significantly increased p21 mRNA expression in a concentration-dependent pattern, and by 24 h reached a 7-fold induction. Results of a kinetics experiment showed that maximum induction was reached after 72 h of indole treatment (Figure 4B). We examined the effect of DIM on p21 mRNA stability by the actinomycin D co-treatment method to block *de novo* transcriptional initiation. DIM treatment caused no significant difference in the rates of mRNA degradation (data not shown). Taken together, these results indicate that the induction of p21 mRNA with DIM is due to increased transcription of this gene and not to increased stability of mRNA.

To determine whether the effect of DIM on p21 induction was dependent on protein synthesis, MCF-7 cells were treated with the translation inhibitor, cycloheximide. Results of Northern blot analyses (Figure 5) showed that induction of the p21 transcript by DIM was not blocked by concomitant treatment with cycloheximide (50 μM) for 24 h, indicating that DIM-mediated induction of p21 transcription occurred in the absence of *de novo* protein synthesis. These results suggest that the initial regulation of p21 transcription is mediated, in part, by interaction of DIM with one or more pre-existing cellular components involved in the transcriptional control of this gene. However, the fact that p21 mRNA levels continued to increase with up to 72 h of DIM treatment (Figure 4) would be consistent with a role of translational events in the later stages of induction.

DIM stimulation of p21 expression is independent of p53

The p53 tumor-suppressor protein is a transcription factor that mediates the transactivation of a number of genes involved in the control of cell proliferation including p21. The p21 promoter contains two p53 binding sites (21) that are critical for the cellular response to DNA damage. We observed, however, no significant change in p53 protein levels following DIM treatments (Figure 2). Furthermore, we observed an increase of p21 transcript and protein in MDA-MB-231 cells (Figure 6) in which wild-type p53 is not expressed (25). These results indicate that DIM-induced expression of p21 was independent of p53.

Deletion analysis of the p21 promoter shows that Sp1/Sp3 binding elements are responsive to DIM

To identify the region within the p21 promoter responsible for DIM-mediated transcriptional activation, MCF-7 cells were transiently transfected with a series of p21-promoter constructs containing deletions from –2326 bp upstream of the p21 transcription start site and terminating at +55 bp (Figure 7). MCF-7 cells were treated with different concentrations of DIM for 24 h and cell lysates were analyzed for CAT activity. As shown in Figure 7, DIM stimulated transcriptional activity of p21-CAT constructs containing deletions ending at –2326, –883, –585 and –291 bp. DIM induced the –291 bp construct by 2–3-fold, similar to its effect on the full length

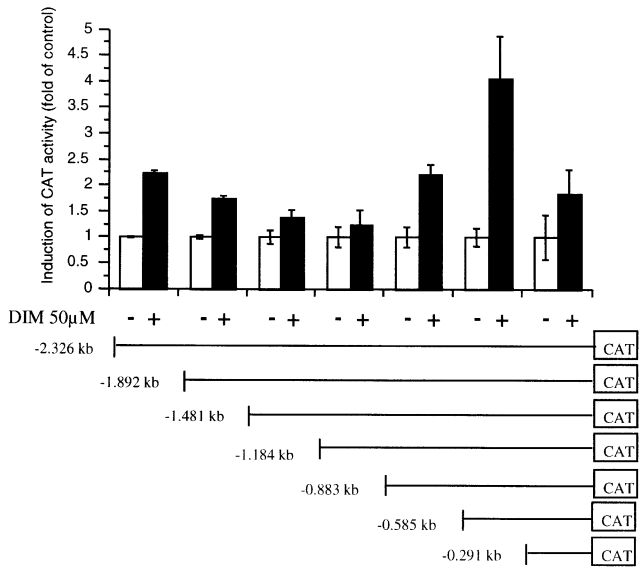


Fig. 7. 5′-Deletion analysis of the p21 promoter. MCF-7 cells were transiently transfected with a series of p21-CAT reporter plasmids that contain the indicated 5′-deletions of the p21 promoter. Cells were treated with either DMSO or DIM (50 μM) for 24 h, and the CAT specific activity was determined as the CAT activity/μg of protein present in the mixture. The values reported here were fold inductions over control (mean ± SD) from three independent experiments with duplicate.

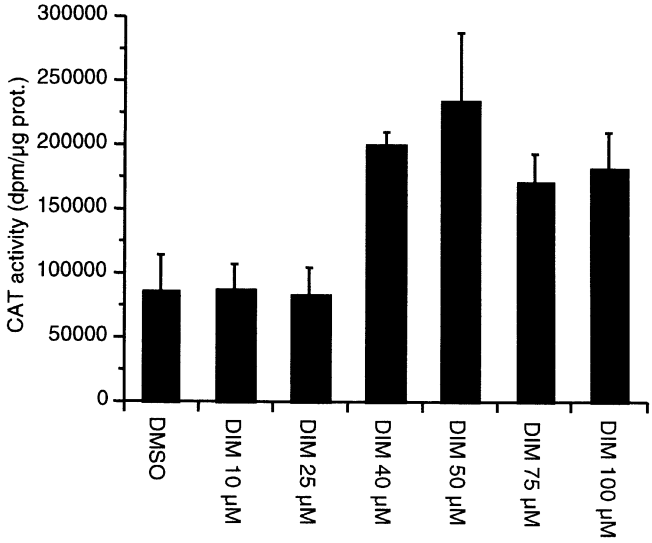


Fig. 8. The Sp1 reporter activity in MCF-7 cells treated with DIM. MCF-7 cells were transiently transfected with Sp1-CAT reporter plasmid containing three Sp1 consensus binding elements. Cells were treated with DMSO or DIM (50 μM) for 24 h and CAT activities were determined as described above. Data shown were the mean ± SD from three independent experiments with duplicate samples.

promoter construct suggesting that the DIM-regulated element is contained within the small region of the p21 promoter.

The region between –119 bp and the transcriptional start site of the p21 gene contains six Sp1 binding sites, and plays a major role in the regulation of p21 transcription in response to a variety of agents (16). Therefore, we examined the effect of DIM on the activity of an Sp1-CAT reporter that contains three consensus Sp1 DNA binding sites. We observed that DIM treatment of transfected cells induced the activity of this construct by 2–3-fold (Figure 8). The level of induction was similar to the inducing effect of DIM on the full length p21

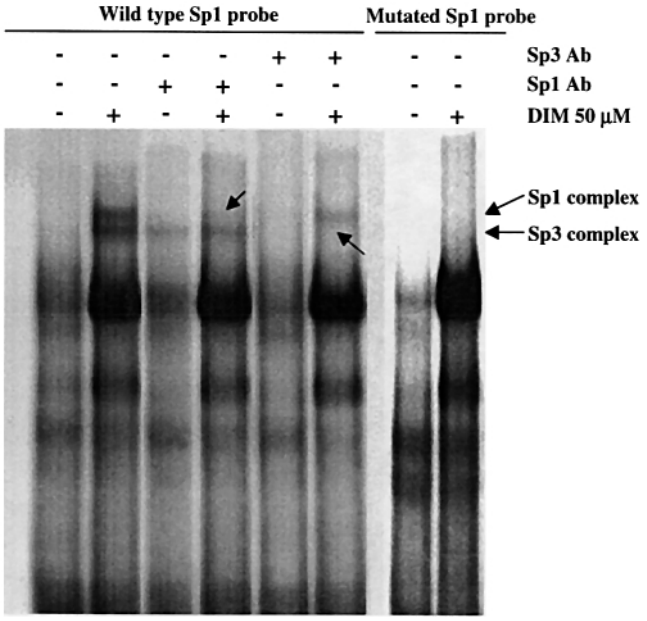


Fig. 9. The stimulation of the binding activity of Sp1/Sp3 to their cognate DNA binding sequence with DIM treatment. Nuclear extracts were prepared from MCF-7 cells that were treated with DMSO or DIM (50 μM) for 48 h. The nuclear fractions (20 μg) were incubated with specific antibodies for Sp1 or Sp3 for 20 min and further incubated with radiolabeled Sp1 wild-type or mutated oligonucleotides for additional 15 min. The protein–DNA complexes were electrophoretically fractionated on non-denaturing 6% polyacrylamide gels. Gels were dried and exposed to X-ray film for radioautography. Arrows indicate the locations of Sp1 or Sp3 protein–DNA complexes.

promoter construct, consistent with a primary role of activated Sp1 binding sites in p21 transcriptional activation.

DIM enhances the binding activities of Sp1/Sp3 proteins to their DNA responsive elements

To determine if DIM may affect the DNA binding activities of Sp1/Sp3, we conducted electrophoretic mobility shift analyses (EMSA) using annealed ^{32}P-labeled Sp1 consensus oligonucleotides. As shown in Figure 9, we detected three DIM-induced protein–DNA binding complexes. Super-shift experiments using specific antibodies against Sp1 or Sp3 indicated that the largest protein–DNA complex contained Sp1 and a smaller complex contained Sp3. The third and smallest complex was unaffected by Sp1/Sp3 antibodies and was produced in experiments using a mutated Sp1 oligonucleotide as the probe, thus indicating non-specific binding (Figure 9). In contrast, the shifted Sp1/Sp3 bands were not produced with the mutated probe. When these bands were competed with different amounts of unlabeled excess wild-type or mutated Sp1 oligonucleotides, we found, as expected, that addition of excess unlabeled probe successfully competed out the shifted Sp1/Sp3 bands (Figure 10). Also, addition of excess unlabeled mutant probe had no effect on the shifted bands (data not shown). These results indicated that DIM induced the specific binding of Sp1 and Sp3 to the consensus Sp1-responsive element of DNA. Taken together with the results of the promoter deletion studies, these findings indicate that DIM-induced expression of p21 involves the activation of binding of Sp1 and Sp3 transcription factors to the proximal Sp1-responsive elements of the p21 promoter.

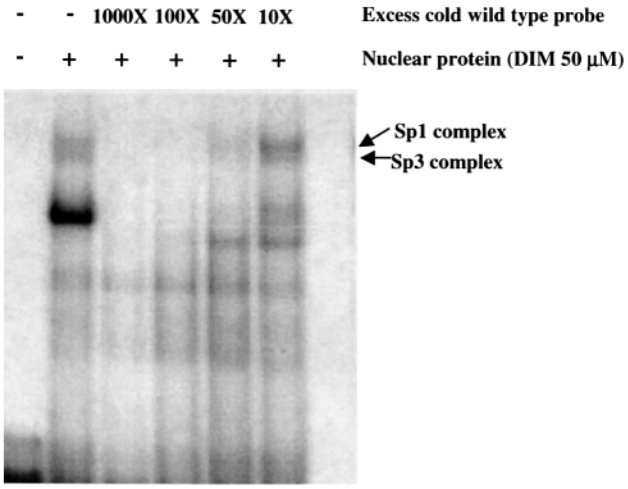


Fig. 10. Competitive gel-shift analysis of the binding of Sp1/Sp3 to their DNA element. Nuclear protein samples were prepared from MCF-7 cells treated with DMSO or DIM (50 μM) for 48 h. Nuclear extracts (20 μg) were pre-incubated with excess amounts of unlabeled wild-type or mutated oligonucleotides. Following a 20-min incubation, radiolabeled, wild-type Sp1 oligonucleotide probes were added and incubated for another 20 min. The protein–DNA complexes were resolved by electrophoresis and gels were dried and exposed to X-ray films. Arrows indicate the locations of Sp1 or Sp3 protein–DNA complexes.

Discussion

In the present study, we observed a rapid induction of G_1 cell cycle arrest that coincided with p21 induction in MCF-7 cells and a concomitant inhibition of CDK2-mediated phosphorylation of Rb. The rapid induction of p21 was controlled primarily at the transcriptional level without *de novo* protein synthesis. Further analyses with promoter deletion and EMSA indicated that Sp1/Sp3 binding activity plays a major role in DIM-mediated p21 induction.

Cell cycle progression is exquisitely regulated by the balance of CDKs and CKIs through an intricate network of growth-inhibitory and growth-stimulatory transduction signals. Control of proliferation in mammalian cells is primarily accomplished in G_1 (27). After G_1, cells become largely independent of extracellular signals and progress automatically through subsequent cell cycle stages (28). Thus, the G_1 CDKs are likely to play a particularly important role in the integration of proliferation control signals with the cell cycle machinery. The major G_1 phase CDKs are CDK2, CDK4 and CDK6. Their activity is positively regulated by cyclins and negatively regulated by CKIs. p21 was the first identified CKI designated as a CDK-interacting protein (CIP), using yeast two-hybrid screen with CDK2 as the bait (15), with broader specificity for CDKs. p21 has a very high affinity for cyclin E/CDK2 complexes (29–30) *in vitro* and >95% of the active CDK2 in normal diploid fibroblasts is associated with p21 (31). *In vitro* kinase analyses demonstrated that p21 was a critical inhibitor of CDK2 (32).

Our results showed that DIM rapidly increased p21 protein expression, reaching a maximum at 6 h that was maintained over 72 h. Consistent with p21 induction kinetics, G_1 cell cycle arrest occurred as early as 6 h. Concurrently, cDK2 activity was strongly reduced for phosphorylation of Rb protein *in vitro*. These results suggest that DIM-mediated induction p21 expression is responsible for the decrease of CDK2 activity and subsequent cell cycle arrest.

In our experiments, the increase of p21 protein occurred well before maximum p21 mRNA levels were reached (>72 h). p21 protein levels are known to be regulated at several levels, including post-translational degradation, as well as transcriptional activation, although the exact mechanism of p21 degradation is controversial (33). p21 is an unstable protein with half life of <30 min (33). Phosphorylation of p21 is reported to increase protein stability and result in a steady state of p21 levels. It was reported that a Taxol-induced increase in p21 protein levels was due to the increase of p21 protein stability resulting from phosphorylation (34). Our results indicate that DIM may regulate p21 expression by several mechanisms, as well.

Expression of p21 is regulated at the transcriptional level by both p53-dependent and -independent mechanisms (36). We demonstrated that DIM increased p21 mRNA expression in both MCF-7 cells, with wild-type p53, and MDA-MB-231 cells, with mutated p53, indicating that induction of p21 is independent of p53 status. This induction was not ablated by co-treatment of cycloheximide, a protein synthesis inhibitor, at least for the first 24 h in our system, arguing that activation of p21 is due to a direct or primary interaction between the p21 promoter and pre-existing transcription factors or associated co-activators. As p21 mRNA induction reached maximum at 72 h, it is possible that newly synthesized proteins also contribute to the induction of this gene expression.

In addition to two p53-responsive elements, there are specific *cis*-acting elements located within the p21 promoter that include binding sites for AP2, C/EBPa, C/EBPb, STATs, RAR, BRACA1, Sp1 and Sp3 transcription factors (16). Our analysis of deletion constructs of the p21 promoter indicated that the response to DIM could be localized to a proximal upstream –291 bp plasmid construct. No known response elements have been identified between –291 to –119 bp (16). However, the six Sp1 binding sites (Sp1-1 to Sp1-6) lie within the DIM-responsive –119 bp construct. These Sp1 binding sites play a major role in the regulation of p21 transcription in response to several chemotherapeutic agents (16,26,37–40).

Sp1 is a member of a multigene family that includes Sp1, Sp2, Sp3 and Sp4 (41). It is a ubiquitously expressed transcription factor that regulates expression of a large number of constitutive and induced mammalian genes by binding to specific GC-rich elements through C-terminal zinc-finger motifs. Sp1 and Sp3 can be differentially involved in p21 promoter activation responding to various inducers in different cell systems. Whereas the tumor-suppressor protein, BRACA1, controls p21 transactivation via Sp1-1 and Sp1-2 sites and is p53-independent (38), the Sp1-3 site has been shown to be required for p21 induction by progesterone (44), TGFβ (49), phorbol ester (PMA), okadaic acid (37), Ca^{2+} (45) and histone deacetylase inhibitors, such as butyrate (39), trichostatin A (TSA) (42) and suberoylanilide hydroxamic acid (SAHA) (26). The induction of p21 by okadaic acid and PMA is mediated mainly through Sp1 in U937 leukemic cells (37), whereas the co-activator, CBP/p300, is required for progesterone-mediated induction of p21 in HeLa cells (44). Butyrate-, TSA- and SAHA-mediated transactivation of p21 promoter requires both Sp1 and Sp3, but does not alter the DNA binding activities of Sp1/Sp3 (26,39,42). Whereas interaction of Sp1 with Smad proteins mediated induction of the p21 promoter by TGFβ (40,45), only Sp3 was required to regulate the inducibility of the p21 promoter by calcium (43). These reports strongly

suggest that Sp1 and Sp3 transcritional activities are differentially modulated in different cellular settings.

Our EMSA analyses showed that DIM induced a marked increase in Sp1 and Sp3 binding to Sp1 consensus sequence. We detected the induction of two specific Sp1–DNA and Sp3–DNA complexes following DIM treatment, indicating that DIM enhanced the binding activity of Sp1/Sp3 protein to Sp1-responsive elements in MCF-7 cells. Specific antibodies against Sp1 or Sp3 ablated the shifted signals, presumably due to blockade of DNA binding. These complexes were also successfully competed with excess unlabeled wild-type Sp1 probe, but not with mutated Sp1 oligonucleotides, indicating the specificity of these complexes. Thus, the induction of p21 with DIM treatment appears to result from increased binding of Sp1 and Sp3 to the p21 promoter.

Increased binding of Sp1/Sp3 to the p21 promoter may result from several mechanisms. The enhancement of Sp1/Sp3 binding may be due to the increased expression of Sp1/Sp3 protein induced by DIM. During Caco-2 cell differentiation, Sp1/Sp3 binding activity was increased, partially because of the increase of Sp1 protein level (46). Changes in phosphorylation or other posttranslational modifications of Sp1/Sp3 protein also may contribute to the increase of the binding activity. It is reported that Sp1 has multiple *O*-linked *N*-acetylglucosamine residues and that these sugar moieties appear to enhance the ability of Sp1 to activate transcription *in vitro* (47). Phosphorylation of Sp1 has been reported to stimulate (48–50) or downregulate (51) the DNA binding activity of Sp1. Although several kinases exhibit activity with Sp1, neither the phosphorylation sites, nor the kinase responsible for *in vivo* activation of Sp1 has been identified. Alternatively, the accessibility of the chromatin structure to Sp1/Sp3 transcription factors can also be involved in the binding activity. Many studies have shown that p21 gene mutations are rare in tumor cells, whereas the p21 promoter contains a putative CpG island, which is methylated, an epigenetic modification (35). CpG island methylation and deacetylation of histone are involved in the creation of transcription suppressive domains (54). Inactivation of tumor-suppressor genes by hypermethylation in the promoters is proposed to be due to chromatin remodeling, thereby blocking the access of transcription factors. Methylation of the cytosine residues in the Sp1/Sp3 target sequences can inhibit transactivation by Sp1 family members (53). The lack of p21 expression appears to be correlated with the hypermethylation of Sp1/Sp3 binding sites within the p21 promoter in both cell lines (55) and primary tumors (56), and the protein expression was induced by 5′-aza-deoxycytidine, a demethylating reagent (55,56). We are currently attempting to determine the precise mechanism by which DIM stimulates Sp1/Sp3 activity in the p21 promoter.

Our previous study showed that I3C, the DIM precursor, can rapidly induce a G_1 cell cycle arrest of human MCF-7 cells that is accompanied by the selective inhibition of CDK6 expression and 50% increases in levels of p21 and p27 (4). I3C decreases CDK6 transcription by targeting the Sp1 transcription factor binding to a composite Ets-Sp1 DNA site within the I3C regulated region of the CDK6 promoter (52). I3C causes a disruption of Sp1 interaction with the CDK6 promoter. The proximal Ets-like site near the I3C-responsive Sp1 site and the necessity of both sites for I3C inhibitory effect suggest that an Ets-like family member(s) may be a candidate transcription factor interacting with Sp1. The failure of I3C to inhibit the transcription of an Sp1-CAT reporter driven by three consensus Sp1 sites suggests that chromatin structure plays a role in I3C responsiveness of the CDK6 promoter (52). Although DIM treatment also induces a G_1 cell cycle arrest in MCF-7 cells, it did not show the effect on CDK6 protein expression. Instead, it strongly increased p21. DIM also induced Sp1-CAT reporter activity, which is unresponsive to I3C, and this inducing activity is similar to the effect on p21-CAT promoter constructs. The differences in activities of DIM and I3C indicate clearly that these two indoles exert their transcriptional regulatory effects through distinct mechanisms and are gene specific.

Our results showing that the induction of p21 protein and mRNA by DIM closely coincided with the G_1 cell cycle arrest, implicate p21 as a direct target for cell cycle control in human breast cancer cells. Thus, our results have identified a distinct inhibitory pathway that establishes a direct link between cell cycle regulation by DIM and the selective control of human breast cancer cell growth.

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