

Induction of Apoptosis by Vinblastine via c-Jun Autoamplification and p53-Independent Down-Regulation of p21^{WAF1/CIP1}

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ABSTRACT

Vinblastine treatment in all cell lines examined causes a robust increase in c-Jun protein expression and phosphorylation and a corresponding increase in activator protein-1 (AP-1) transcriptional activity. We show in KB-3 carcinoma cells that this is due to a strong autoamplification loop involving the proximal AP-1 site in the c-Jun promoter, resulting in highly increased c-Jun mRNA and c-Jun protein. Inhibitors of RNA transcription and protein translation blocked both vinblastine-induced c-Jun expression and apoptotic cell death, suggesting that apoptosis is dependent, at least in part, on transcription/translation. Small interfering RNA (siRNA) to c-Jun was used to interrupt the amplification cycle and was found to be highly effective, reducing vinblastine-induced c-Jun expression at both the mRNA and protein levels by 90%. Apoptosis and caspase-3 activation

were significantly inhibited in c-Jun siRNA-treated cells. To uncover potential mechanisms of c-Jun-mediated cell death and protection by c-Jun siRNA, candidate target genes were examined. Chromatin immunoprecipitation revealed preferential association of c-Jun with the p21 (cyclin-dependent kinase inhibitor) gene promoter after vinblastine treatment. In KB-3 cells, which have compromised p53 function, and in p53-null cells but not in p53 wild-type cells, vinblastine caused down-regulation of p21 expression concomitant with increased c-Jun expression, suggesting a role for c-Jun in negative regulation of the p21 promoter independent of p53. These results provide strong evidence that c-Jun induction in response to vinblastine plays a proapoptotic role in part via down-regulation of p21, promoting cycling and subsequent cell death of mitotically impaired cells.

The vinca alkaloid vinblastine is an important antitumor agent used for the treatment of testicular cancer and Hodgkin's and non-Hodgkin's lymphomas and in combination therapy for carcinomas of the lung, bladder, and for several other cancers (Rowinsky and Donehower, 1998). Vinblastine binds to tubulin subunits and inhibits tubulin polymerization, thus disrupting the dynamic instability of spindle microtubules (Jordan and Wilson, 2004). This leads to


mitotic arrest and subsequent cell death by apoptosis, either soon after metaphase arrest, after a delay, or after resumption of an impaired cell cycle entailing DNA endoreduplication (Khan and Wahl, 1998; Casenghi et al., 1999). The mechanisms that trigger apoptosis after metaphase arrest are under active investigation. Antimitotic drugs such as vinblastine activate several signal transduction pathways that may promote apoptosis. Two prominent and perhaps universal responses are those that lead to the activation of c-Jun NH₂-terminal kinase (JNK) (Fan and Chambers, 2001) and to phosphorylation of Bcl-2 and Bcl-xL (Ruvolo et al., 2001). Although some evidence has been presented to suggest that JNK catalyzes Bcl-2/Bcl-xL phosphorylation occurring in response to antimitotic drugs (Yamamoto et al., 1999), more recent work has indicated that a distinct kinase is responsible (Du et al., 2005).

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ABBREVIATIONS: JNK, c-Jun NH₂-terminal protein kinase; AP-1, activating protein 1; DMSO, dimethyl sulfoxide; RT, reverse transcription; siRNA, small interfering RNA; PCR, polymerase chain reaction; VBL, vinblastine; CHAPS, 3-[[3-cholamidopropyl]dimethylammonio]1-propane-sulfonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; AMC, amino-4-methyl coumarin; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone.

We have documented previously that vinblastine induces JNK activation, c-Jun expression and phosphorylation, and AP-1 activation in KB-3 carcinoma cells (Berry et al., 2001; Fan et al., 2001; Brantley-Finley et al., 2003). The increase in c-Jun expression is blocked by the JNK inhibitor SP600125, suggesting that c-Jun induction occurs via a JNK-dependent pathway (Brantley-Finley et al., 2003). Various approaches have been used to decipher the role of this pathway in the cellular response to this drug, but these have not produced a consistent answer. For example, whereas a dominant-negative to c-Jun rendered KB-3 cells more vinblastine-resistant (Fan et al., 2001), suggesting a proapoptotic role, c-Jun knockout fibroblasts, predicted to be more resistant, were equally as sensitive as wild-type cells (Obey et al., 2005). In addition, stable or inducible overexpression of c-Jun renders cells markedly resistant to vinblastine (Obey et al., 2005; Duan et al., 2007). These results may reflect cell type-specific differences in c-Jun function and probably reflect the fact that c-Jun plays a role in both cell proliferation and cell death and can functionally participate in many different protein complexes.

The mechanism(s) underlying the robust induction of c-Jun protein observed in vinblastine-treated cells has not been established. Several possibilities exist. One is through transcriptional regulation. The human c-Jun promoter has several regulatory elements responsive to different signal transduction pathways. An AP-1 site at -71 to -64 is particularly responsive to c-Jun/ATF2 heterodimers and confers the ability of the c-Jun promoter to be autoregulated by its product (Angel et al., 1988). Another is through enhanced protein stability. For example, c-Jun is targeted for ubiquitin-mediated degradation by inactive JNK, and JNK-mediated phosphorylation protects against degradation (Fuchs et al., 1998).

In this article, we show that the increase in c-Jun expression in response to vinblastine is through a transcriptional autoamplification mechanism involving the proximal AP-1 site in the c-Jun promoter. This suggested the use of small interfering RNA (siRNA) to block the amplification cycle, and this approach effectively eliminated c-Jun mRNA and protein induction in response to vinblastine. Apoptosis was significantly inhibited in c-Jun siRNA-treated cells compared with control cells, indicating that c-Jun induction plays an important proapoptotic role, and we present evidence implicating the cyclin-dependent kinase inhibitor, p21, as a key target.

Materials and Methods

Materials. ATF2, JNK1, JNK2, and actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); c-Jun (NH₂ terminus) antibody was from Transduction Laboratories (San Jose, CA); c-Jun (Ser-63) and ATF2 (Thr-71) phosphospecific antibodies and p53 antibody were from Cell Signaling Technology (Waltham, MA); p21 antibody was from Calbiochem (San Diego, CA); and GAPDH monoclonal antibody was from Ambion (Austin, TX). The dual luciferase reporter assay system was from Promega (Madison, WI). Fetal bovine serum was from Hyclone (Logan, UT), and other cell culture reagents were from Invitrogen (Carlsbad, CA). c-Jun promoter luciferase reporter plasmids were kindly provided by Dr. X.-F. Wang (Duke University Medical Center, Durham, NC). Unless otherwise stated, other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The KB-3 human cervical carcinoma cell line was maintained in monolayer culture at 37°C and 5% CO₂ in Dulbecco's

modified Eagle's medium supplemented with 10% fetal bovine serum, 4.5 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. HCT116 colon carcinoma cells, both wild-type and p53-null, were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum.

Cell Extraction and Immunoblotting. Whole-cell extracts were prepared, and immunoblotting was performed as described previously (Berry et al., 2001) using 15 µg of protein per lane for samples derived from experiments involving siRNA and 50 µg of protein per lane otherwise. Quantitation of relative protein expression from immunoblot films was determined by scanning densitometry using an HP ScanJet 6200C scanner (Hewlett Packard, Palo Alto, CA) and ImageQuant software (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

AP-1 Luciferase Assay. Cells (1.5×10^5 cells/well) were plated in 12-well plates 1 day before transfection and then cotransfected with 1 µg of 7× pAP-1-Luc (firefly luciferase under control of seven copies of an AP-1 consensus element) (Stratagene, La Jolla, CA) and 0.1 µg of pRL-TK (*Renilla reniformis* luciferase under control of thymidine kinase promoter) (Promega) using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). After 24 h, cells were treated with vehicle (0.1% DMSO) or 30 nM vinblastine for various times and harvested for the determination of firefly and *R. reniformis* luciferase activities using the Dual-Luciferase Assay System (Promega) and a TD-20/20 Luminometer (Turner Biosystems, Sunnyvale, CA). Luciferase reporter constructs containing the human c-Jun promoter region -79 to +170, either wild-type or with the AP-1/CRE site at -71 to -64 mutated were used in similar experiments. The results are expressed as average relative firefly luciferase activity normalized to *R. reniformis* luciferase activity.

Real-Time Polymerase Chain Reaction. Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed according to the manufacturers' protocols using reagents from Bio-Rad Laboratories (Hercules, CA) and the Prism 7700 Sequence Detection System and software of Applied Biosystems (Foster City, CA). Total RNA was isolated using RNeasy extraction kit (Qiagen, Valencia, CA). Total RNA (1 µg) was used to synthesize cDNA using the iScript cDNA kit to quantify transcripts for c-Jun, and the control housekeeping gene GAPDH. Real-time RT-PCR reactions were performed using the iTaq SYBR Green Supermix with ROX and specific primers designed using the Taqman Probe and Primer Design function (ignoring the probe) of the Primer Express version 1.5 software. The c-Jun (5'-GAGAG-GAAGCGCATGAGGAA-3' and 5'-CCAGCCGGGCGATTC-3'; accession number NM_002228, National Center for Biotechnology Information database) and GAPDH primers (5'-GAAGGTGAAG-GTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'; accession number NM_002046, National Center for Biotechnology Information database) were synthesized by IDT DNA (Coralville, IA). Standard curves for each assay were generated using 4-fold serial dilutions of a cDNA pool containing all samples. Standard curves ranged from 20.0 to 0.08 ng (RNA equivalents of cDNA). Optimal primer concentrations were determined to be 160 nM for c-Jun and 300 nM for GAPDH to reach at least 91% amplification efficiency. Relative gene expression in 1.25 ng of RNA equivalents of cDNA for each individual sample was calculated using standard curves, plotted as log of the nanograms of input RNA versus cycle number. Results are presented as the ratio of c-Jun to GAPDH expression relative to a standard curve for each assay.

Cell Death ELISA. Untreated and vinblastine-treated KB-3 cells were diluted to a concentration of 5×10^4 cells/ml for measurement of apoptosis using the Cell Death Detection ELISA kit (Roche, Penzberg, Germany).

Optimization of siRNA Transfection. siRNA transfection conditions were optimized using Silencer GAPDH siRNA control (Ambion). Fluorescein labeling of validated GAPDH siRNA using 6-carboxy-fluorescein was performed using Label IT Nucleic Acid Labeling kit following the manufacturer's protocol (Mirus Corpo-

ration, Madison, WI). Transfection of fluorescent-labeled siRNA was performed using KB-3 cells grown on 8-chamber culture slides (BD Falcon; BD Biosciences, San Jose, CA). Cells at 40% confluence were transfected with 50 nM fluorescein-labeled GAPDH siRNA using Oligofectamine (2 μ l per 250 μ l of Opti-MEM low-serum media) (Invitrogen). Twenty-four hours after transfection, cells were fixed using 4% paraformaldehyde, and slides were analyzed as described previously (Bene et al., 2004).

Transfection of c-Jun siRNA. KB-3 cells were plated in 24-well plates to achieve 30 to 40% confluence by the next day. Cells were washed twice with OPTIMEM low-serum media and transfected with 50 nM concentration of a pool of four siRNAs targeting different regions against c-Jun (SMART pool; Dharmacon, Lafayette, CO) or a pool of four nontargeting control siRNAs recommended by the manufacturer (siCONTROL; Dharmacon). After 24 h, cells were treated with vehicle (0.1% DMSO) or 30 nM vinblastine for 24 h. For longevity of siRNA action, KB-3 cells were transfected with the pool of c-Jun siRNAs for 24, 36, 48, or 72 h before application of 30 nM vinblastine for another 24 h.

Caspase-3 Assay. After treatment, cells were pelleted, washed twice in phosphate-buffered saline and lysed in 20 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A at 4°C. After centrifugation (16,000g, 10 min), caspase-3 activity in the supernatant was assayed with DEVD-amino-4-methyl coumarin (AMC) as substrate. Extracts (50 μ g of protein) were incubated in 0.25 ml of 100 mM HEPES, pH 7.4, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, and 50 μ M DEVD-AMC for 60 min at 30°C. The amount of liberated fluorescent AMC was determined using a Spectrofluorometer (PerkinElmer Life and Analytical Sciences, Waltham, MA) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Stennicke and Salvesen, 2000).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation was performed as described previously (de Belle et al., 2000) with slight modifications. All solutions contained the following protease and phosphatase inhibitors: EDTA-free protease inhibitor cocktail tablet (Roche), 20 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ M okadaic acid, and 1 mM phenylmethylsulfonyl fluoride. After cross-linking of cells with formaldehyde, nuclear extracts were prepared and sonicated to shear chromatin to an average size of ~500 base pairs. Samples were precleared with Protein A/G Sepharose beads, and c-Jun antibody (1–100 dilution) (Santa Cruz Biotechnology) was added to 1.5 mg of protein sample. A part of each captured immunocomplex was used for immunoblot analysis to confirm the presence of c-Jun in the chromatin pull-down. For the remaining sample, cross-links in the immunoprecipitated chromatin were reversed by heating with proteinase K at 65°C overnight, and DNA was purified by the MinElute Reaction Cleanup column (Qiagen) and resuspended in water. The chromatin immunoprecipitation (ChIP)-captured DNA sample and sonicated nuclear extract consisting of genomic DNA were subject to RT-PCR with p21 promoter-specific primers (IDT DNA), which amplify 446 base pairs of the 2101 to 2547 region of the human p21 promoter (accession number U24170) (p21 promoter sense: 5'-CTC ACA TCC TCC TTC TTC AG-3'; p21 promoter antisense: 5'-CAC ACA CAG AAT CTG ACT CCC-3'). The PCR profile was 3 min at 95°C (denaturation); 30 cycles of 30 s at 95°C; 30 s at 54°C (annealing temperature); 45 s at 72°C (primer extension); and 5 min at 72°C (final extension). PCR products were analyzed by gel electrophoresis using 1.2% agarose.

Results

Vinblastine Induced c-Jun Expression at Both the Protein and mRNA Levels with Concomitant AP-1 Activation. KB-3 cells were treated with 30 nM vinblastine for periods up to 48 h, and cell extracts were analyzed by immu-

noblotting for c-Jun, phospho-c-Jun, ATF2, and phospho-ATF2 (Fig. 1A). c-Jun protein and phospho-c-Jun expression were induced with a maximum at 16 to 24 h. ATF2 was present in untreated cells and phospho-ATF2 was detected at 8 to 32 h. Previous results have indicated that JNK is responsible for phosphorylation of c-Jun and ATF2 observed under these conditions (Fan et al., 2000; Stone and Chambers, 2000; Brantley-Finley et al., 2003). Consistent with these results, vinblastine increased AP-1-dependent transcription with a similar time course, as determined by the use of a luciferase reporter driven by several copies of an AP-1 consensus element (Fig. 1B). To determine whether the increase in c-Jun protein expression was at the mRNA level, c-Jun mRNA was measured by real-time RT-PCR. c-Jun mRNA, determined as a ratio with respect to GAPDH mRNA, increased 10-fold with a maximum after 16 h of vinblastine treatment (Fig. 1C).

The proximal AP-1 site (–71 to –64) in the c-Jun promoter has been shown to be sufficient for a maximal response to most stimulatory signals and is preferentially bound by c-Jun/ATF2 heterodimers (Angel et al., 1988; van Dam et al., 1993). We therefore transfected cells with a luciferase reporter controlled by a –79 to +170 sequence of the c-Jun promoter. Vinblastine strongly stimulated the reporter, whereas a reporter under the control of a mutated promoter lacking a functional AP-1 site was almost completely nonresponsive to vinblastine (Fig. 1D). These results suggest that vinblastine induces c-Jun at the transcriptional level through the proximal AP-1 site in the c-Jun promoter.

Vinblastine-Induced c-Jun Expression Was Blocked by Inhibitors of Transcription or Translation. To test the possibility that vinblastine-induced c-Jun expression may be dependent on both transcription and translation, KB-3 cells were pretreated with cycloheximide or actinomycin D for 30 min and then treated with 30 nM vinblastine for 16 h. Whole-cell extracts were prepared and subjected to immunoblotting for c-Jun and phospho-c-Jun. As shown in Fig. 2A, the inhibitors completely blocked vinblastine-induced expression of c-Jun and phospho-c-Jun. We next determined whether inhibition of c-Jun synthesis influenced vinblastine-induced apoptosis. KB-3 cells were pretreated with cycloheximide or vehicle for 30 min and then treated with 30 nM vinblastine or vehicle for 48 h. Apoptosis was quantitatively assessed as described under *Materials and Methods* (Fig. 2B). Apoptosis was induced by vinblastine alone as expected. Cycloheximide alone also induced apoptosis. In contrast, the combination of both vinblastine and cycloheximide led to a significantly decreased level of apoptosis relative to vinblastine alone. These results suggest that protein translation is required for vinblastine-induced apoptosis.

Vinblastine-Induced c-Jun Expression at Both the mRNA and Protein Levels Was Blocked by Specific siRNAs to c-Jun. Because the increase in c-Jun expression in response to vinblastine occurs at the transcriptional level, this provided an opportunity to test whether induction could be blocked via siRNA. Although siRNA approaches have been used extensively to reduce the expression of constitutively expressed mRNAs, their use to block mRNA induction occurring in response to specific stimuli has not been so widely reported. Transfection efficiency was optimized using fluorescein-labeled GAPDH siRNA. Representative images of transfected cells are shown in Supplementary Fig. S1. Inspection of 200 cells over several fields indicated that trans-

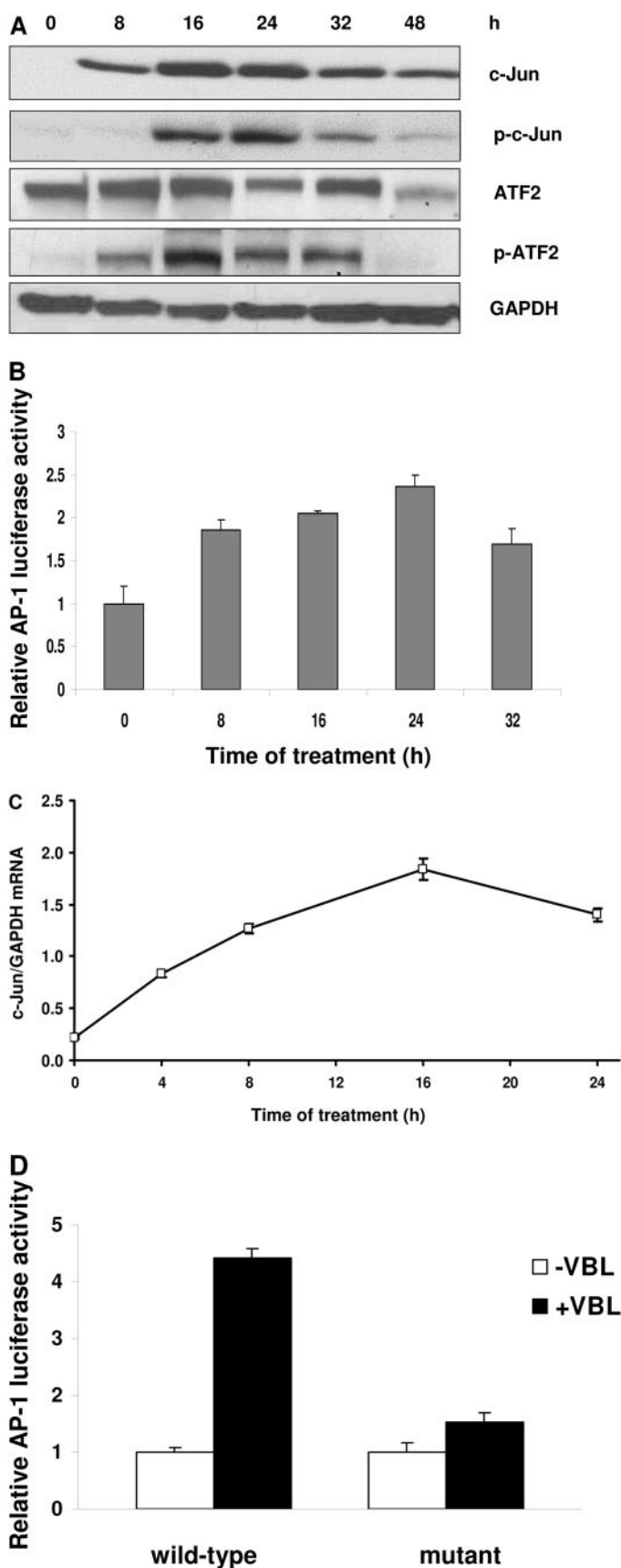


Fig. 1. Vinblastine induces c-Jun protein and mRNA expression and stimulates AP-1 activity via an autoregulatory mechanism. **A**, KB-3 cells were treated with 30 nM vinblastine for the indicated times, and whole-cell extracts were prepared and subjected to immunoblotting for c-Jun,

fection efficiency was approximately 70% under optimal conditions (50 nM siRNA and transfection time of 24 h). Effective reduction of GAPDH expression was confirmed by immunoblotting (Supplementary Fig. S1). These same conditions were used to transfect KB-3 cells with either a pool of four siRNAs specific for c-Jun or nontargeting control siRNAs, which have no homology with any known sequence in the human genome. After 24-h transfection, cells were treated with 30 nM vinblastine for an additional 24 h, and whole-cell extracts were subjected to immunoblotting with c-Jun antibody. As shown in Fig. 3A, pretransfection with c-Jun siRNA effectively eliminated the robust induction of c-Jun occurring in response to vinblastine. Quantitation of protein expression by densitometry indicated that c-Jun siRNA decreased c-Jun induction by 90%. In contrast, the expression levels of several other proteins in the JNK pathway, including ATF2, JNK1, and JNK2, were unaltered by siRNA treatment. To evaluate the longevity of c-Jun siRNA action, cells were transfected with siRNA for increasing duration, from 24 to 72 h, and then stimulated with vinblastine for 24 h. Figure 3B demonstrates that c-Jun siRNA remained highly effective in its ability to block c-Jun induction for periods up to 72 h before the addition of vinblastine. Under the same experimental conditions, control siRNA had no effect on c-Jun expression induced by vinblastine (data not shown). To detect and confirm changes at the mRNA level, c-Jun mRNA was measured by real-time RT-PCR. As shown in Fig. 3C, pretransfection with c-Jun siRNA essentially completely eliminated the increase in c-Jun mRNA by vinblastine.

Vinblastine-Induced Apoptosis Was Inhibited in Cells Transfected with c-Jun siRNA. Having established a highly effective method to block vinblastine-induced c-Jun expression, we next evaluated the effect of c-Jun siRNA on vinblastine-induced apoptosis (Fig. 3D). In the absence of vinblastine, a very low basal level of apoptosis was observed that was similar for untransfected cells or cells transfected with control or c-Jun siRNA. When cells were treated with vinblastine, apoptosis was highly significantly reduced in cells pretransfected with c-Jun siRNA compared with untransfected cells or cells pretransfected with control siRNA (Fig. 3D). To further document the effect of siRNA on vinblastine-induced apoptosis, caspase-3 assays were conducted. As shown in Fig. 3E, caspase-3 activity was detected in KB-3 cells at 24 h and increased over the next 24 h, as observed previously (Fan et al., 2001). At the 24- and 36-h time points, caspase-3 activity was increased slightly but

phospho-c-Jun, ATF2, and phospho-ATF2. GAPDH was used as a loading control. **B**, KB-3 cells were treated with 30 nM vinblastine for the indicated times, and AP-1 transcriptional activity was determined using a luciferase reporter system, as described under *Materials and Methods*. Results are expressed as ratios of Firefly/*R. reniformis* luciferase activities (mean \pm S.D., $n = 6$). **C**, KB-3 cells were treated with 30 nM vinblastine for the times indicated, and RNA was prepared and c-Jun mRNA levels determined by quantitative real-time RT-PCR, as described under *Materials and Methods*. Results are presented as the ratio of c-Jun to GAPDH expression relative to a standard curve for each assay (mean \pm S.D., $n = 6$). **D**, Mutation in AP-1/CRE site of c-Jun promoter abrogates vinblastine-induced AP-1 activity. KB-3 cells were transiently transfected with pRL-TK-Luc and p79/170c-Jun-Luc (wild-type or AP-1 mutant) in a 5:1 ratio. After 24 h, transfected cells were treated with 30 nM vinblastine (+VBL) or vehicle (0.1% DMSO, -VBL) for 16 h. Cells were harvested, and extracts were prepared for the determination of luciferase activity as described under *Materials and Methods*. Results are expressed as ratios of Firefly/*R. reniformis* luciferase activities (mean \pm S.D., $n = 6$).

not significantly by control siRNA (p value < 0.09 at 24 and 36 h). In contrast, significantly lower caspase-3 activity was observed in KB-3 cells transfected with c-Jun siRNA (p value < 0.02 at 24 h, < 0.0004 at 36 h). By 48 h after treatment, caspase-3 activity was comparable in control and c-Jun siRNA-transfected KB-3 cells and not significantly different. These results indicate a marked decrease in apoptosis and a delay in caspase-3 activation in response to vinblastine when c-Jun induction is blocked, implicating a proapoptotic role for c-Jun in this context. The reduced apoptosis at 48 h may be a direct consequence of the earlier delay in caspase-3 activation or, because caspase-3 activity returned to normal, may reflect the coexistence of caspase-independent apoptotic pathways.

p21 as a Key Target of c-Jun. To search for target genes that might mediate the proapoptotic effects of c-Jun, we used ChIP and hybridization to AP-1 arrays to identify promoters preferentially bound by c-Jun in vinblastine-treated KB-3 cells. c-Jun was readily immunoprecipitated from KB-3 cell chromatin, with much greater relative abundance after vinblastine treatment as expected and with no c-Jun signal when antibody was omitted (Fig. 4A). ChIP-enriched DNA was subjected to hybridization to AP-1 arrays as described previously (Hayakawa et al., 2004). The results of this study will be reported separately. One putative target gene uncovered was the CDK inhibitor p21^{WAF1/CIP1}. ChIP-enriched DNA was used for PCR amplification using primers corresponding to the p21 promoter, as described under *Materials and Methods*. The p21 promoter sequence was highly en-

riched in the immunoprecipitate from vinblastine-treated cells and undetectable in the immunoprecipitate from control cells or when antibody was omitted (Fig. 4B, lanes 3, 4, and 5). The p21 promoter sequence was detectable with equal abundance when total chromatin from untreated cells or vinblastine-treated cells was used in parallel experiments (Fig. 4B, lanes 1 and 2). Previous studies have indicated that c-Jun can regulate the p21 promoter, and several reports have indicated that c-Jun can directly repress p21 (Tsao et al., 1996; Wang et al., 2000). Consistent with this possibility and with the demonstration of an association of c-Jun with the p21 promoter (Fig. 4B), we observed a time-dependent decrease in p21 expression in vinblastine-treated KB-3 cells (Fig. 4C). Indeed, the pattern of p21 expression was reciprocal to that of c-Jun. Figure 4C also shows p53 expression, which, as reported previously (Fan et al., 2001), is found in relatively low levels in KB-3 cells and undergoes a transient reduction in expression after vinblastine treatment.

Effect of Vinblastine on p21 Expression in p53 Wild-Type and p53-Null HCT116 Cells. c-Jun has also been implicated in negative regulation of p21 in an indirect manner through negative regulation of p53. Thus, evidence has been presented that c-Jun binds to the p53 promoter and represses p53 transcription, which in turn causes decreased association of p53 with the p21 promoter and decreased p21 expression (Schreiber et al., 1999). KB-3 cells are a human cervical carcinoma HeLa subline, and as such, they are considered compromised for p53 function by virtue of expression of the HPV E6 oncoprotein. Although the data of Fig. 4C show a reciprocal relationship between c-Jun and p21 expression after vinblastine treatment, they do not address whether p53 acts as an intermediary regulator. To answer this question, we used HCT116 cells, both wild-type and p53-null. The cell lines were treated with vinblastine for different periods up to 48 h, and cell extracts were subjected to immunoblotting. The 24- and 48-h time points are presented in Fig. 5. In HCT116 wild-type cells, vinblastine robustly induced c-Jun expression with a maximum at 24 h and a decrease in expression at 48 h. However, p53 and p21 levels remained unaltered. In p53-null cells, vinblastine induced c-Jun expression to a similar extent and with a similar time course as observed in wild-type cells (Fig. 5). However, in p53-null cells, p21 levels declined markedly with vinblastine treatment. Taken together, these results suggest that vinblastine-induced c-Jun may down-regulate p21 only in cells that lack wild-type p53 function.

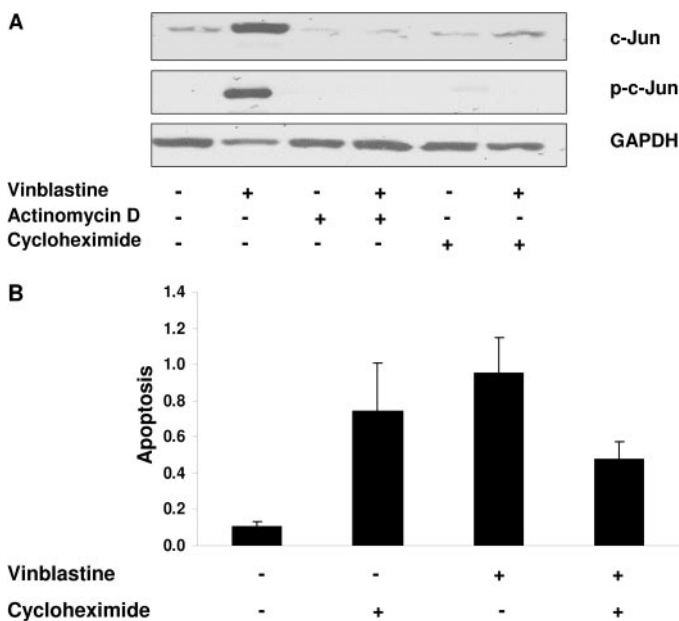


Fig. 2. Vinblastine-induced c-Jun expression/phosphorylation and apoptosis are blocked by inhibitors of transcription or translation. A, KB-3 cells were pretreated with cycloheximide (50 $\mu\text{g/ml}$) or actinomycin D (0.3 $\mu\text{g/ml}$) for 30 min and then treated with vehicle (0.1% DMSO) or 30 nM vinblastine for 16 h. Whole-cell extracts were prepared and subjected to immunoblotting for c-Jun, phospho-c-Jun, or GAPDH. B, vinblastine-induced apoptosis requires protein synthesis. KB-3 cells were pretreated with cycloheximide (50 $\mu\text{g/ml}$) or vehicle for 30 min and then treated with 30 nM vinblastine or vehicle for 48 h. The relative extent of apoptosis was assessed using a Cell Death Detection ELISA assay as described under *Materials and Methods*. Results are shown as mean \pm S.D. ($n = 3$) and are representative of two independent experiments. The difference in apoptosis of cells treated with vinblastine alone compared with cells treated with vinblastine plus cycloheximide was statistically significant (p value < 0.02 by two-tailed t test).

Discussion

Microtubule inhibitors in general exert their lethality by causing mitotic arrest and subsequent cell death, most commonly by apoptosis. However, the molecular mechanisms that link mitotic arrest to cell death are far from understood. One of the complicating factors is that microtubule inhibitors have been documented to activate or perturb many different signaling pathways, including those involving mitogen-activated protein kinases, p53, nuclear factor- κB , Raf-1, Bcl-2 proteins, and others (Bhalla, 2003; Mollinedo and Gajate, 2003). Despite this range of responses, one feature common among all cell lines examined is activation of the JNK pathway, first documented in KB-3 cells (Osborn and Chambers, 1996). Although JNK can phosphorylate a diverse spectrum

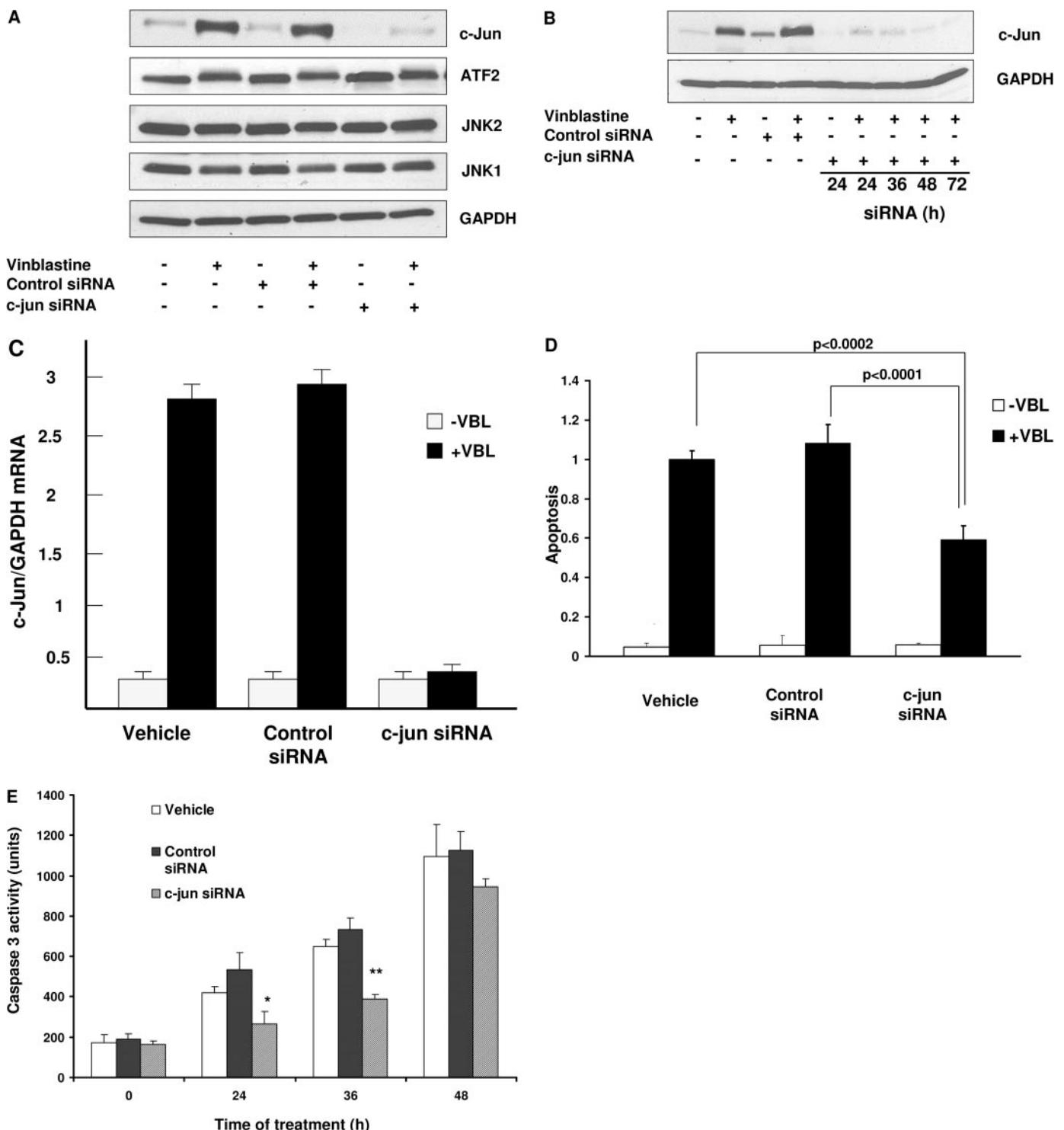


Fig. 3. Down-regulation of c-Jun by siRNA inhibits vinblastine-induced apoptosis. **A**, inhibition of vinblastine-induced c-Jun expression by c-Jun siRNA. KB-3 cells were untransfected or transfected with 50 nM control siRNA or c-Jun siRNA for 24 h and then treated with 30 nM vinblastine (VBL) or vehicle (0.1% DMSO) for 24 h. Cell extracts were subjected to immunoblotting for detection of c-Jun, ATF2, JNK1, JNK2, and GAPDH. **B**, longevity of c-Jun siRNA. KB-3 cells were untransfected, transfected with 50 nM control siRNA for 24 h, or transfected with c-Jun siRNA for 24, 36, 48, or 72 h and then treated with 30 nM vinblastine (VBL) or vehicle (0.1% DMSO) for 24 h, as indicated. Cell extracts were subjected to immunoblotting for detection of c-Jun or GAPDH. **C**, inhibition of vinblastine-induced c-Jun mRNA by c-Jun siRNA. KB-3 cells were transfected and treated as in **A**, and RNA was prepared and c-Jun mRNA levels determined by quantitative real-time RT-PCR, as described under *Materials and Methods*. Results presented are the ratio of c-Jun to GAPDH expression relative to the standard curve (mean \pm S.D., $n = 6$). **D**, vinblastine-induced apoptosis is decreased by inhibition of c-Jun expression by c-Jun siRNA. KB-3 cells were untransfected or transfected with 50 nM control siRNA or c-Jun siRNA for 24 h and then treated with 30 nM vinblastine (VBL) or vehicle (0.1% DMSO) for 48 h. The relative extent of apoptosis was assessed using a Cell Death Detection ELISA assay, as described under *Materials and Methods*. Results are shown as mean \pm S.D. ($n = 10$, -VBL; $n = 6$, +VBL) and are representative of two independent experiments. p Values, determined by two-tailed t test, are indicated. **E**, delayed activation of caspase-3. KB-3 cells were untransfected or transfected with 50 nM control siRNA or c-Jun siRNA and then treated with vehicle or 30 nM vinblastine for 24, 36, or 48 h. Caspase-3 was assayed as described under *Materials and Methods*. Results represent mean \pm S.D., $n = 4$. *, p value < 0.02 , and **, p value < 0.0004 , relative to control untransfected cells by two-tailed t test.

of substrates, JNK activation usually entails phosphorylation of c-Jun and activation of AP-1-dependent transcription (Davis, 2000). We have shown previously (Berry et al., 2001) and confirmed in this study that treatment of KB-3 cells with vinblastine leads to c-Jun induction and AP-1 activation. The aims of this study were to define the mechanism of c-Jun induction and to determine its role in apoptosis. The results support a model for regulation at the transcriptional level and provide evidence for a proapoptotic role involving p21 regulation.

The robust increase in c-Jun expression observed in vinblastine-treated cells is JNK-dependent, because we have shown previously that specific inhibition of JNK by SP600125 completely blocks induction (Brantley-Finley et al., 2003). JNK can regulate c-Jun levels both by increasing

c-Jun transcription (Karin, 1995) and/or by increasing c-Jun stability (Fuchs et al., 1998). Although the present data do not exclude the latter as a contributory factor, the evidence strongly supports a transcriptional autoamplification mechanism: (1) c-Jun expression is blocked by inhibitors of transcription or translation; (2) c-Jun mRNA levels increase before and parallel with the increase in c-Jun protein; (3) vinblastine activates the c-Jun promoter via the proximal AP-1 site; and (4) siRNA to c-Jun blocks c-Jun induction at both the mRNA and protein levels. Given these data, it seems likely that the increase in c-Jun expression proceeds via JNK-mediated phosphorylation of pre-existing c-Jun and ATF2, which combine to activate the c-Jun promoter at the proximal AP-1 site, leading to an increase in c-Jun mRNA and protein, which is in turn phosphorylated by JNK, resulting in further amplification of this autoregulatory loop. Although other stressful stimuli that result in c-Jun induction, such as UV irradiation, have been shown previously to operate via such a mechanism (Angel et al., 1988; Karin, 1995), these results are the first to provide definitive evidence that microtubule inhibitors also increase c-Jun levels through an analogous autoregulatory mechanism.

Vinblastine and other antimetabolic drugs promote a robust increase in c-Jun expression not only in KB-3 cells but in all cell lines examined to date (C. Lyle and T. Chambers, unpublished observations), suggesting that it is a key response to microtubule inhibition. However, in the absence of an effective and specific way to block c-Jun function or expression, the role of this pathway has been difficult to decipher. The most compelling results were derived from stable expression of TAM-67, a dominant-negative c-Jun mutant, which rendered KB-3 cells more resistant to vinblastine (Fan et al., 2001). Given the present results that vinblastine induces c-Jun mRNA expression, an opportunity was presented to test siRNA as an approach to block expression and probe function. In general, siRNAs have been used to silence constitutively expressed mRNAs, and there are few reports describing the knockdown of an mRNA induced by a stress stimulus (Devi, 2006). In our system, c-Jun siRNA was extremely effective in reducing the increase in c-Jun mRNA and protein in vinblastine-treated cells. Under these conditions, vinblastine-induced apoptosis was significantly decreased to approximately 60% of control levels (Fig. 3D). These results indicate that c-Jun plays a major proapoptotic role. However, the fact that there remains significant cell death when c-Jun induction is repressed suggests the existence of alternate pathways. Indeed, a major effect of c-Jun

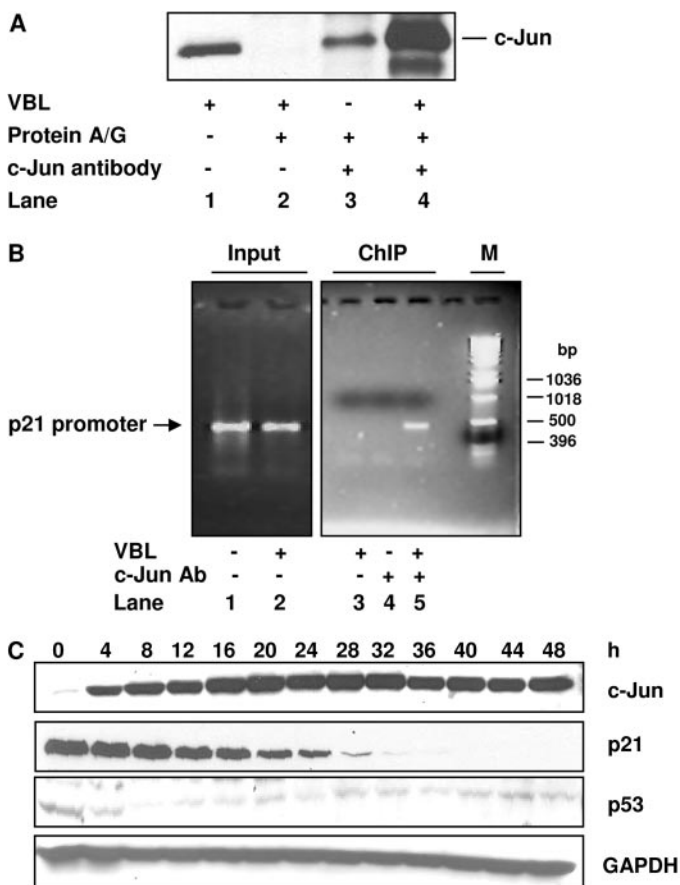


Fig. 4. Regulation of p21 by c-Jun. **A**, immunoblotting for c-Jun in ChIP products. KB-3 cells were untreated or treated with vinblastine (30 nM, 24 h), and chromatin was prepared and subjected to immunoprecipitation with c-Jun antibody followed by immunoblotting for c-Jun. Lane 1, whole-cell extract after vinblastine treatment as positive control; lane 2, precipitation conducted without c-Jun antibody; lanes 3 and 4, immunoprecipitates of chromatin prepared from control and vinblastine-treated cells, respectively. **B**, ChIP assay for in vivo association of c-Jun with p21 promoter. Chromatin or ChIP-captured DNA was subjected to PCR with p21 promoter-specific primers. Lane 1, total chromatin from untreated cells; lane 2, total chromatin from vinblastine-treated cells (VBL, 30 nM, 24 h); lane 3, immunoprecipitate of chromatin from vinblastine-treated cells conducted without c-Jun antibody; lanes 4 and 5, immunoprecipitates of chromatin prepared from control and vinblastine-treated cells, respectively. **M**, molecular mass standards. **C**, vinblastine-induced down-regulation of p21 protein expression. KB-3 cells were treated with vehicle (0.1% DMSO for 24 h) or 30 nM vinblastine for the times indicated, and cell lysates were subjected to immunoblotting for c-Jun, p21, p53, and GAPDH.

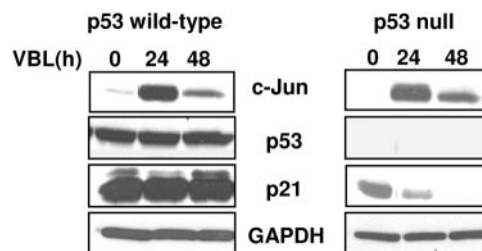


Fig. 5. Influence of p53 on vinblastine-induced down-regulation of p21. HCT116 wild-type or HCT116 p53-null cells were treated with 100 nM vinblastine for the times indicated, and cell lysates were subjected to immunoblotting for c-Jun, p53, p21, and GAPDH. Note that the p53 immunoblots in each panel were processed under identical conditions, and thus the signals generated are directly comparable.

depletion seemed to be in delay, not overall inhibition, of caspase-3 activation (Fig. 3E).

To gain insight into the mechanism of c-Jun in vinblastine-induced apoptosis, we sought to identify candidate target genes. Using chromatin immunoprecipitation and promoter array hybridization, as described previously (Hayakawa et al., 2004), one of the promoters identified by this method was that of p21 (N. Upreti and T. Chambers, unpublished observations). Chromatin immunoprecipitation confirmed an association of c-Jun with the p21 promoter after vinblastine treatment (Fig. 4B). c-Jun has been shown capable in different studies of up-regulation or repression of p21 transcription. In one study, c-Jun transactivated the p21 promoter in collaboration with Sp1 but was unable to transactivate the p21 promoter in the absence of Sp1 (Kardassis et al., 1999). In several other studies, c-Jun was shown to repress p21 transcription (Tsao et al., 1996; Wang et al., 2000). We observed down-regulation of p21 concomitant with c-Jun up-regulation in KB-3 cells, suggesting that c-Jun occupancy causes repression of the p21 promoter.

The down-regulation of p21 by c-Jun may be a mechanism to prevent cell cycle arrest after aberrant mitotic exit, promoting cycling of impaired cells and thus facilitating an apoptotic outcome. In this regard, the function of c-Jun in the response to microtubule inhibition may be analogous to that in the UV response, in which a major role of c-Jun is to promote cell cycle re-entry of UV-damaged cells (Shaulian et al., 2000). However, in the UV response, c-Jun acts as a negative regulator of p53 and indirectly causes p21 down-regulation. This prompted us to determine whether the down-regulation of p21 in response to vinblastine occurred in a p53-dependent or -independent manner. We found that in cells with wild-type p53 function, vinblastine failed to down-regulate p21, whereas in p53-null cells, p21 down-regulation was observed, similar to the results with KB-3 cells, which have compromised p53 functionality. Although a broader spectrum of cells will need to be examined before a general consensus can be formed, it is interesting to note that the binding sites for c-Jun in the p21 promoter overlap with those of p53. Thus, the distal promoter region, at -1394 to -2301, contains several binding sites for p53, and within this region, there is also a binding site for c-Jun (Chung et al., 2002; Gartel and Radhakrishnan, 2005). Thus, p53 and c-Jun may compete for binding, and this may explain why c-Jun is an effective regulator only in the absence of wild-type p53. A testable hypothesis is that c-Jun may play a greater role in p21 regulation in cells with compromised p53 function, which includes the majority of cancer cells. It is interesting that previous studies have shown that recruitment of myc to the p21 promoter blocks p21 induction by p53 (Seoane et al., 2002). Thus, competitive interaction of transcription factors seems to be a key mechanism for regulation of p21 transcription.

In summary, we have demonstrated that siRNA is an effective means to knock down c-Jun mRNA induced in response to vinblastine treatment, and we presented evidence for a proapoptotic role that may be mediated, at least in part, through p21 down-regulation. The demonstration of highly effective silencing of a gene induced by a stressful stimulus represents a powerful application of siRNA. The approach described here may be of considerable usefulness in further

exploration of the role of c-Jun in the response to microtubule inhibition and other systems in which c-Jun induction plays a key role.

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