ELSEVIER

Biochemical Pharmacology 62 (2001) 581–591

Biochemical Pharmacology

AP-1 activation and altered AP-1 composition in association with increased phosphorylation and expression of specific Jun and Fos family proteins induced by vinblastine in KB-3 cells

April Berry, Mary Goodwin, Cindy L. Moran, Timothy C. Chambers*

Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Mail Slot 516, 4301 W. Markham St., Little Rock, AR 72205-7199, USA

Received 12 September 2000; accepted 7 December 2000

Abstract

Vinblastine and other microtubule inhibitors are important antitumor agents that cause mitotic arrest, and induce apoptosis through poorly understood mechanisms, in a wide variety of cell lines. The activating protein 1 (AP-1) transcription factor is a major target of the c-Jun NH_2-terminal kinase (JNK) signaling pathway, which is activated by microtubule inhibitors. Therefore, we examined the effect of vinblastine on AP-1 composition and activity in human KB-3 carcinoma cells. Vinblastine caused highly selective effects on AP-1 proteins in a concentration- and time-dependent manner. Specifically, c-Jun, expressed at a low level in control cells, was greatly increased and phosphorylated, Jun D was phosphorylated, Jun B underwent phosphorylation and subsequently became undetectable, and Fra 1 expression was also greatly increased. In contrast, Fra 2, c-Fos, and Fos B were relatively unchanged by vinblastine. Changes in AP-1 preceded caspase 3 activation and, therefore, occurred prior to the commitment phase of apoptosis. With the exception of c-Jun, which was not affected by paclitaxel, the same alterations in AP-1 proteins occurred after exposure to vincristine, paclitaxel, and colchicine, demonstrating that these are general responses to microtubule inhibition. Supershift assays demonstrated that in control cells, AP-1 binding activity was mediated by Jun D/Fra 2 heterodimers, whereas after vinblastine treatment, AP-1 complexes also containing c-Jun and Fra 1 were present, suggesting that induction of these latter proteins by vinblastine is functionally significant. Consistent with these observations, vinblastine stimulated AP-1-dependent luciferase reporter gene transcription. These findings suggest that alterations in AP-1 composition and activity may be key events in the early response of KB-3 cells to microtubule inhibitors. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Vinblastine; AP-1; Jun proteins; Fos proteins; Apoptosis; Microtubule inhibitors

1. Introduction

The Vinca alkaloid vinblastine has broad antineoplastic activity and is used to treat breast and testicular carcinomas, Hodgkin's and non-Hodgkin's lymphomas, Kaposi's sarcoma, and several other cancers [reviewed in Refs. 1 and 2]. Vinblastine causes metaphase arrest by binding to tubulin and inhibiting its polymerization which, in turn, induces cell death by apoptosis. Apoptosis is characterized morphologically by cell shrinkage and chromosomal condensation and fragmentation, and biochemically by activation of a program of macromolecular destruction effected by caspases [3]. The cytotoxicity of vinblastine and other microtubule inhibitors largely correlates with their ability to induce metaphase arrest [4]. The biochemical mechanisms responsible for directing mitotically arrested cells to an apoptotic pathway remain unclear. However, several signaling and checkpoint pathways have been implicated, most notably those involving p53, Bcl-2, and JNK [reviewed in Refs. 5 and 6]. Understanding these mechanisms is of critical importance for improving the effectiveness and selectivity of antimitotic drugs, and for the development of methods to overcome resistance to these agents.

Both p53-dependent and -independent modes of cell death following treatment of cells with microtubule inhibitors have been described [5,6]. However, because of the

* Corresponding author. Tel.: +1-501-686-5755; fax: +1-501-686-8169

E-mail address: ChambersTimothyC@exchange.uams.edu (T.C. Chambers).

Abbreviations: JNK, c-Jun NH_2-terminal kinase; AP-1, activating protein 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; and TK, thymidine kinase.

0006-2952/01/$ – see front matter © 2001 Elsevier Science Inc. All rights reserved.
PII: PII S0006-2952(01)00694-3

varying model systems and experimental conditions employed, the relationship between p53 status and sensitivity to these agents remains poorly defined. Recent results have indicated that in normal cells, p53 does not function as a sensor of microtubule damage. Rather, in this setting, p53 operates to prevent DNA re-replication in cells undergoing mitotic slippage, a process of adaptation into a G_1-like state which follows aberrant exit from metaphase arrest [7]. In cancer cells, there is no significant correlation between p53 status and sensitivity to antimitotic drugs [8]. The anti-apoptotic Bcl-2 protein has also been implicated in apoptosis following microtubule damage. Microtubule inhibitors stimulate Bcl-2 phosphorylation, which is associated with Bcl-2 inactivation and thus apoptosis promotion [9,10].

Another important mediator of apoptotic signals is the JNK subgroup of mitogen-activated protein kinases [reviewed in Ref. 11]. Many apoptotic stimuli cause JNK activation, and a great deal of evidence has accumulated to suggest that stress-induced JNK activation acts as a pro-apoptotic signal. For example, cells with targeted deletion of the JNK1 and JNK2 genes are resistant to UV-induced apoptosis [12]. In neuronal cells, withdrawal of nerve growth factor results in apoptosis, which is mediated by JNK signaling to c-Jun [13]. Microtubule inhibitors activate JNK in a wide variety of cell lines [14–19] and inhibition of JNK protects cells from their cytotoxicity [17,18], suggesting that JNK contributes to apoptosis induction by these agents. Interestingly, recent evidence has identified JNK as the kinase responsible for vinblastine- and paclitaxel-induced Bcl-2 phosphorylation [20,21], strengthening the notion that JNK signaling is pro-apoptotic in this context.

The best-characterized substrate of JNK is c-Jun, a component of the AP-1 transcription factor. AP-1 is a term describing dimeric transcription factors belonging to the basic region leucine zipper (bZIP) group of DNA binding proteins. Conventional AP-1 is composed of either homodimers of Jun family members (c-Jun, Jun B, Jun D), or heterodimers of Jun proteins with Fos family members (c-Fos, Fos B, Fra 1, Fra 2) [reviewed in Refs. 22–24]. Jun-Jun and Jun-Fos dimers preferentially bind to a common site, the TRE or AP-1 site, which has the sequence TGAC/GTCA. AP-1 activity is controlled primarily by the abundance and phosphorylation status of these proteins. JNK phosphorylates two main sites, Ser63 and Ser73, in the transactivation domain of c-Jun. AP-1 is activated in response to diverse extracellular stimuli and plays a critical role in many fundamental biological processes including development, proliferation, differentiation, and cellular stress responses [22–24].

An AP-1 complex capable of binding to a TRE can potentially be composed of one of several dozen different dimer combinations. Although AP-1 induction by paclitaxel has been reported [25,26], to our knowledge there has not been a systematic study of the effect of microtubule inhibitors on the expression and phosphorylation of all major AP-1 family members in any given system. Evidence is accumulating to suggest that different types of AP-1 are functionally distinct and may activate different target genes [22,23]. Therefore, it has become increasingly important to identify the protein components involved. In this study, we have demonstrated that vinblastine and other microtubule inhibitors cause highly selective alterations in the expression and phosphorylation of specific AP-1 proteins, leading to the formation of novel AP-1 complexes and increased AP-1 activity. These changes preceded caspase 3 activation, suggesting that alteration in AP-1 may be an early and important event in the response to microtubule inhibition.

2. Materials and methods

2.1. Materials

Antibodies used for immunoblotting were from the following sources: polyclonal antibodies to Jun D, Fra 1, Fos B, and actin, and the monoclonal antibody to Jun B, were obtained from Santa Cruz Biotechnology; monoclonal c-Jun antibody was obtained from Transduction Laboratories; polyclonal antibodies to Fra 2 and c-Fos were obtained from Geneka Biotechnology, Inc.; and phospho-(Ser-63)-specific c-Jun antibody was from New England Biolabs. The AP-1 Nu-Shift kit, with additional Fos and Jun family antibodies for supershift analysis, was obtained from Geneka. [γ-^{32}P]ATP and the enhanced chemiluminesence detection system were from Amersham International. Cell culture reagents were obtained from GIBCO-BRL Life Technologies. Unless otherwise stated, all other reagents were purchased from the Sigma Chemical Co.

2.2. Cell culture

The KB-3 cell line, which expresses low levels of wild-type p53, was provided by Dr. M.M. Gottesman (National Cancer Institute) and has been described elsewhere [27]. KB-3 cells were treated at 70% confluency; cells that became non-adherent were combined with those that remained adherent. All stock solutions of drugs were made at 10 mM in DMSO. The final concentration of DMSO was constant at 0.1% in all experiments, with controls receiving vehicle alone.

2.3. Preparation of whole cell and nuclear extracts and phosphatase treatment

Following treatment, cells were scraped gently into the medium, pelleted, washed in ice-cold phosphate-buffered saline, and lysed in 4 vol. of 25 mM HEPES (pH 7.5), 0.3 M NaCl, 0.2% SDS, 0.5% sodium deoxycholate, 0.2 mM EDTA, 0.5 mM DTT, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.1% Triton X-100, 20 μg/mL of aprotinin, 50 μg/mL of leupeptin, 10 μM pepstatin, 0.1 μM okadaic acid, and 1 mM PMSF. After 15 min on ice, extracts were

sonicated (3 × 10 sec), insoluble material was removed (20 min, 100,000 *g*), and the protein concentration in the supernatant was determined using the BioRad Protein Assay. Extracts were stored at –20° until required. Nuclear extracts were prepared, and treatment with acid phosphatase was performed, as previously described [15].

2.4. Immunoblot analysis

Whole cell or nuclear extracts were fractionated by SDS–PAGE, transferred to polyvinylidene difluoride membrane (Micron Separations, Inc.), and probed with the appropriate antibody at 1:1000 dilution, with the exception of the Jun D antibody which was used at 1:5000. Primary antibody was detected by horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system (Amersham). In some experiments, the primary antibody was neutralized by preabsorption with the corresponding peptide antigen (0.2 μg/mL) prior to immunoblotting. Unless otherwise indicated, 50 μg of protein/lane was subjected to immunoblotting.

2.5. Caspase 3 assay

Extracts were prepared by sonication of cells for 10 sec in 0.3 mL of 20 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 2 mM DTT, 0.1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 μg/mL of leupeptin, and 1 μg/mL of pepstatin A. The supernatants obtained after centrifugation (16,000 *g*, 10 min, 4°) were used to determine the caspase 3 activity by fluorometric assay using the substrate Ac-Asp-Glu-Val-Asp-AMC (DEVD-AMC). Extracts containing 100 μg protein were incubated in a final volume of 0.25 mL containing 100 mM HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 50 μM substrate. After incubation for 30 min at 30°, the liberated fluorescent group (amino-4-methyl courmarin, AMC) was monitored using a spectrofluorometer (Perkin Elmer) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Results are expressed as means ± SD of three determinations.

2.6. EMSA

AP-1-specific DNA binding activity was determined by EMSA using the c-Jun/AP-1 Nushift kit according to the directions of the manufacturer (Geneka). The kit included a nuclear extract from Swiss 3T6 Albino cells as a positive control for c-Jun/AP-1 binding activity. Briefly, 10 μg of nuclear extract was incubated in a final volume of 16 μL containing 4 μL of binding buffer, 2 μL of stabilization buffer, and 1 μL of a ^{32}P-labeled AP-1 oligonucleotide probe (approx. 0.1 ng, 10^5 cpm). Variable additions to the reaction mixture included 2 μL of antibody to individual AP-1 proteins, 2 μL of corresponding antibody peptide epitope, 2 μL (10 ng) of unlabeled wild-type oligonucleotide probe as competitor, or 2 μL of mutant probe as a non-competing control. After 15 min at 30°, the mixture was subjected to 5% acrylamide Tris/glycine/EDTA gel electrophoresis which was dried and exposed to Hyperfilm MP (Amersham). The AP-1 probe (5′-CGC TTG A*TG AGT CA*G CCG GAA-3′, with the AP-1 consensus sequence indicated in italics) was labeled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified on a MicroSpin™ G25 column according to directions (Geneka).

2.7. Transfection and AP-1 luciferase assay

Cells (2 × 10^5/well) were plated in a 6-well plate and transfected with 2 μg TRE2-Luc (firefly luciferase under control of 2 copies of the TRE) together with 2 μg pRL-TK-Luc (*Renilla* luciferase under control of the TK promoter for constitutive expression) using 20 μL of Superfect Reagent (Qiagen). After 32 hr, cells were treated with vehicle (0.1% DMSO), vinblastine, or TPA, and after 16 hr were harvested for determination of firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega). The results were expressed as average relative firefly luciferase activity, normalized to *Renilla* luciferase activity to control for transfection efficiency. Data are means ± SD of four independent experiments.

3. Results

3.1. Selective expression and phosphorylation of AP-1 family proteins in KB-3 cells by vinblastine

We first determined whether there were concentration-dependent effects of vinblastine on AP-1 protein expression by immunoblotting of whole cell extracts prepared from KB-3 cells treated for 16 hr. As shown in Fig. 1A. c-Jun protein expression was just detectable in control cells, increased slightly upon treatment with 0.01 μM vinblastine, and was robustly induced at 0.1 μM and higher concentrations. In addition, a mobility shifted form of c-Jun (P-c-Jun) was observed at the higher concentrations. Jun B, on the other hand, was readily detectable in control cells, and a mobility shifted form (P-Jun B) was observed at 0.01 μM and higher concentrations of vinblastine. An additional lower molecular weight species was also observed. However, this band was still detected when the Jun B antibody was preabsorbed with corresponding peptide antigen prior to immunoblotting (data not shown), and is therefore denoted as non-specific (NS). Jun D showed a more complex pattern in control whole cell extracts with three immunoreactive bands (Fig. 1A). The slowest migrating (upper) species was identified as Jun D based on its molecular mass of 39 kDa, the fact that immunoreactivity was lost when the antibody was preabsorbed with peptide antigen (data not shown), and because this same species was present in nuclear extracts (see below). In contrast, the central species (denoted NS in Fig. 1A) was still present when antibody

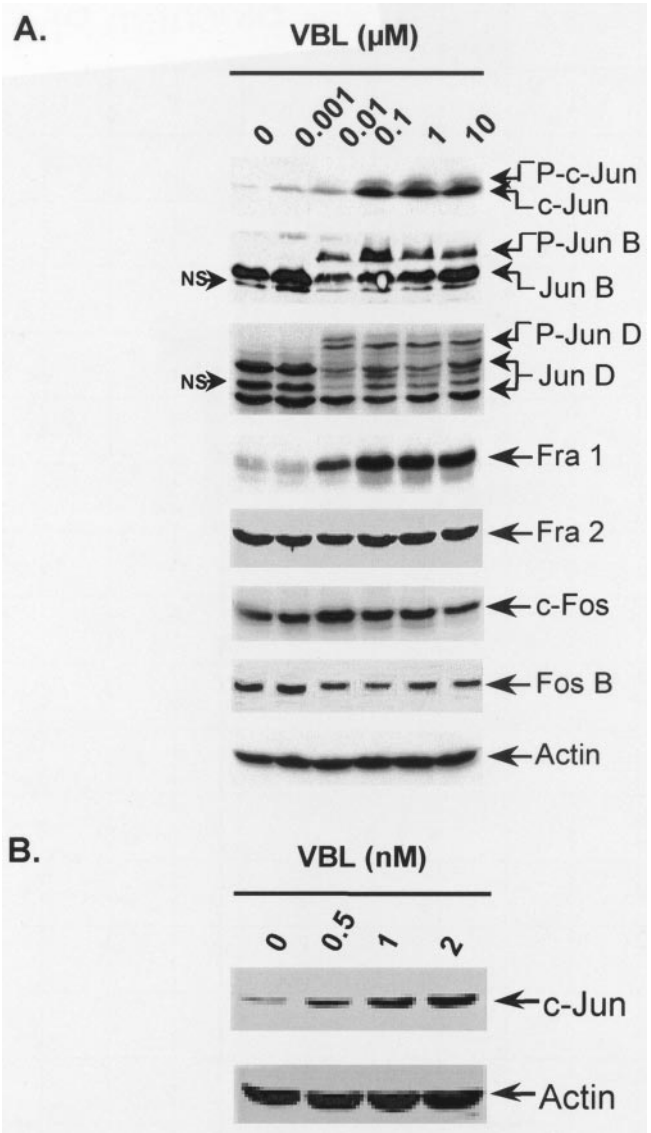

Fig. 1. Concentration-dependent effects of vinblastine on AP-1 proteins. (A) KB-3 cells were untreated or treated with the indicated concentration of vinblastine for 16 hr. Whole cell extracts were prepared, and samples were subjected to SDS–PAGE separation and immunoblotting with the indicated antibodies. P indicates the phosphorylated form. NS indicates non-specific immunoreactivity (i.e. not competed away with the corresponding peptide antigen). (B) c-Jun expression in KB-3 cells treated for 48 hr with the indicated concentrations of vinblastine. Identical results were obtained in an independent experiment. Actin was used as a loading control in both panels.

preabsorbed with the corresponding peptide antigen was used for immunoblotting; it was not detected in nuclear extracts and is, therefore, considered non-specific. The fastest migrating (lower) band also specifically reacted with the Jun D antibody and is also labeled as Jun D for this reason. However, its expression in nuclear extracts was variable, and its apparent molecular mass (34 kDa) is significantly lower than that of authentic Jun D. The identity of this protein remains unclear. It is apparent that 39-kDa Jun D undergoes a mobility shift consistent with phosphorylation at 0.01 μM vinblastine or higher concentrations. The mobility of the non-specific band also shifted in response to vinblastine. In terms of the Fos family, Fra 1 was poorly expressed in control cells, but a robust increase in expression was observed at 0.01 μM vinblastine, increasing at 0.1 μM and higher concentrations. Other family members (Fra 2, c-Fos, and Fos B) were relatively unchanged in response to vinblastine (Fig. 1A). Actin was used as a control and was also unchanged.

The IC_{50} of KB-3 cells for vinblastine in a 4-day cell viability assay was 0.5 nM. To determine whether low but lethal vinblastine concentrations nearer the IC_{50} also affect AP-1 protein expression, c-Jun expression was examined after treating cells for 48 hr with 0.5, 1.0, and 2 nM vinblastine. As shown in Fig. 1B, c-Jun expression was induced in a concentration-dependent manner after exposure to low concentrations of vinblastine.

To confirm that the more slowly migrating immunoreactive bands represent phosphorylated forms of Jun proteins, we prepared nuclear extracts from control and vinblastine-treated cells and treated them with acid phosphatase prior to electrophoresis. Nuclear extracts were used because the whole cell extracts were prepared in a buffer incompatible with phosphatase activity. As shown in Fig. 2, the more slowly migrating forms of Jun B and Jun D were absent following phosphatase treatment, confirming that they were due to phosphorylation, whereas the mobility of the parent bands was unaffected. Similarly, the shifted c-Jun species (P-c-Jun) was also sensitive to phosphatase treatment (data not shown).

To determine the time-dependent effects of vinblastine on AP-1 protein expression, KB-3 cells were treated with 0.1 μM vinblasine, the lowest concentration tested that produced maximum effects (Fig. 1), and whole cell extracts were prepared at regular intervals for up to 36 hr. The results are presented in Fig. 3 and are consistent with, and thus confirm, those of Fig. 1. Expression of c-Jun and Fra 1 was induced starting at about 4 hr of treatment and was maintained for up to 36 hr. Phosphorylation-induced mobility shifts occurred in c-Jun, Jun B, and Jun D beginning at around 10 hr of treatment. Interestingly, Jun B expression eventually decreased until the protein was undetectable. Fra 2, c-Fos, and Fos B remained relatively unchanged even after 36 hr of treatment with vinblastine, and actin was also unchanged. These results show that there is selective alteration in the expression and phosphorylation of AP-1 family proteins by vinblastine in KB-3 cells.

3.2. Caspase 3 activation in relation to alterations in AP-1 proteins

The question arises of whether the vinblastine-induced changes in AP-1 proteins precede the execution phase of apoptosis, or occur later, perhaps as a consequence of apoptosis. This temporal relationship clearly has an important

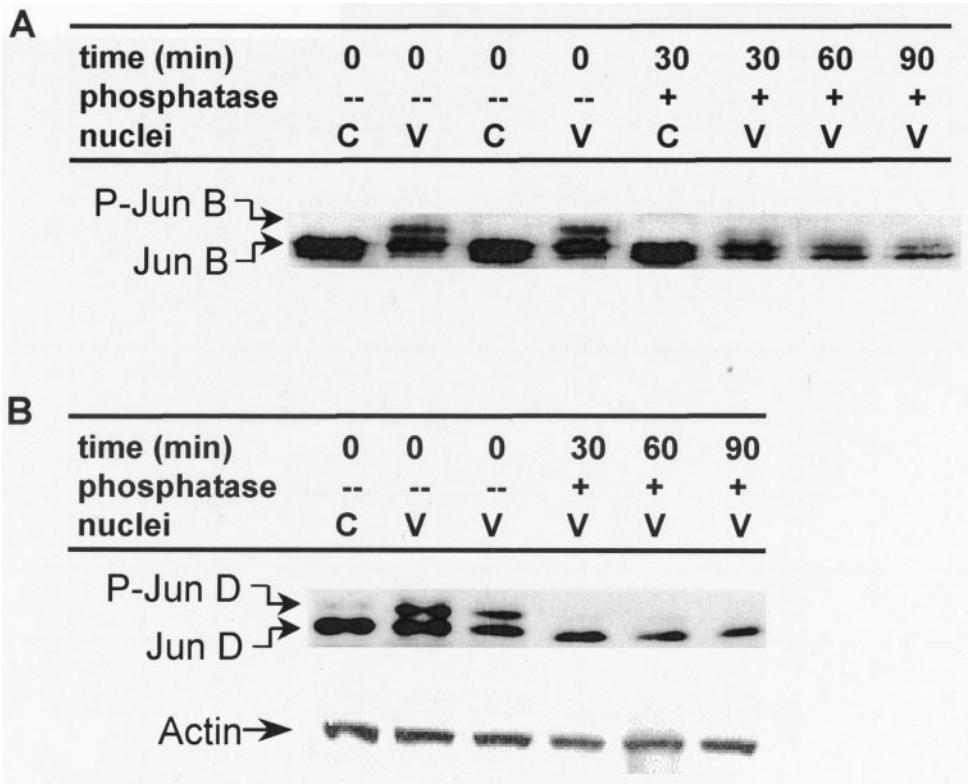

Fig. 2. Mobility shifts due to phosphorylation. Nuclear extracts from control (C) or vinblastine treated (V) (0.1 μM, 16 hr) cells were incubated in the absence (–) or presence (+) of acid phosphatase, for the times indicated. Incubation mixtures containing 10 μg protein/lane were subjected to SDS–PAGE and were immunoblotted for Jun B (panel A) or Jun D (panel B). Actin expression as a control is also shown for samples in panel B.

bearing on the potential role of alterations in AP-1 in the cellular response to vinblastine. Therefore, caspase 3 activity was evaluated by direct enzymatic assay as described in "Materials and methods." As shown in Fig. 4, the increase in caspase 3 activity occurred mainly between 24 and 48 hr after the addition of vinblastine, consistent with previous results showing caspase 3 cleavage at 30 hr and later times [15]. Therefore, alterations in AP-1 are relatively early events occurring before the execution phase of apoptosis. Further studies will be required to demonstrate a functional relationship between AP-1 activation and caspase 3 activation.

3.3. Effect of other microtubule inhibitors on AP-1 protein expression

To determine whether the effects of vinblastine on AP-1 proteins were drug-specific, or induced also by other microtubule inhibitors, KB-3 cells were treated with vinblastine, vincristine, paclitaxel, or colchicine, each at 30 and 100 nM. As shown in Fig. 5A, vincristine, paclitaxel, and colchicine all induced phosphorylation of Jun B and Jun D and expression of Fra 1, to a similar extent as vinblastine, showing that these are general responses to microtubule inhibitors. Interestingly, the microtubule-destabilizing agents vincristine and colchicine, like vinblastine, promoted a robust increase in expression and phosphorylation of c-Jun, whereas the microtubule-stabilizing agent paclitaxel failed to do so. One possible explanation is that c-Jun induction/phosphorylation by paclitaxel was transient rather than sustained and declined at 16 hr. However, time–course experiments revealed that paclitaxel did not induce c-Jun protein expression at any time up to 36 hr (data not shown) in contrast to vinblastine (Fig. 3). To confirm the failure of paclitaxel to stimulate c-Jun phosphorylation, we utilized a phospho-(Ser63)-specific c-Jun antibody, which recognizes the protein phosphorylated at a major JNK site. As shown in Fig. 5B, vinblastine promoted a strong increase in expression of total and phosphorylated c-Jun as indicated by strong immunoreactivity to both antibodies, whereas total and phosphorylated c-Jun remained at basal levels after paclitaxel treatment.

3.4. Effect of vinblastine on AP-1 DNA binding activity and composition

We next determined whether the alterations in AP-1 proteins by vinblastine observed by immunoblotting resulted in changes in AP-1 binding activity or AP-1 complex formation. For this purpose, we used EMSA and supershift analysis. Initially, we confirmed the specificity of the binding assay reagents making use of a nuclear extract from 3T6 Swiss Albino cells, which express c-Jun containing AP-1 complexes (see "Materials and methods"). The mobility of

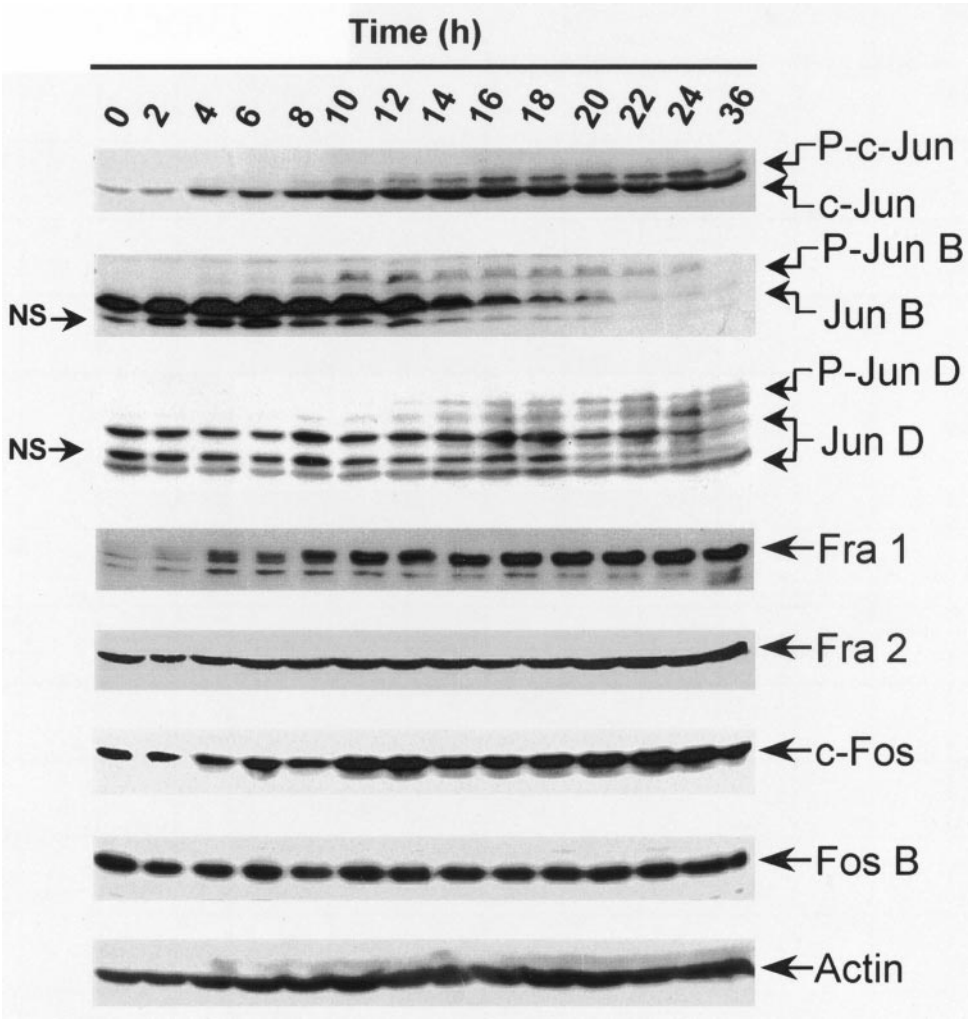

Fig. 3. Time-dependent effects of vinblastine on AP-1 proteins. KB-3 cells were treated with 0.1 μM vinblastine for the times indicated, whole cell extracts were prepared, and samples were subjected to SDS–PAGE separation and immunoblotting with the indicated antibodies. P indicates the phosphorylated form. NS indicates non-specific immunoreactivity. Identical results were obtained in an independent experiment.

the labeled AP-1 probe was shifted in the presence of the 3T6 nuclear extract, and a 100-fold molar excess of wild-type, but not mutant, unlabeled probe competed with binding (Fig. 6A, lanes 1–3). c-Jun antibody included in the binding reaction created a supershift in mobility (lane 4), and the supershifted species was no longer formed if the antibody was first neutralized with the corresponding peptide antigen (lane 5). These results demonstrated the specificity and utility of these reagents for evaluating AP-1 binding and composition.

Nuclear extracts were then prepared from control and vinblastine-treated KB-3 cells. The vinblastine treatment conditions (100 nM, 16 hr) were chosen specifically to correspond to those resulting in maximal changes in AP-1 proteins, based on results in Fig. 3. In the absence of antibodies, AP-1 DNA binding activities were comparable in nuclear extracts from control and treated cells (Fig. 6, panels B and D). This is not unexpected because a major effect of vinblastine is to promote phosphorylation of Jun proteins which is known not to affect DNA binding activity [24], and not all AP-1 proteins expressed may be participating in AP-1 complex formation (see below). Indeed, to answer the question as to the makeup of AP-1 complexes in control cells, and to evaluate possible changes in response to vinblastine, we performed supershift analysis using antibodies from several sources. Figure 6B shows results with antibodies provided by Geneka. In nuclear extracts from control cells, we observed a strong supershift with Jun D antibody, but no supershift with antibodies to c-Jun, Jun B, c-Fos, or Fos B. In nuclear extracts from vinblastine-treated cells, a similar pattern was observed, with the exception of the c-Jun antibody, which produced a supershift (Fig. 6B). The presence of c-Jun in AP-1 complexes from vinblastine-treated, but not control, cells was confirmed using a different c-Jun antibody, from Transduction Laboratories, which proved more effective for this type of analysis (Fig. 6C). Antibodies from Santa Cruz against Fos family proteins were utilized in Fig. 6D. These results show an absence of c-Fos and Fos B in the AP-1 complexes from both extracts, consistent with results using Geneka antibodies (Fig. 6B).

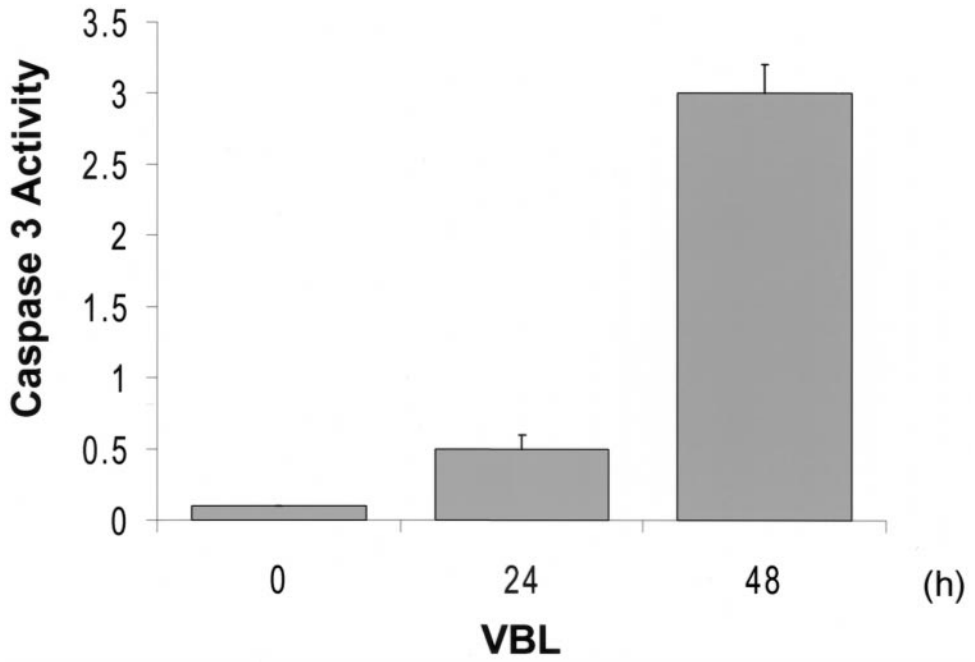

Fig. 4. Caspase 3 activation by vinblastine. KB-3 cells were treated with 0.1 μM vinblastine for the times indicated, and cell lysates were prepared and subjected to caspase 3 assay, as described in "Materials and methods." Assays, in arbitrary units, were performed in triplicate, and results are presented as means ± SD.

Supershifts were observed in the presence of Fra 2 antibody in nuclear extracts from both control and vinblastine-treated cells, and a supershifted band was observed in the presence of Fra 1 antibody in the extract from vinblastine-treated cells but not in the control extract (Fig. 6D). These results indicate that vinblastine, while not altering total AP-1 binding activity, does alter AP-1 composition. In control cells, AP-1 binding activity appears to be mediated primarily by Jun D/Fra 2 heterodimers, and after vinblastine treatment, Jun D/Fra 2 dimers persist and are accompanied by new AP-1 complexes containing c-Jun and Fra 1, consistent with the induction of these latter proteins by vinblastine (Fig. 1).

3.5. Stimulation of AP-1-dependent transcriptional activity by vinblastine

While phosphorylation of the transactivation domains of Jun proteins does not alter their DNA binding properties, such phosphorylation leads to increased transcriptional activity [22–24]. Because a major effect of vinblastine is to stimulate phosphorylation of Jun proteins, we directly evaluated AP-1-dependent transcriptional activity using transient transfection of a luciferase reporter system, as described in "Materials and methods." Treatment with vinblastine, either at 30 or 100 nM, stimulated AP-1 luciferase activity over 3-fold (Fig. 7). The increase was comparable to that obtained with TPA, a well-known activator of AP-1 (Fig. 7).

4. Discussion

In this study, we demonstrated highly selective alterations in specific members of the Jun and Fos family by vinblastine. Major changes included increased expression and phosphorylation of c-Jun, phosphorylation of Jun B and Jun D, and increased expression of Fra 1, whereas c-Fos, Fos B, and Fra 2 were largely unaffected. Antibody supershift analyses revealed that the vinblastine-inducible proteins, c-Jun and Fra 1, were competent to participate in novel AP-1 complex formation, resulting in altered AP-1 composition in treated cells. AP-1-dependent transcriptional activity was increased significantly by vinblastine, consistent with the observed changes in abundance and phosphorylation status of these AP-1 family members. AP-1 modulation by vinblastine was a relatively early event occurring prior to caspase 3 activation.

With the exception of c-Jun, which was not affected by paclitaxel, the same pattern of alterations in AP-1 proteins was observed after exposure of the cells to vincristine, paclitaxel, and colchicine, demonstrating that these are general responses to microtubule inhibitors. The fact that paclitaxel failed to induce c-Jun expression or to stimulate phosphorylation was surprising in view of our previous results using a phospho-(Ser73)-specific c-Jun antibody, which suggested that paclitaxel stimulated the phosphorylation of endogenous c-Jun [15]. We believe the basis for this apparent discrepancy is that the antibody used in the previous study exhibits non-specific immunoreactivity, and recognizes not only c-Jun phosphorylated at Ser73, but also

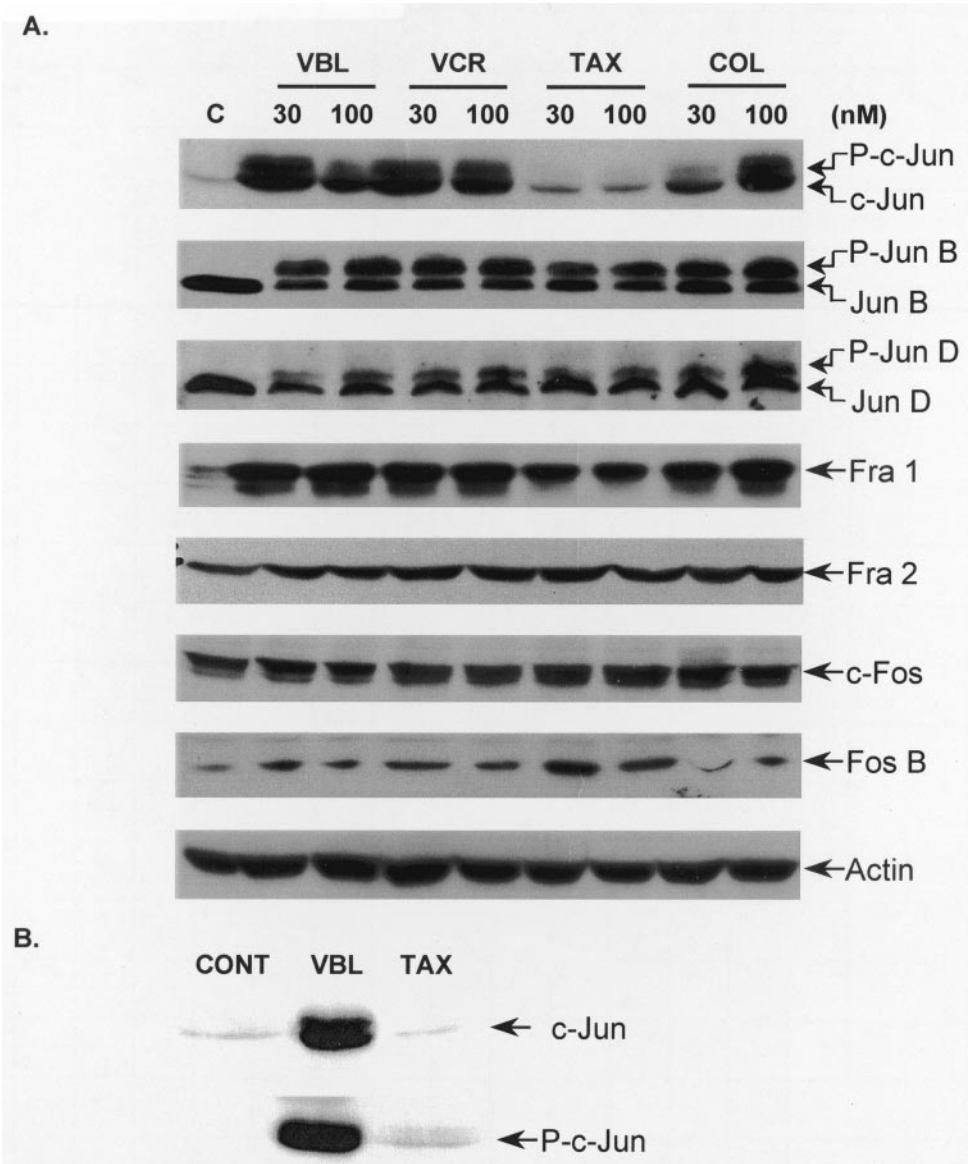

Fig. 5. Effect of microtubule inhibitors on AP-1 protein expression and phosphorylation. (A) KB-3 cells were untreated (C) or treated with 30 or 100 nM vinblastine (VBL), vincristine (VCR), paclitaxel (TAX), or colchicine (COL) for 16 hr. Whole cell extracts (or for Jun D analysis, nuclear extracts) were prepared, and samples were subjected to SDS–PAGE separation and immunoblotting with the indicated antibodies. P indicates the phosphorylated form. (B) Extracts from control (CONT), vinblastine-treated (VBL, 100 nM, 16 hr) or paclitaxel-treated (TAX, 100 nM, 16 hr) cells were subjected to immunoblotting with phosphorylation-independent c-Jun antibody, as in panel A, or with phospho(Ser63)-specific c-Jun antibody (P-c-Jun). These results are representative of three experiments.

other phosphorylated Jun proteins. The positive response in the previous work [15] was likely due, not to c-Jun phosphorylation, but to phosphorylation of Jun D or Jun B, all of which migrate on SDS gels at similar positions. We have confirmed that paclitaxel fails to stimulate c-Jun phosphorylation by using a superior antibody in this study (Fig. 5B). Thus, the microtubule-stabilizing agent paclitaxel differs from the microtubule-destabilizing agents with regard to effects on c-Jun in this system. It is evident that in response to vinblastine, c-Jun phosphorylation and expression increased in a coordinated way (Fig. 3). This was not unexpected because a strong amplification loop is known to exist where c-Jun can regulate its own expression through an AP-1-like site in its promoter that is particularly responsive to activated c-Jun/ATF-2 dimers [24]. Vinblastine increases c-Jun mRNA expression (Berry A and Chambers TC, unpublished observations), indicating that there is a transcriptional or posttranscriptional component to increased c-Jun protein expression. In addition, c-Jun becomes stabilized after phosphorylation by JNK [28], and this may also contribute to the parallel increase in both protein expression and phosphorylation. We have shown previously that paclitaxel strongly activates JNK in KB-3 cells [15]. Therefore, the low level of phosphorylation of c-Jun by paclitaxel (Fig. 5B) is likely related to low substrate expression. The mechanism(s) leading to increased c-Jun expression by vinblas-

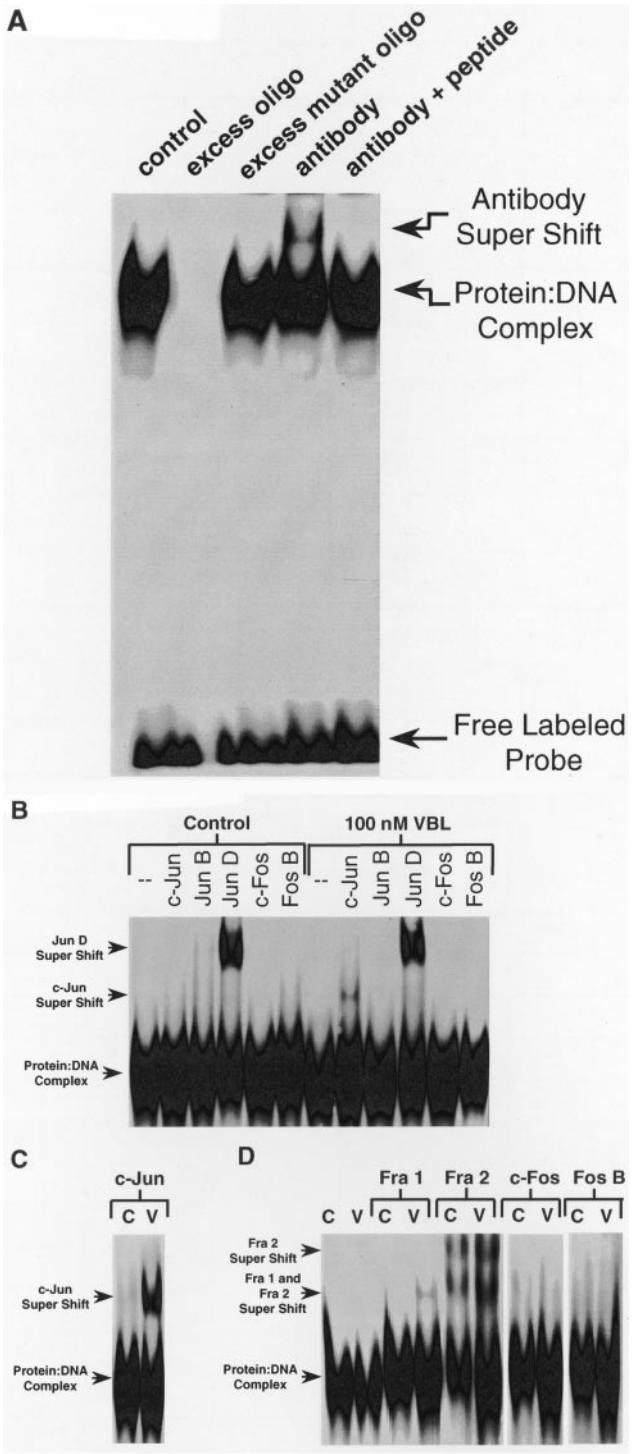

Fig. 6. AP-1 binding and composition by EMSA and supershift. EMSA with a ^{32}P-labeled AP-1 oligonucleotide probe was performed as described in "Materials and methods." (A) Specificity of the reagents was confirmed using a nuclear extract from 3T6 cells with the following additions to the standard binding reaction. Lane 1, no addition; lane 2, 100-fold excess of unlabeled wild-type oligonucleotide probe; lane 3, 100-fold excess of mutated probe; lane 4, c-Jun antibody; and lane 5, c-Jun antibody neutralized with corresponding peptide antigen. The migration positions of the free probe (most of which was washed out of the gel during fixing and drying), protein:DNA complex, and supershifted complex are indicated. (B–D) KB-3 cells were untreated (Control, C) or treated with 100 nM vinblastine for 16 hr (VBL or V), and nuclear extracts were subjected to EMSA in the absence or presence of antibodies to the indicated AP-1 proteins. (B) Antibodies from Geneka. (C) Antibodies from Transduction Laboratories. (D) Antibodies from Santa Cruz. The regions of the gels showing protein:DNA complexes and supershifted complexes are shown. These results were verified in an independent experiment.

tine may be lacking in response to paclitaxel. c-Jun is under complex regulation through both JNK-dependent and -independent pathways [29], and these may be differentially operative in a drug-specific manner. Further work will be required to determine the pathways involved in c-Jun induction by microtubule destabilizers, and the basis for the differential effects of vinblastine and paclitaxel on c-Jun expression.

In this study, we also demonstrated Jun B phosphorylation in response to vinblastine and the other microtubule inhibitors. Following phosphorylation, Jun B levels decreased until the protein was undetectable (Fig. 3). These results are consistent with a recent report, which has shown mitotic phosphorylation and subsequent degradation of Jun B in HeLa cells [30]. Phosphorylation of Jun B was catalyzed by cdc2/cyclin B at Ser23 and Thr150, which triggered protein degradation [30]. Interestingly, the major phosphorylation sites for JNK in the NH_2-terminus of c-Jun and Jun D are not conserved in Jun B [31]; hence, the phosphorylation and degradation of Jun B that we observed in response to vinblastine are likely related to the mitotic phosphorylation described by Bakiri *et al.* [30]. Importantly, Jun B has been described as an antagonist of c-Jun and to exhibit opposing effects on cell proliferation. For example, Jun B represses c-Jun transactivation by forming inactive heterodimers [32] and inhibits c-Jun-mediated transformation [33]. Conversely, c-Jun is needed for normal cell cycle progression, and work with c-jun-/- fibroblasts has shown that a critical role of c-Jun is in negative regulation of p53/p21 [34]. In addition, c-Jun positively regulates cyclin D1 expression, which is required for G_1 progression, and Jun B represses the cyclin D1 promoter [30,34]. Collectively, these findings suggest that a reciprocal relationship exists between Jun B and c-Jun, and that in order for c-Jun to function during the G_1 phase, Jun B, as an antagonist, needs to be degraded. Our results also indicate a reciprocal relationship between Jun B and c-Jun expression in response to vinblastine (Fig. 3), consistent with these observations.

While our results have shown modulation of AP-1 proteins in KB-3 cells by microtubule inhibitors, these findings do not formally address whether these alterations reflect mitotic or apoptotic events. Vinblastine and the other microtubule inhibitors cause arrest at the G_2/M phase of the KB-3 cell cycle, as determined by DNA analysis by flow cytometry and by accumulation of cyclin B1, with subsequent apoptotic cell death as indicated by caspase 3 activation (Fig. 4) and DNA laddering (Fan M, Stone AA, and Chambers TC, unpublished observations). Addition of a microtubule inhibitor to an asynchronous cell culture will,

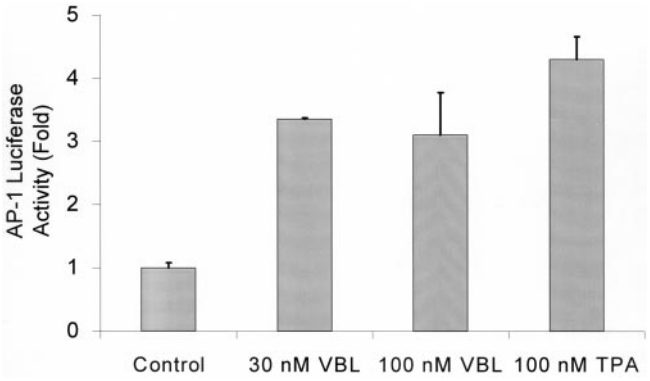

Fig. 7. Activation of AP-1/TRE-dependent transcription by vinblastine. KB-3 cells were transiently transfected with firefly luciferase reporter gene under the control of two copies of TRE (TRE2-Luc) and with control *Renilla* luciferase under the control of a constitutive promoter (pRL-TK-Luc). Cells were then left untreated or were treated for 16 hr with vinblastine (VBL) or TPA as indicated, and luciferase activities were determined. Results (means ± SD, N = 4) are expressed as average relative firefly luciferase activity normalized to *Renilla* activity. See "Materials and methods" for further details. Essentially identical results were obtained in an independent experiment.

over time, generate a mixed population of unaffected, arrested, or apoptotic cells, depending on the position of each cell in the cycle upon drug exposure. This complicates the issue of distinguishing mitotic from apoptotic biochemical events. However, recent results have indicated that a surprising number of cellular responses to microtubule inhibitors, originally thought to reflect apoptotic events, actually appear to represent exaggerated forms of mitotic events. For example, Bcl-2 phosphorylation in response to microtubule inhibitors has been considered a step between microtubule damage and apoptosis [10], but also occurs normally at mitosis [35]. Likewise, JNK activation by microtubule inhibitors, documented as a stress response to microtubule dysfunction by several laboratories [15–19], is now also realized to occur normally at mitosis [20,36]. Another example is Jun B phosphorylation, discussed above, which is likely catalyzed by cdc2/cyclin B at mitosis [30]. Thus, while the exact functions of individual AP-1 proteins in cell cycle control and apoptosis are still being elucidated, aberrant expression or abnormally prolonged phosphorylation of these critical proteins may contribute to apoptotic signaling in response to mitotic arrest.

AP-1 activation by paclitaxel [25] or by taxotere [26] has been described as an early event in the apoptotic response of human B lymphoblasts or gastric cancer cell lines, respectively, suggesting a possible general role not restricted to a specific cell type. Sustained AP-1 activation or the formation of novel AP-1 complexes would represent an attractive mechanism for the activation of specific target genes dictating cell fate in response to microtubule inhibition. In other systems, evidence indicating a role for specific AP-1 target genes in apoptosis is accumulating. In neuronal cells, nerve growth factor withdrawal leads to apoptosis. The JNK/AP-1 pathway clearly plays a critical pro-apoptotic role because inhibition of c-Jun function blocks apoptosis [13]. Recent work has provided a mechanistic basis for these results by showing that apoptosis is triggered by AP-1-dependent FasL transcription [37]. In thymineless death of colon cancer cells, apoptosis is triggered by FasL induction via AP-1 and NF-κB sites in the FasL promoter [38]. AP-1 sites also exist in the promoter regions of other death inducers such as tumor necrosis factor-α [39]. Whether specific pro-apoptotic AP-1 target genes are induced by microtubule inhibitors remains to be determined and is an important goal of future work.

Acknowledgments

We thank Dr. Jonathan Ashwell for providing the TRE luciferase construct. This work was supported by National Institutes of Health Grant CA-75577 (to T.C.C.).

References

[1] Rowinsky EK, Donehower RC. Microtubule-targeting drugs. In: Perry MC, editor. The chemotherapy source book, 2nd ed. Baltimore: Williams & Wilkins, 1998. p. 387–423.
[2] Haskell CM. Antineoplastic agents. In: Haskell CM, editor. Cancer treatment, 4th ed. Philadelphia: W.B. Saunders, 1995. p. 78–165.
[3] Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol 1999; 17:2941–53.
[4] Jordan MA, Thrower D, Wilson L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. Cancer Res 1991;51:2212–22.
[5] Blagosklonny MV, Fojo T. Molecular effects of paclitaxel: myths and reality (a critical review). Int J Cancer 1999;83:151–6.
[6] Wang LG, Liu XM, Kreis W, Budman DR. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. Cancer Chemother Pharmacol 1999;44:355–61.
[7] Lanni JS, Jacks T. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. Mol Cell Biol 1998;18: 1055–64.
[8] Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace AJ, Kohn KW, Fojo T, Bates SE, Rubinstein LV, Anderson NL, Buolamwini JK, van Osdol WW, Monks AP, Scudiero DA, Sausville EA, Zaharevitz DW, Bunow B, Viswanadhan VN, Johnson GS, Wittes RE, Paull KD. An information-intensive approach to the molecular pharmacology of cancer. Science 1997;275:343–9.
[9] Haldar S, Jena N, Croce CM. Inactivation of bcl-2 by phosphorylation. Proc Natl Acad Sci USA 1995;92:4507–11.
[10] Blagosklonny MV, Giannakakou P, El-Deiry WS, Kingston DGI, Higgs PI, Neckers L, Fojo T. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. Cancer Res 1997;57:130–5.
[11] Widmann C, Gibson S, Jarpe MB, Johnson G. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79:143–80.
[12] Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell A, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. Science 2000;288:870–4.
[13] Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–31.
[14] Osborn MT, Chambers TC. Role of the stress-activated/c-Jun NH_2-terminal protein kinase pathway in the cellular response to Adriamy-

cin and other chemotherapeutic drugs. J Biol Chem 1996;271:30950–5.
[15] Stone AA, Chambers TC. Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK, and p38) signaling pathways in human KB-3 carcinoma cells. Exp Cell Res 2000;254:110–9.
[16] Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 1998;273: 4928–36.
[17] Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H, Wimalasena J. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun-N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J Biol Chem 1999;274:8208–16.
[18] Lee L, Li G, Templeton DJ, Ting JPY. Paclitaxel (taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH_2-terminal kinase (JNK/SAPK). J Biol Chem 1998;273: 28253–60.
[19] Shtil AA, Mandlekar S, Yu R, Walter RJ, Hagen K, Tan T-H, Roninson IB, Kong A-NT. Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. Oncogene 1999;18:377–84.
[20] Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. Mol Cell Biol 1999;19:8469–78.
[21] Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA, Chambers TC. Vinblastine-induced phosphorylation of Bcl-2 and Bcl-X_L is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. J Biol Chem 2000;275:29980–5.
[22] Karin M, Liu Z-G, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240–6.
[23] Wisdom R. AP-1: One switch for many signals. Exp Cell Res 1999; 253:180–5.
[24] Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995;270:16483–6.
[25] Amato SF, Swart JM, Berg M, Wanebo HJ, Mehta SR, Chiles TC. Transient stimulation of the c-Jun-NH_2-terminal kinase/activator protein 1 pathway and inhibition of extracellular signal-regulated kinase are early effects in paclitaxel-mediated apoptosis in human B lymphoblasts. Cancer Res 1998;58:241–7.
[26] Kim R, Ohi Y, Inoue H, Toge T. Taxotere activates transcription factor AP-1 in association with apoptotic cell death in gastric cancer cell lines. Anticancer Res 1999;19:5399–406.
[27] Shen DW, Cardarelli C, Hwang J, Cornwell M, Richert N, Ishii S, Pastan I, Gottesman MM. Multiple drug-resistant human KB carcinoma cell lines independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins. J Biol Chem 1986;261:7762–70.
[28] Fuchs SY, Fried VA, Ronai Z. Stress-activated kinases regulate protein stability. Oncogene 1998;17:1483–90.
[29] Marinissen MJ, Chiariello M, Pallante M, Gutkind JS. A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun promoter: a role for c-Jun NH_2-terminal kinase, p38s, and extracellular signal-regulated kinase 5. Mol Cell Biol 1999;19:4289–301.
[30] Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. EMBO J 2000;19:2056–68.
[31] Kallunki T, Deng T, Hibi M, Karin M. c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 1996;87:929–39.
[32] Deng T, Karin M. JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. Genes Dev 1993;7:479–90.
[33] Schütte J, Viallet J, Nau M, Segal S, Fedorko J, Minna J. *jun-B* inhibits and c-*fos* stimulates the transforming and *trans*-activating activities of c-*jun*. Cell 1989;59:987–97.
[34] Schreiber M, Kolbus A, Piu F, Szabowski A, Mohle-Steinlein U, Tian J, Karin M, Angel P, Wagner EF. Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev 1999;13:607–19.
[35] Ling Y-H, Tornos C, Perez-Soler R. Phosphorylation of Bcl-2 is a marker of M phase events and not a determinant of apoptosis. J Biol Chem 1998;273:18984–91.
[36] Fan M, Du L, Stone AA, Gilbert KM, Chambers TC. Modulation of mitogen-activated protein kinases and phosphorylation of Bcl-2 by vinblastine represent persistent forms of normal fluctuations at G_2-M. Cancer Res 2000;60: 6403–7.
[37] Le-Niculescu H, Bonfoco E, Kasuya Y, Claret F-X, Green DR, Karin M. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol 1999;19:751–63.
[38] Harwood FG, Kasibhatla S, Petak I, Vernes R, Green DR, Houghton JA. Regulation of FasL by NF-κB and AP-1 in Fas-dependent thymineless death of human colon carcinoma cells. J Biol Chem 2000; 275:10023–9.
[39] Rhoades KL, Golub SH, Economou JS. The regulation of the human tumor necrosis factor α promoter region in macrophage, T cell, and B cell lines. J Biol Chem 1992;267: 22102–7.