Vinblastine-induced Phosphorylation of Bcl-2 and Bcl-X\textsubscript{L} Is Mediated by JNK and Occurs in Parallel with Inactivation of the Raf-1/MEK/ERK Cascade*

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Vinblastine is an important drug used for treating breast and testicular carcinomas, Hodgkin’s and non-Hodgkin’s lymphomas, Kaposi’s sarcoma, and several other cancers (reviewed in Refs. 1 and 2). The cellular effects of vinblastine are mediated primarily by binding to tubulin subunits, inhibiting tubulin polymerization and leading to disruption of microtubule dynamics. Like other microtubule inhibitors such as paclitaxel, the effects of vinblastine are concentration-dependent. Very low concentrations in the subnanomolar range cause growth inhibition without mitotic arrest, whereas very high concentrations in the 1–100 \( \mu \text{M} \) range cause nonspecific immediate effects in non-proliferating as well as proliferating cells. At clinically relevant concentrations (5–500 nM range), the cytotoxicity of vinblastine appears to be associated with G\textsubscript{2}/M phase arrest, followed by apoptotic cell death (1, 2).

The signaling pathways responsible for promoting cell death in response to microtubule inhibitors are under intense investigation. As first shown for paclitaxel (3), a common feature of microtubule inhibitors is to stimulate phosphorylation of Bcl-2 (4). Phosphorylation occurs in an unstructured loop and involves several sites, the principal ones being Ser\textsuperscript{32} and Ser\textsuperscript{347} (reviewed in Ref. 5). Uncertainty still exists whether phosphorylation increases or decreases the anti-apoptotic function of Bcl-2. Most data support the original hypothesis (3) and suggest that phosphorylation inactivates Bcl-2, thus promoting apoptosis, possibly by freeing Bax from Bcl-2/Bax dimers (6). Evidence has also been presented that Bcl-2 phosphorylation is associated with normal mitotic progression (7, 8). The anti-apoptotic Bcl-2 relative, Bcl-X\textsubscript{L}, also undergoes phosphorylation in response to microtubule inhibition (9), but the functional consequences of this modification remain to be established.

Because of the universal nature of Bcl-2 phosphorylation and its potential role in apoptotic and mitotic regulation, interest has focused on the specific signal transduction pathways and protein kinases involved. Bcl-2 phosphorylation in response to microtubule inhibitors occurs concurrently with hyperphosphorylation and apparent activation of Raf-1 (4, 10). However, Raf-1 does not appear to directly phosphorylate Bcl-2 (11). Other kinases implicated in Bcl-2 phosphorylation include Cdc2/cyclin B1, cAMP-dependent protein kinase, and mitogen-activated protein kinases (MAPKs).\textsuperscript{1} Bcl-2 is not an \textit{in vitro} substrate of Cdc2 and does not co-immunoprecipitate with Cdc2/cyclin B1 complexes (8). One report described stimulation of Bcl-2 phosphorylation by cAMP-dependent protein kinase (12), but other studies have failed to demonstrate Bcl-2 phosphorylation following cAMP-dependent protein kinase activation (13). Among MAPKs, c-Jun NH\textsubscript{2}-terminal kinase (JNK) has been shown to phosphorylate Bcl-2 in COS-7 cells in the presence of constitutively active Rac1 (14). However, other reports have suggested that JNK is not involved in Bcl-2 phosphorylation induced by microtubule inhibitors. For example, JNK activation following microtubule inhibition can occur

**Note**

1 The abbreviations used are: MAPKs, mitogen-activated protein kinases; JNK, c-Jun NH\textsubscript{2}-terminal protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MEKK, MEK kinase; GST, glutathione \( S \)-transferase; FPLC, fast protein liquid chromatography; JIP-1, JNK-interacting protein-1; MKK, MAPK kinase; ASK, apoptosis signal-regulating kinase; SEK, stress-activated protein kinase/ERK kinase.
much earlier than Bcl-2 phosphorylation (15, 16); upstream regulators of the JNK pathway failed to induce Bcl-2 phosphorylation (17); and inhibition of JNK by dominant-negative proteins did not affect paclitaxel-induced Bcl-2 phosphorylation (17). No evidence currently exists on the sites of phosphorylation or kinases involved in Bcl-X<sub>L</sub> phosphorylation in response to microtubule inhibition, although JNK has been implicated in Bcl-X<sub>L</sub> phosphorylation in response to ionizing radiation (18).

We originally demonstrated JNK activation by vinblastine (19) and extended this observation to show that microtubule inhibitors activate components of the JNK pathway while inactivating ERK and p38 in KB-3 cells (20). In the present study, we sought to determine whether JNK is responsible for vinblastine-induced phosphorylation of Bcl-2 and Bcl-X<sub>L</sub>. To achieve this goal, we inhibited the expression of JNK1 and JNK2 isoforms by using highly specific phosphorothioate antisense oligonucleotides. The antisense oligonucleotides specifically and effectively depleted JNK isoform expression and in combination inhibited JNK-mediated phosphorylation of endogenous c-Jun and ATF-2 in intact cells treated with vinblastine. In addition, the antisense oligonucleotides very effectively inhibited vinblastine-induced phosphorylation of Bcl-2 and also inhibited Bcl-X<sub>L</sub> phosphorylation. Moreover, we have found that the characteristic hyperphosphorylation of Raf-1 that occurs in response to vinblastine corresponds not to activation, but rather to inactivation of Raf-1 enzymatic activity for MEK1.

These results suggest that a JNK-dependent pathway regulates vinblastine-induced phosphorylation of Bcl-2 and Bcl-X<sub>L</sub>, which occurs in parallel with inactivation of Raf-1/MEK/ERK.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies to JNK1, JNK2, ERK1/2, Raf-1, and Bcl-2 and the GST-c-Jun-(1–79) fusion protein substrate were obtained from Santa Cruz Biotechnology, and antibody to Bcl-X<sub>L</sub> was from Transduction Laboratories. Phospho-specific polyclonal antibodies to ERK, c-Jun, and AFT-2 were obtained from New England Biolabs, Inc., and antibody to T7 tag was from Novagen. GST-MEKi(K97A)-agarose beads were from Stressgen Biotech Corp. I<sup>γ-32P</sup>ATP was obtained from Amersham Pharmacia Biotech. Fetal bovine serum was from HyClone Laboratories, and other cell culture reagents were from Life Technologies, Inc. Unless otherwise stated, other reagents were from Sigma.

**Preparation of Cell Extracts, Acid Phosphatase Treatment, and Immunoblotting**—Whole cell and nuclear extracts were prepared, and fractions of 0.4 ml were collected. Fractions were assayed for JNK activity as described above.

**Results**

**JNK1 and JNK2 Isoforms Are Activated by Vinblastine**—Vinblastine and other microtubule inhibitors, which induce G<sub>2</sub>/M arrest and apoptosis in many cell types, have been shown to activate JNK (16, 19, 20) and to induce phosphorylation of Bcl-2 (3, 4) and Bcl-X<sub>L</sub> (9). In the present study, we used an antisense oligonucleotide approach to determine whether JNK is responsible for vinblastine-induced phosphorylation of these Bcl-2 family proteins. Using a standard immunocomplex assay (20), control KB-3 cells exhibited barely detectable JNK activity, whereas cells treated with vinblastine had readily detectable JNK activity (Fig. 1A). To determine whether vinblastine differentially activated JNK isoforms in KB-3 cells, we subjected cell extracts from treated cells to fractionation on a Mono Q column as described under “Experimental Procedures.” Two peaks of JNK activity were resolved upon elution with a salt gradient (Fig. 1B). Aliquots of fractions encompassing both peaks were subjected to immunoblotting with anti-JNK1 polyclonal antibody (sc-474) and anti-JNK2 monoclonal antibody (sc-7345) (Fig. 1C). The anti-JNK1 antibody recognized proteins of 46 kDa (major) and 54 kDa (minor) in the first peak, whereas the anti-JNK2 antibody failed to recognize any protein in peak 1. Conversely, JNK2 immunoreactivity (at 54 kDa) was observed across peak 2. A chromatographically distinct form of p46<sup>JNK1</sup> contributes to the leading edge of peak 2. These results indicate that both major forms of JNK in KB-3 cells, namely p46<sup>JNK1</sup> and p54<sup>JNK2</sup>, are activated by vinblastine.

**Inhibition of JNK Expression by Antisense Oligonucleotides**—To determine whether we could specifically inhibit JNK isoform expression, we used antisense oligonucleotides directed toward JNK1 and JNK2. Cells were transfected for 4.5 h with JNK1 or JNK2 antisense oligonucleotides or the corresponding scrambled oligonucleotides in the presence of Lipofectin as
described under “Experimental Procedures.” Treatment with AS-JNK1 or AS-JNK2 led to reduced expression of the corresponding JNK isoforms in a dose-dependent manner (Fig. 2A), which was maximal 60–68 h after removal of the oligonucleotides (data not shown). In addition, AS-JNK1 specifically inhibited the expression of JNK1, but did not affect the expression of JNK2, p38, or ERK; and likewise, AS-JNK2 showed strict specificity (Fig. 2B). Scrambled oligonucleotides were without effect. Because JNK1 and JNK2 often play redundant roles, and both were activated by vinblastine, we investigated whether we could simultaneously inhibit their expression. As shown in Fig. 2C, a combination of 150 nM AS-JNK1 plus 150 nM AS-JNK2 inhibited the expression of both JNK1 and JNK2. Although the effectiveness of AS-JNK1 was reduced slightly in the presence of AS-JNK2, the overall reduction in total JNK expression with this combination was ~85% as determined by densitometric scanning. Higher oligonucleotide concentrations (~400 nM total), whether antisense or scrambled, generated nonspecific toxicity, which may also have been due to the higher concentration of Lipofectin required for efficient uptake; but 300 nM total oligonucleotide was well tolerated with no signs of cytotoxicity up to 96 h. Therefore, we chose as an optimum condition for the remaining experiments a combination of 150 nM AS-JNK1 and 150 nM AS-JNK2, with 150 nM Scr-JNK1 and 150 nM Scr-JNK2 as the control, with cells harvested 60 h post-transfection.

Effect of Antisense Oligonucleotides on Vinblastine-induced Phosphorylation of c-Jun, ATP-2, Bel-2, Bel-X<sub>L</sub>, and Raf-1—Cells were transfected with AS-JNK1/2 or Scr-JNK1/2 for 4.5 h and maintained for 44 h to allow JNK protein levels to diminish. Cells were then either untreated or treated with 30 nM vinblastine for 1 h, followed by incubation in drug-free medium for 15 h, and harvested (60 h post-transfection). JNK-mediated NH<sub>2</sub>-terminal phosphorylation of endogenous c-Jun and ATP-2 was evaluated using appropriate phospho-specific antibodies, and Bel-2, Bel-X<sub>L</sub>, and Raf-1 phosphorylation was examined by immunoblotting. As shown in Fig. 3A, the vinblastine-induced phosphorylation of c-Jun and ATP-2, observed under control conditions in the presence of scrambled oligonucleotides, was inhibited by AS-JNK1/2. Vinblastine-induced Bcl-2 phosphorylation, characterized by the presence of several immunoreactive bands of slower mobility, was also inhibited by AS-JNK1/2 treatment. The extent of inhibition of c-Jun, ATP-2, and Bcl-2 phosphorylation by the JNK antisense oligonucleotides was 75–85% as determined by densitometry (Fig. 3C). Immunoblotting with anti-Bcl-X<sub>L</sub> antibody showed two bands of 30 and 28 kDa in extracts from control cells; and after vinblastine treatment, the 30-kDa but not the 28-kDa species underwent a mobility shift (Fig. 3A). A doublet for Bel-X<sub>L</sub> at 28–30 kDa, with vinblastine-mediated phosphorylation of the higher molecular mass species, has been described previously in studies of several other cell lines (9). The identity of the 28-kDa species is currently uncertain. The vinblastine-induced mobility shift of 30-kDa Bel-X<sub>L</sub> was partially reversed by AS-JNK1/2 such that both unphosphorylated and phosphorylated species were present, corresponding to 65% inhibition of Bel-X<sub>L</sub> phosphorylation (Fig. 3C). Importantly, the phosphorylation-induced mobility shift of Raf-1 by vinblastine was completely unaffected by AS-JNK1/2, showing that the oligonucleotides exhibit selective effects on phosphorylation and indicating that Raf-1 phosphorylation is not dependent on JNK signaling. The expression of JNK1 and JNK2 in these samples is also shown in Fig. 3A to confirm reduced expression by AS-JNK1/2 under the experimental conditions. The gel mobility shifts in Bcl-2, Bel-X<sub>L</sub>, and Raf-1, as well as immunoreactivity with the anti-phospho-c-Jun antibody, were lost after treatment with extracts of acid phosphatase, confirming they are due to phosphorylation (Fig. 3B; data not shown). Essentially identical results to those shown in Fig. 3A were obtained in two additional experiments, and quantitation of the results from these studies is presented in Fig. 3C.

JIP-1 Expression Distinguishes Nuclear and Non-nuclear JNK Signaling—The results presented above suggest that in response to vinblastine, activated JNK phosphorylates both nuclear (c-Jun, ATF-2) and non-nuclear (Bel-2, Bel-X<sub>L</sub>) substrates. JIP-1 was originally identified as an inhibitor of JNK signaling (22) and is now known to act as a scaffold coordinat- ing components in the mixed lineage kinase/MKK7/JNK module (23). When overexpressed, JIP-1 causes cytoplasmic retention of JNK and prevents its access to nuclear targets (22). We prepared cells stably expressing JIP-1 to determine whether such an approach would selectively block nuclear versus non-nuclear JNK substrate phosphorylation. Two independent transfectants were chosen, JIP-1-1 and JIP-1-37, which expressed comparable levels of JIP-1 as determined by T7 tag immunoreactivity (Fig. 4). When cells were treated with vinblastine, JIP-1 underwent a mobility shift consistent with its phosphorylation. JIP-1 has previously been shown to be phosphorylated by JNK when expressed in Chinese hamster ovary cells (22). Vinblastine-induced phosphorylation of c-Jun and ATF-2 was inhibited in both JIP-1 transfectants, whereas Raf-1, Bel-2, and Bel-X<sub>L</sub> phosphorylation was unaffected (Fig.
4). These results are consistent with the known mechanism of action of overexpressed JIP-1 and indicate that JNK access to nuclear substrates is specifically restricted in the presence of overexpressed JIP-1.

**Bcl-2/Bcl-XL Phosphorylation Occurs in Parallel with Inactivation of the Raf-1/MEK/ERK Pathway**—Despite the fact that microtubule inhibitors induce phosphorylation of both Raf-1 and Bcl-2, the link between these parameters has remained unclear. To determine whether the mobility shift in Raf-1 parallels changes in Raf-1 activity, we subjected extracts from control and vinblastine-treated cells to Raf-1 immunocomplex assay as described under “Experimental Procedures.” Vinblastine-induced phosphorylation of Raf-1, Bcl-2, and Bcl-XL was observed as before (Fig. 3); and in addition, we observed inactivation of ERK as shown by loss of expression of phosphorylated/activated ERK1/2 versus unchanged total ERK1/2 expression (Fig. 5A). Thus, Raf-1 phosphorylation coincides with inactivation of ERK after vinblastine treatment. Direct immunocomplex assay of Raf-1 was performed with GST-MEK as substrate. As shown in Fig. 5B, in complete reaction mixtures performed in duplicate, Raf-1 enzymatic activity for MEK substrate was observed only in extracts from untreated cells, not in extracts from vinblastine-treated cells. Appropriate controls were performed and showed only background phosphorylation when anti-Raf-1 antibody was omitted during immunoprecipitation or when substrate was omitted from the reaction mix-
tecture (Fig. 5B). These results demonstrate that vinblastine-induced Raf-1 phosphorylation coincides with inactivation of both Raf-1 and ERK.

**DISCUSSION**

The signals that direct cells toward an apoptotic pathway following G2/M phase arrest induced by microtubule inhibitors are not known. However, the findings that these agents induce phosphorylation of the anti-apoptotic protein Bcl-2 and that phosphorylation may disable Bcl-2 function suggest a potential mechanism. The identification of the protein kinase(s) responsible for this type of Bcl-2 phosphorylation is therefore of critical importance for our understanding of mitotic and apoptotic regulation. In this study, we showed that vinblastine-induced phosphorylation of endogenous Bcl-2 is almost completely inhibited by antisense oligonucleotides targeting JNK1 and JNK2. The antisense oligonucleotides utilized were highly specific for inhibition of JNK isoform expression and effectively inhibited vinblastine-induced phosphorylation of two known JNK substrates, c-Jun and ATF-2. These results provide compelling evidence that the JNK pathway is responsible for Bcl-2 phosphorylation in response to vinblastine.

**Fig. 4. Stable expression of JIP-1 inhibits nuclear JNK substrate phosphorylation.** Vector-transfected KB-3 cells or two independent lines stably expressing JIP-1 were either untreated or treated with 30 nM vinblastine (VBL) for 1 h, followed by incubation in drug-free medium for 15 h, and harvested. Cell extracts were prepared and subjected to immunoblotting with antibody to T7 tag to detect JIP-1 or with antibody to phospho-c-Jun, phospho-ATF-2, Raf-1, Bcl-2, or Bcl-XL. Phosphorylated species are indicated by $P$.

**Fig. 5. Vinblastine-induced Bcl-2 and Bcl-XL phosphorylation occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade.** KB-3 cells were either untreated (Control) or treated with 30 nM vinblastine (VBL) for 1 h, followed by incubation in drug-free medium for 15 h, and harvested. Cell extracts were prepared and subjected to immunoblotting with phospho-specific or phosphorylation-independent ERK1/2 antibodies (A) and to Raf-1 immunocomplex assay (B) as described under “Experimental Procedures.” In B, anti-Raf-1 antibody (Ab) or MEK1 substrate was omitted as indicated, and complete reactions were performed in duplicate. An autoradiograph is shown. Phosphorylated species are indicated by $P$. Molecular mass standards (in kilodaltons) are indicated on the left.

The results are consistent with other reports that have used independent approaches to implicate JNK in Bcl-2 phosphorylation. In an earlier study, Maundrell et al. (14) showed that JNK phosphorylated Bcl-2 both in vitro and in Bcl-2-overexpressing COS-7 cells that coexpress JNK and active Rac1. Based on mutagenesis and phosphopeptide sequence analysis, four sites (Thr$^{56}$, Ser$^{70}$, Thr$^{74}$, and Ser$^{87}$) were identified that are present in a putative unstructured flexible loop. Each of these sites occurs NH$_2$-terminal to a proline residue, a well established recognition determinant for MAPKs (24). Srivastava et al. (25) demonstrated paclitaxel-mediated phosphorylation of Bcl-2 at Ser$^{70}$ in breast cancer cells that was inhibited by dominant-negative JNK. A very recent study also concluded that JNK is responsible for paclitaxel-induced Bcl-2 phosphorylation in Jurkat cells overexpressing Bcl-2 (26). Major sites were identified as Thr$^{69}$, Ser$^{70}$, and Ser$^{87}$. Ser$^{70}$ was also found as a major site of Bcl-2 phosphorylation in G$_2$/M phase enriched cell populations (26), and other studies have also suggested that Bcl-2 phosphorylation occurs during normal mitotic progression (7, 8). These results collectively suggest the intriguing possibility that JNK activation by microtubule inhibitors represents a persistent form of normal mitotic enzyme activation, with Bcl-2 as a key target.

Our data contrast with several reports indicating that kinases other than JNK catalyze Bcl-2 phosphorylation in response to microtubule inhibition. In one study, evidence against a role for JNK was based on different kinetics of JNK activation and Bcl-2 phosphorylation (15). In another report, it was concluded that JNK was not responsible for Bcl-2 phosphorylation because transient expression of putative upstream components, Ha-Ras or MEKK1, failed to induce Bcl-2 phosphorylation in UP293 cells (13). However, the expression of these plasmids may not imitate the appropriate signaling pathway for Bcl-2 phosphorylation by JNK. In addition, many stimuli that activate JNK such as DNA-damaging agents fail to induce phosphorylation of Bcl-2 (e.g. Ref. 4), suggesting that substrate accessibility may be a key factor enabling JNK to phosphorylate Bcl-2 under appropriate cellular conditions. Further evidence against a role for JNK was suggested by transient expression of a combination of dominant-negatives to components in the JNK pathway, namely dnASK1, dnSEK1, and dnJNK1, which failed to inhibit paclitaxel-induced Bcl-2 phosphorylation in ovarian carcinoma cells (17). Importantly, however, Yamamoto et al. (26) reported a similar result, but
found that a different combination, namely dnASK1, dnMKK7, and dnJNK1, was very effective in inhibiting paclitaxel-induced Bcl-2 phosphorylation. These results suggest that very specific three-kinase modules may be involved in Bcl-2 phosphorylation and that only specific dominant-negative combinations are inhibitory. Based on the sequences around the identified phosphorylation sites in the loop region of Bcl-2 (14, 25, 26), direct phosphorylation by AMP-dependent protein kinase (12) or protein kinase C (27) is also unlikely because proximal basic residues required by these kinases (24) are lacking.

Bcl-XL was also phosphorylated in KB-3 cells after vinblastine treatment (Fig. 2). Poruchynsky et al. (9) previously reported Bcl-XL phosphorylation in several malignant cells lines in response to paclitaxel and vinblastine. Our finding that Bcl-XL phosphorylation is partially inhibited by antisense oligonucleotides to JNK (Fig. 3) suggests that JNK may at least in part be responsible. Further work will be required to substantiate this conclusion. Although the sites phosphorylated in response to microtubule inhibitors are presently unknown, Kharbanda et al. (18) recently reported JNK-mediated phosphorylation of Thr47 and Thr15 in Bcl-XL after exposure of U937 cells to ionizing radiation. A non-phosphorylatable form of Bcl-XL was more effective than the wild type in blocking apoptosis (18), suggesting, perhaps like Bcl-2, that phosphorylation inhibits its anti-apoptotic activity.

In addition to phosphorylation of Bcl-2 and Bcl-XL, microtubule inhibitors characteristically cause a hyperphosphorylation of Raf-1. A major question is whether Raf-1 hyperphosphorylation is activating and, if so, whether Bcl-2 phosphorylation requires Raf-1 activation. Although many reports have demonstrated a phosphorylation-induced mobility shift in Raf-1 after microtubule disruption (4, 9, 10, 13), Raf-1 enzymatic activity remains to be determined whether the functional consequences of vinblastine-induced phosphorylation of Bcl-2, Bcl-XL, c-Jun, and ATF-2 are complementary or oppositional. Thus, a major challenge will be to determine how these diverse downstream signals are coordinated to allow cells to respond appropriately to microtubule inhibition.

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