0026-895X/01/5902-254–262$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:254–262, 2001

Vol. 59, No. 2
143/881149
Printed in U.S.A.

Involvement of Asp-Glu-Val-Asp-Directed, Caspase-Mediated Mitogen-Activated Protein Kinase Kinase 1 Cleavage, c-Jun N-Terminal Kinase Activation, and Subsequent Bcl-2 Phosphorylation for Paclitaxel-Induced Apoptosis in HL-60 Cells

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Received March 10, 2000; accepted October 22, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Paclitaxel is a novel anticancer drug that has demonstrated efficacy toward treating several malignant tumor types. Here, we demonstrate that c-Jun NH_2-terminal kinase (JNK), but not p38 mitogen-activated protein kinase or extracellular signal-regulated kinase 1/2, was persistently activated by paclitaxel or other microtubule-damaging agents within human leukemia HL-60 cells. Overexpression of a dominant-negative mutant, mitogen-activated protein kinase kinase 1 (MEKK1-DN) or treatment with JNK-specific antisense oligonucleotide prevented paclitaxel-induced JNK activation, Bcl-2 phosphorylation and apoptosis. Furthermore, we found that the full-length MEKK1 was cleaved to a 91-kDa carboxyl-terminal fragment at the earlier time of apoptosis induced by microtubule-damaging agents. This cleavage, however, occurred consistently with JNK activation and Bcl-2 phosphorylation, but preceded DNA fragmentation in cells in response to paclitaxel activity. The caspase inhibitor Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO), but not Ac-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO), effectively blocked MEKK1 cleavage, JNK activation, Bcl-2 phosphorylation, and subsequent apoptosis. Subcellular fractionation revealed that the 91-kDa C-terminal MEKK1 fragment was translocated to cytosol. Notably, the MEKK1 fragment could be coimmunoprecipitated with anti-JNK antibodies, suggesting that a signaling complex of C-terminal MEKK1/stress-activated protein kinase/extracellular-signal regulated kinase 1/JNK formed during apoptosis induced by microtubule-damaging agents. Taken together, our results suggest that disruption of cytoarchitecture by paclitaxel triggers a novel apoptosis-signaling pathway, wherein an active DEVD-directed caspase (DEVDase) initially cleaves MEKK1to generate a proapoptotic kinase fragment that is able to activate JNK and subsequent Bcl-2 phosphorylation, finally eliciting cell death.

c-Jun N-terminal kinase (JNKs), known as stress-activated protein kinases (SAPKs), is a member of the family of mammalian mitogen-activated protein (MAP) kinase that mediates intracellular signals originated from diverse extracellular stimuli, including growth factors, cytokines, UV light, heat shock, and a variety of anti-cancer drugs (Chen et al., 1996; Osborn and Chambers, 1996; Verheij et al., 1996). The activation of JNK involves a protein kinase cascade wherein mitogen-activated protein kinase kinase-1 (MEKK1) (Lange-Carter et al., 1993) activates SAPK kinase-1 (SEK1) (Yan et al., 1994) and ultimately activates JNK. The activation of the JNK cascade in cells leading to a wide range of cellular functions including inflammation, cell growth, and cell death. Recently, a number of studies have referred to extensive efforts attempting to decipher the role of JNK in apoptosis. Various well known chemotherapeutic drugs, such as doxorubicin, vinblastine, VP-16, camptothecin, and paclitaxel have been demonstrated to be capable of activating JNK (Seimiya et al., 1997), these drugs being critical for the apoptosis-triggering program for different cell lines. The expression of a dominant-negative mutant of MEKK1 or JNK prevented the induction of apoptotic cell death by UV-C, γ-irradiation, or anticancer drugs (Faris et al., 1996). These observations suggest an essential role for JNK in the regulation of apoptosis induced by diversified stimuli.

This work was supported by National Science Council of Taiwan Grant NSC 89-2320-B-002-015.

ABBREVIATIONS: JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAP, mitogen-activated protein; MEKK, mitogen-activated protein kinase kinase; SAPK, stress-activated protein kinase; SEK, stress-activated protein kinase kinase; DEVDase, Asp-Glu-Val-Asp-directed caspase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; DN, dominant negative; Dex, dexamethasone; VCR, vincristine; VBL, vinblastine; ERK, extracellular signal-regulated kinase.

Once activated, JNK phosphorylates several transcription factors, including c-Jun, ATF-2, and Elk-1 (Gupta et al., 1995), thereby regulating gene expression. In some cases, however, activation of these transcription factors seems to be indirect and to be insufficient to account for the JNK-mediated apoptosis, suggesting that certain alternative downstream targets for JNK are involved in the apoptosis mechanism. The antiapoptotic gene Bcl-2 protects cancer cells from apoptosis induced by a variety of anticancer agents (Kim et al., 1997). Bcl-2 protein is known to function upstream to block the activity of a family of proteins, known as caspases, that cleave and degrade a number of substrates located after aspartic acid residues (Nicholson et al., 1995). The heterodimerization status of Bcl-2, with its proapoptotic family members such as Bax, Bak, or Bad, modulates apoptosis by regulating caspase activity (Oltvai and Korsmeyer, 1994). Recently, paclitaxel and other microtubule-interfering agents have been found to induce Bcl-2 phosphorylation, thus altering its heterodimerization with Bax (Haldar et al., 1995), ultimately encouraging the cell to undergo apoptosis. Interestingly, several kinases have been implicated in this phosphorylation event, including c-Raf-1 (Blagosklonny et al., 1997), protein kinase A (Srivastava et al., 1998), and JNK (Maundrell et al., 1997). These studies suggest that JNK or other kinases trigger apoptosis program in cells may mediate through the phosphorylation of Bcl-2 protein.

Although some caspases are regulated, in part, by the antiapoptotic protein Bcl-2, other caspases are regulated via unknown mechanisms. Caspase substrates have been identified, including poly(ADP-ribose) polymerase (Lazebnik et al., 1995), lamin (Lazebnik et al., 1995), fodrin (Cryns et al., 1996), protein kinase C δ (Emoto et al., 1995), retinoblastoma protein (An and Dou, 1996), DNA-dependent protein kinase (Casciola-Rosen et al., 1995), and the protease itself. Some caspase substrates were cleaved to functionally inactive species; others, in contrast, were cleaved to functionally active species. Interestingly, the JNK kinase kinase MEKK1 could be cleaved by DEVDase early in the genotoxin-induced apoptotic response (Widmann et al., 1998). It was found that the initial cleavage of MEKK1 generated a proapoptotic kinase fragment that was able to activate JNK and subsequent caspases to facilitate the onset of apoptosis. These findings provide an important insight into the apoptotic mechanism by which MEKK1 plays a critical role in the balance between antiapoptotic and proapoptotic pathways.

Paclitaxel is an effective agent in the treatment of breast, ovarian, lung, and head and neck cancers (Sarosy and Reed, 1993). Paclitaxel promotes tubulin polymerization, thus altering the dynamic equilibrium of the assembling and disassembling of microtubules, and causing mitotic arrest for dividing cells (Horwitz, 1992). The precise mechanisms of paclitaxel-induced cytotoxicity and apoptosis, however, have not been elucidated completely. Recently, several studies have found that JNK activation induced by paclitaxel treatment is required for apoptosis (Amato et al., 1998; Lee et al., 1998). Other studies, however, have argued against this finding (Wang et al., 1999). Such controversial findings prompted us to study the role of JNK in paclitaxel-induced Bcl-2 phosphorylation and apoptosis in greater detail. We were also interested in examining whether caspase-dependent MEKK1 cleavage is involved in paclitaxel-induced apoptosis signaling. Our studies delineate a signal pathway wherein, upon paclitaxel treatment, MEKK1 could be initially cleaved by DEVDase, which in turn triggers JNK activity and subsequent Bcl-2 phosphorylation, finally leading to a positive loop to increase more caspase activity during apoptotic cell death.

Materials and Methods

Cell Culture and Chemicals. HL-60 cells, a human promyelocytic leukemic cell line, were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in a humidified 5% CO_2 atmosphere and cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Paclitaxel, vincristine, and vinblastine were purchased from Sigma Chemical Co. (St. Louis, MO).

Hypodiploid Cell Assay. Cells were harvested and washed with PBS, and hypodiploid cells were analyzed by flow cytometer as described as previously (Kuo et al.,1998).

DNA Fragmentation Assay. HL-60 cells were treated with various drugs or combined with oligonucleotide for different periods of time. After that, treated cells were harvested and washed with PBS, and DNA fragmentation was analyzed by agarose gel electrophoresis as described previously (Kuo et al., 1999).

Establishment of HL-60/MEKK1-DN Clones. Transfection was created by electroporation (model T800; BTX, San Diego, CA) of HL-60 cells with glucocorticoid-inducible pSRα-MEKK (K432 M) vector (a gift from Dr. Michael Karin of the Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA). Briefly, cells were suspended in 1 ml of HEPES-buffered saline containing plasmid DNA and then received electric treatment as follows: electric amplitude, 900 V; pulse width, 99 ms. After 10 min on ice, the cells were transferred to fresh complete medium and cultured for 24 h before addition of hygromycin. To avoid problems with clonal variation, the transfected cells were selected for hygromycin for 4 weeks, and all of the clones were pooled.

Immunoprecipitation and Kinase Activity Assays. Cell lysis and immune complex kinase assays were performed as described previously (Shiah et al., 1999). HL-60 cells were treated with different drugs, washed twice with ice-cold PBS, and lysed in buffer containing 20 mM HEPES, pH 7.4, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The soluble extracts were prepared by centrifugation at 14,500 rpm for 15 min at 4°. After normalization of protein concentration, equal amounts of protein were incubated with protein A-Sepharose and anti-JNK1 (1 μg; C17; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERK1 (1 μg; C16; Santa Cruz Biotechnology), or anti-p38 (1 μg; N20; Santa Cruz Biotechnology) for 3 h at 4°. The immune complexes were washed twice with lysis buffer and the once with kinase assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 20 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100), after which they were resuspended in 20 μl of kinase assay buffer containing 5 μCi of [γ-^{32}P]ATP, 30 μm cold ATP, and 2 μg of substrate and incubated for 20 min at 30°. Reactions were terminated by the addition of the SDS sample buffer and boiling for 5 min. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. Glutathione-*S*-transferase-c-jun (1/79) was used as a substrate for JNK1, myelin basic protein was used for assaying ERK1, and ATF-2 was used as a substrate for p38.

Antisense Oligonucleotides Treatment. The rationale of JNK1 antisense oligonucleotide design and treatment is based on the report by Shiah et al. (1999). The JNK1-specific antisense (5′-GTCACGCTTGCTTCTGCTCATGAT-3′) and sense (5′-ATCATGAGCAGAAGCAAGCGTGAC-3′) phosphorothioates were synthesized and purified by high-performance liquid chromatography (Genset Co.). These sequences represent amino acids −1 to +7 of JNK1. The

oligonucleotides were dissolved in distilled and sterilized water and added into culture medium. After treatment with the oligonucleotides for 16 h, cells were analyzed the JNK1 activity and JNK1 protein level.

Western Blot Analysis. Western blot was measured by the method described previously (Kuo et al., 1998). Briefly, cell lysates were prepared, electrotransferred, and then immunoblotted with anti-JNK1, anti-Bcl-2, and anti-MEKK1 (C22) antibodies (Santa Cruz Biotechnology). Detection was performed with Western blotting reagent ECL (Amersham), and the chemiluminescence was exposed by the filters on Kodak X-Omat film (Kodak, Rochester, NY).

In Vivo Phosphorylation of Bcl-2. HL-60 cells were incubated in medium for 4 h in the presence of 250 μCi of [^{32}P]orthophosphoric acid per ml and further incubated with or without paclitaxel or plus DEVD-CHO or YVAD-CHO for 4 h before harvesting. At the end of the labeling period, the cells were washed and harvested in ice-cold PBS. The cells were lysed for 30 min on ice in 1 ml radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 120 mM naCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS). The lysates were centrifuged at 900*g* for 30 min at 4°C. Bcl-2 immunoprecipitation was conducted at 4°C on the resulting supernatant with anti-Bcl-2 antibody. After centrifugation and washing, the immunoprecipitated Bcl-2 was resolved by 10% SDS-PAGE, electrotransferred onto NC filters, and autoradiographed.

DEVDase Activity Assay. DEVDase activity was measured by the method described previously (Shiah et al., 1999). In brief, cytosolic extracts were prepared by repeated cycles of freezing and thawing in 300 μl of extraction buffer (12.5 mM Tris, pH 7.0, 1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Cell lysates (100 μg) were then diluted with 1 ml of assay buffer (50 mM Tris, 1 mM EDTA, and 10 mM EGTA, pH 7.0) and incubated at 37° for 30 min in dark with 10 μM fluorescence substrate, Ac-DEVD-AMC. The fluorescence of the cleaved substrate was determined using a spectrofluorometer (Hitachi F-3000) set at excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Preparation of Fractionated Proteins. Cells were collected, washed with ice-cold PBS, and then suspended in buffer A (2 mM EDTA, 10 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min, an equal volume of buffer B (0.5 M sucrose, 0.1 M KCl, 10 mM $MgCl_2$, 2 mM CaCl2, 2 mM EDTA, and 10 mM Tris-HCl, pH 7.5) was added. The nuclear-rich fraction was pelleted by centrifugation (2,700 rpm for 10 min). The supernatant was removed and placed in a separate tube and again centrifuged (43,000 rpm for 90 min). The supernatant was collected as the cytosol-rich fraction, and the pellet was dissolved in buffer C (8 mM CHAPS, 150 mM NaCl, 0.1 M sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5) and incubated at 4°C for 2 h. The membrane-rich fraction was then collected by centrifugation (43,000 rpm for 60 min). Each fraction was separated by SDS-PAGE, and full-length MEKK1 and its fragment were detected by immunoblotting analysis.

Results

The Effect of Paclitaxel on JNK Activation, Bcl-2 Phosphorylation, Cell-Cycle Progression and Apoptosis. To explore whether the JNK signaling pathway is activated within HL-60 cells in response to paclitaxel, we examined JNK activity by using an immunocomplex kinase assay. Figure 1A, upper blot, indicates that, after 1 μM paclitaxel treatment, JNK activity was obviously detectable and increased at 2 h and was sustained for up to 12 h. Western blot analysis revealed that this JNK activation was not caused by enhanced expression of JNK protein (Fig. 1A, lower blot). It has already been recognized that paclitaxel has the ability to enhance the phosphorylation state of antiapoptotic protein Bcl-2, and that this can be easily detected using Western blotting analysis because of the slower blot-migrating form of Bcl-2. Figure 1B clearly reveals that the phosphorylated forms of Bcl-2 are initially detected at 2 h, their presence gradually increasing during the period of paclitaxel treatment. To determine whether the slower-migrating forms of Bcl-2 represented a phosphorylated form of the protein, we

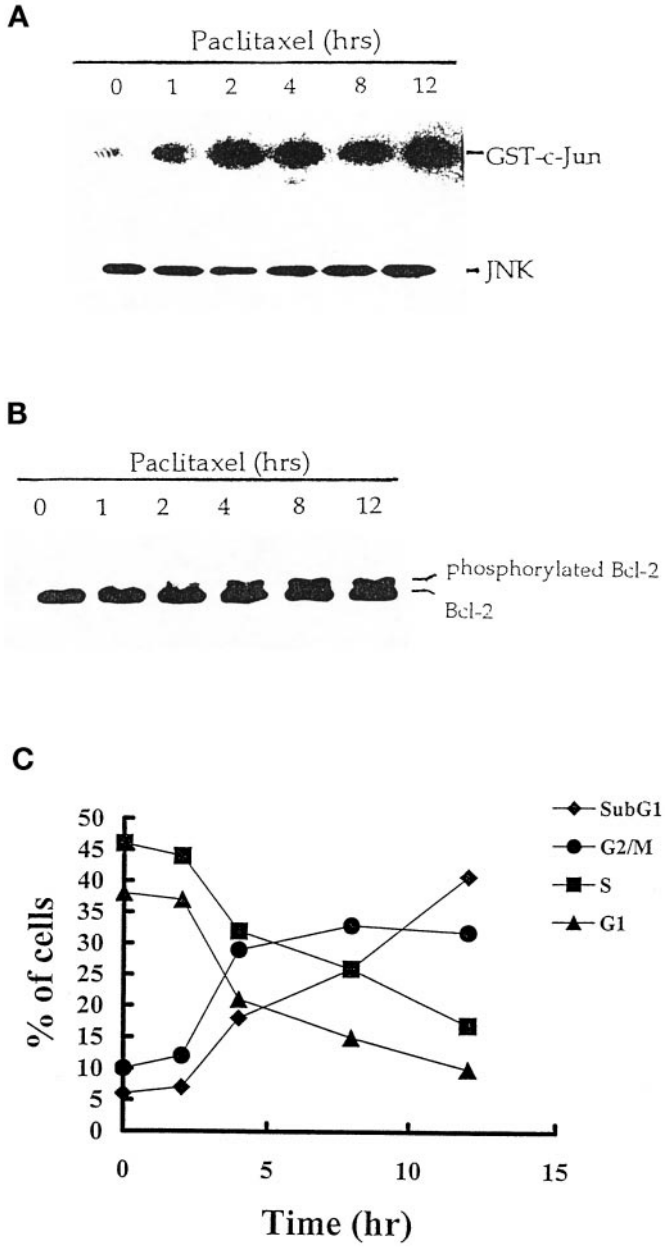

Fig. 1. A, JNK activity was induced by paclitaxel. HL-60 cells were treated with 1 μM paclitaxel for indicated times, after which cells were harvested and the cytosolic fraction analyzed for JNK activity (upper blot) or for JNK protein level (bottom panel). The kinase activities were determined by immunocomplex kinase assay, and the protein levels were determined by Western blot as described under *Materials and Methods*. B, Western blot analysis of the phosphorylation of Bcl-2 by paclitaxel. Cells were treated with 1 μM paclitaxel for indicated times. C, effect of paclitaxel on cell cycle progression and apoptosis of HL-60 cells. Cells were treated with 1 μM paclitaxel for indicated times, then aliquots from control and drug-treated cultures were removed and fixed in 80% ethanol. The fixative solution was stained with the fluorescent propidium iodide for DNA content. Estimates of the cell cycle phase distribution were calculated using the Multicycle program. The data are the representative of two independent experiments.

exposed paclitaxel-treated HL-60 cells to potato acid phosphatase. This treatment clearly resulted in the loss of the slower-migrating forms of the protein and a concomitant increase in the faster-migrating protein species (data not shown). Flow cytometric analysis showed that paclitaxel treatment caused a slight increase in G_2/M phase cells and apoptotic cells (sub-G_1 cells) at 4 h, and both phases of cells were evidently increased after 8 h treatment (Fig. 1C). These observations revealed that the kinetics of JNK activation preferentially correlated with the degree of Bcl-2 phosphorylation, but preceded the occurrence of apoptosis and G_2/M arrest.

The Role of JNK Activation in Bcl-2 Phosphorylation and Apoptosis. To verify the possible role of JNK in paclitaxel-induced Bcl-2 phosphorylation and apoptosis, we transfected and expressed a dominant-negative MEKK1 (MEKK1-DN) plasmid [pSRα-MEKK1 (K432 M)], which was under control by the glucocorticoid-inducible promoter, within HL-60 cells. We have previously shown that the transfectants successfully expressed a dominant-negative form of MEKK1 protein in the presence of inducer dexamethasone (Dex) (Shiah et al., 1999). In this context, we subsequently treated both the MEKK1-DN-transfected HL-60 (HL-60/MEKK-DN) and parental HL-60 cells with paclitaxel or other microtubule-damaging agents such as, vincristine (VCR) or vinblastine (VBL), and then examined JNK activity and mechanism of Bcl-2 phosphorylation and apoptosis.

In the absence of Dex, HL-60/MEKK-DN cells expressed a 4.2-, 6.5-, and 7.3-fold level of JNK activity compared with normal basal conditions in response to 1 μM, respectively, paclitaxel (TAX.), VCR, and VBL (Fig. 2A, upper blot). By contrast, in the presence of Dex, HL-60/MEKK-DN expressed only basal level JNK activity under treatment with the same microtubule-damaging drugs (Fig. 2A, upper blot). Notably, we observed that these above-mentioned microtubule-damaging agents could all induce remarkable Bcl-2 phosphorylation in HL-60/MEKK-DN cells in the absence of Dex (Fig. 2A, lower blot). By contrast, these drugs all failed to induce the phosphorylation of Bcl-2 in HL-60/MEKK-DN cells in the presence of Dex (Fig. 2A, lower blot). Consistent with these observations, in the absence of Dex, HL-60/MEKK-DN cells became resistant to apoptosis induced by those microtubule-damaging drugs, as quantified by the annexin V-positive cells (Fig. 2B).

Acknowledging that MEKK1-DN may not specifically inhibit JNK activity, we thus treated cells with the JNK1-specific antisense oligonucleotide to block JNK activity. Initially, HL-60 cells were treated with 25 μM JNK1-specific antisense oligonucleotide for 16 h before the addition of 1 μM paclitaxel for various time periods depending upon the specific experimental protocol. Upon treatment with JNK1 antisense oligonucleotide for periods of up to 16 h, intracellular levels of JNK1 protein decreased as a consequence, and no evident cytotoxicity was observed for HL-60 cells (data not shown). Under such conditions, immunocomplex kinase assay reveals that paclitaxel-elicited JNK activity could be blocked by JNK antisense oligonucleotide but not by its sense oligonucleotide analog (Fig. 3A, upper blot). Western blot analysis showed that the paclitaxel-induced phosphorylated form of Bcl-2 was reduced by the presence of JNK antisense oligonucleotide (Fig. 3A, middle blot). To further confirm that the phosphorylation of Bcl-2 is indeed blocked by JNK antisense, the [^{32}P]orthophosphoric acid-labeled cells exposed to paclitaxel and sense or antisense oligonucleotide was immunoprecipitated with anti-Bcl-2 antibody and resolved by 10% SDS-PAGE. Consistently, the JNK-specific antisense, but not sense oligonucleotide, effectively diminished the paclitaxel-induced ^{32}P-labeled Bcl-2 (Fig. 3A, bottom blot). Agarose gel electrophoresis showed that paclitaxel-induced DNA fragmentation was effectively prevented by JNK antisense oligonucleotide presence (Fig. 3B).

These results clearly suggest that the JNK kinase cascade is required for paclitaxel-mediated Bcl-2 phosphorylation and apoptotic cell death for human leukemic HL-60 cells.

DEVDase-Mediated MEKK1 Cleavage Induced by Paclitaxel. It has been demonstrated that MEKK1 plays a critical role in regulating both antiapoptotic and proapoptotic signals depending upon the stimuli integrated within the cells. Once MEKK1 has been cleaved by a caspase-3-like protease, this will generate a proapoptotic signal and, in turn, trigger a caspase feedback loop. Under such a premise, immunoblot analysis revealed that the 91-kDa MEKK1 fragment, a C-terminal kinase domain (Widmann et al., 1998),

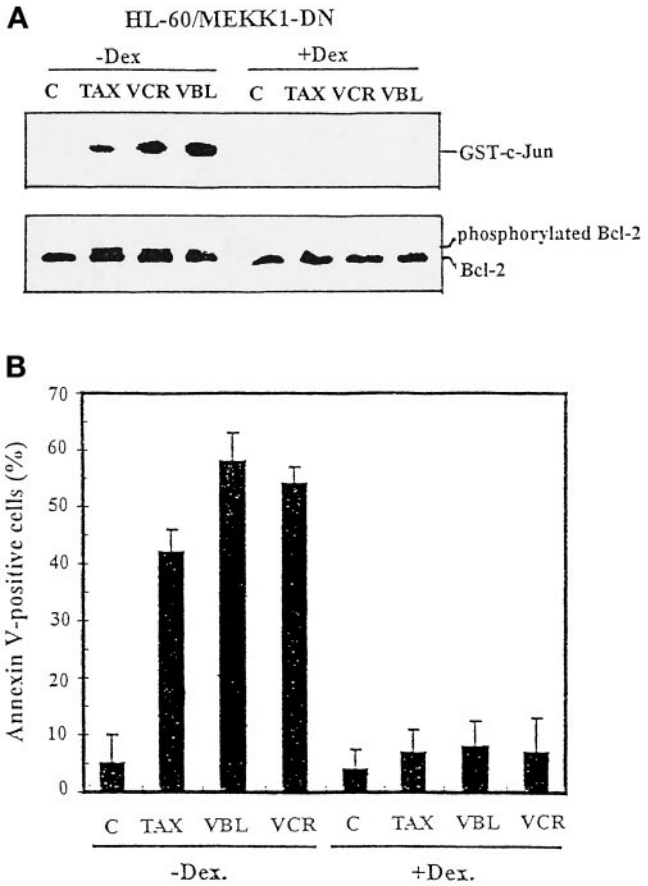

Fig. 2. A, effect of inducible MEKK1-DN on JNK activity induced by microtubule-damaging agents (upper blot) and Bcl-2 phosphorylation (lower blot). Transfected MEKK1-DN HL-60 cells were incubated with or without 1 μM Dex for 16 h. Cells were then treated with 1 μM paclitaxel (TAX), vinblastine (VBL), or vincristine (VCR) for 4 h. Cells were collected, and cell lysates were assayed for JNK activity and Bcl-2 phosphorylation by immunocomplex kinase assay and Western blot, respectively. B, inhibition of microtubule-damaging agents-induced apoptosis by expresion of MEKK1-DN. Transfected cells were treated with TAX, VBL, or VCR for 10 h, then were subjected to analyze the apoptosis. Apoptosis was determined by stained with anti-annexin V antibody and analyzed with flow cytometry.

could be detected as early as 2 h after paclitaxel treatment (Fig. 4A), because we used an antibody which specifically recognizes the epitope corresponding to amino acids 663 to 684 mapping at the C-terminal of MEKK1. Interestingly, the paclitaxel-mediated MEKK1 cleavage proceeded in parallel with JNK activation (Fig. 1A). DEVD-directed caspase activity assay revealing that slight, but significant, DEVDase activity could be initially detected 2 h after paclitaxel treatment (Fig. 4B). The results cited above suggest that paclitaxel induces MEKK1 cleavage, which correlates with the timing of caspase activation, JNK activation, and Bcl-2 phosphorylation.

The Role of DEVDase in Paclitaxel-Induced JNK Activation, Bcl-2 Phosphorylation, and Apoptosis. To address the question of whether or not DEVDase-mediated MEKK1 cleavage plays a role in regulating paclitaxel-induced JNK activation, Bcl-2 phosphorylation, and apoptosis, we used the caspase tripeptide inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO to examine these responses. Figure 5A indicates that pretreatment of cells with 75 μM Ac-DEVD-CHO, effectively inhibited paclitaxel-induced cellular responses, including MEKK1 cleavage (upper blot), JNK activation (middle blot), Bcl-2 phosphorylation (bottom blot), and apoptotic cell death. Under the same conditions, however, another caspase tripeptide inhibitor, Ac-YVAD-CHO, failed to block the above-reported, paclitaxel-induced cellular alterations (Fig. 5B). Also, Ac-DEVD-CHO treatment inhibited VBL- or VCR-induced MEKK1 cleavage, JNK activation, Bcl-2 phosphorylation, and apoptosis (Fig. 5C); by contrast, Ac-YVAD-CHO failed to do so (data not shown).

The results reported above provide an intriguing clue to the identity and function of the DEVD-CHO-inhibitable caspase involved in paclitaxel-induced MEKK1 cleavage, which lies upstream of the site of JNK activation and the subsequent Bcl-2 phosphorylation.

MEKK1 Fragment Linkage with JNK in Paclitaxel-Treated Cells. The caspase inhibitor Ac-DEVD-CHO blocked MEKK1 cleavage and downstream JNK activity, suggesting that the MEKK1 C-terminal kinase fragment may mediate the activating signal to JNK. Initially, we asked whether the intracellular distribution of full-length MEKK1 and a MEKK1 C-terminal 91-kDa fragment was different for cells treated with paclitaxel compared with control conditions. Figure 6A indicates that the most abundant full-length MEKK1 protein was detected in the membrane fraction of cells not having been treated with paclitaxel. By contrast, a

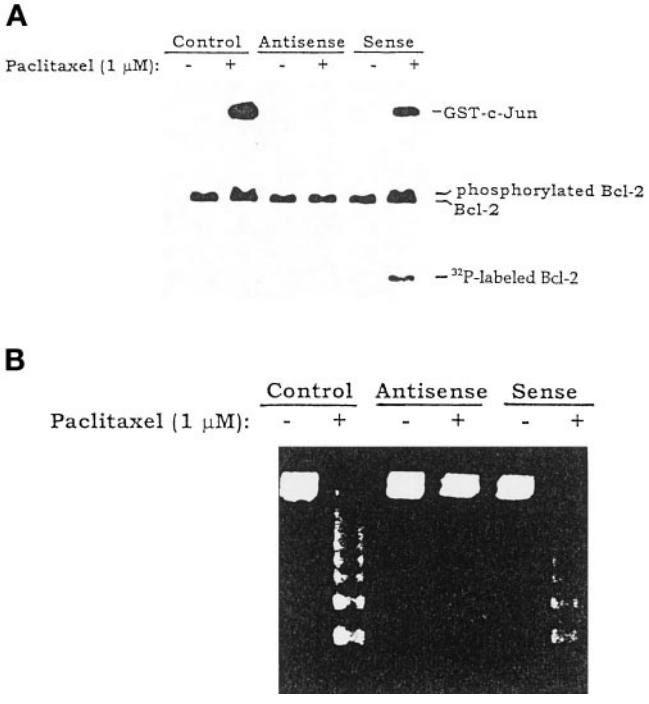

Fig. 3. Effect of JNK1-specific antisense oligonucleotide on paclitaxel-induced JNK activity (A, upper blot), Bcl-2 phosphorylation (A, middle and bottom blots), and apoptosis (B). HL-60 cells were treated with 25 μM JNK-specific antisense or sense oligonucleotide phosphorothioates for 16 h before the addition of 1 μM paclitaxel for another 4 h. JNK kinase activity was performed as described under *Materials and Methods*. For detecting Bcl-2 phosphorylation, either using Western blot to examine the slower migrating form of Bcl-2 proteins (A, middle blot) or using [^{32}P] orthophosphoric acid to label cells and radioimmunoprecipitation with anti-Bcl-2 antibody (A, *bottom blot*). The analysis of ^{32}P-labeled Bcl-2 is described under *Materials and Methods*. The apoptosis assay was performed with DNA fragmentation analyzed by 1.8% agarose gel electrophoresis.

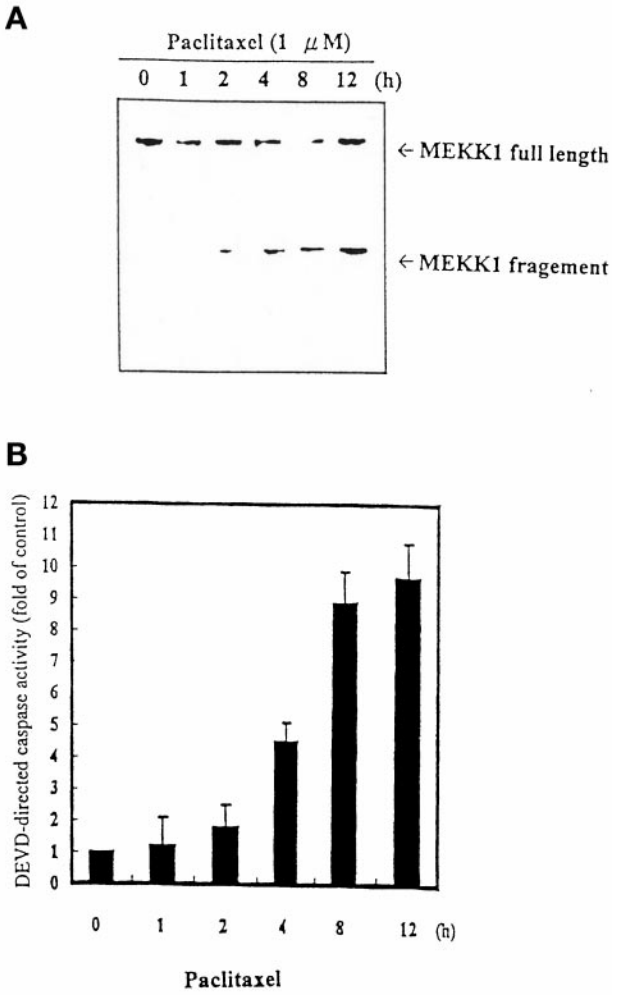

Fig. 4. Effect of MEKK1 cleavage and DEVDase activity in HL-60 cells treated with paclitaxel. A, cells were treated with 1 μM paclitaxel for indicated times. Cellular lysates were subjected to Western blotting by using anti-MEKK1 antibody, which recognizes the epitope corresponding to amino acids 663 to 684 mapping at the C-terminal of MEKK1. B, cells were treated as described in A. Cell lysates were then prepared and performed to DEVDase activity assay as described under *Materials and Methods*. (Columns, means of three independent experiments; bars, S.D.

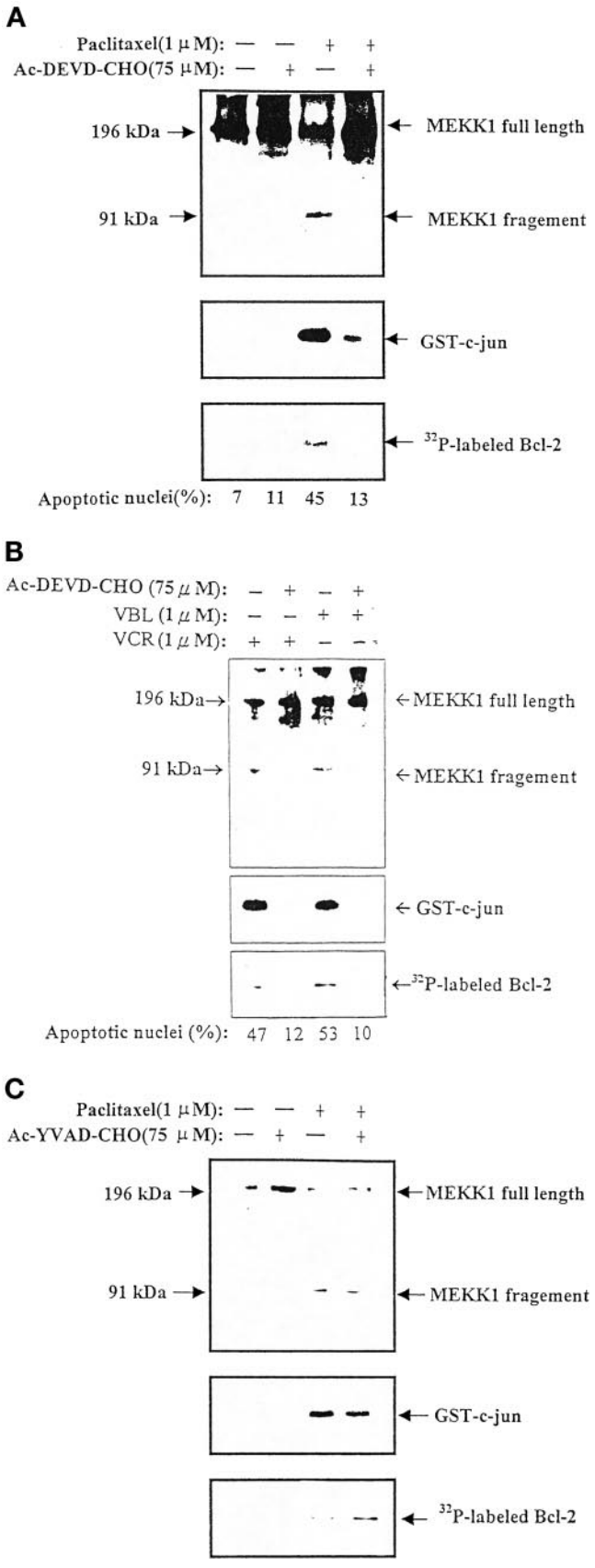

Fig. 5. Effect of caspase inhibitors on MEKK1 cleavage, JNK activity, Bcl-2 phosphorylation, and subsequent apoptosis in cells after treatment with microtubule-damaging agents. A, inhibition of Ac-DEVD-CHO on paclitaxel-induced MEKK1 cleavage, JNK activation, Bcl-2 phosphorylation, and apoptosis. HL-60 cells were pretreated with or without 75 μM Ac-DEVD-CHO for 2 h, and then were treated with 1 μM paclitaxel for another 4 h. After that, cell lysates were prepared and subjected to Western blotting, JNK activity assay, and apoptosis assay. The analysis of ^{32}P-labeled Bcl-2 described under *Materials and Methods*. B, inhibition of Ac-DEVD-CHO on VBL or VCR-induced MEKK1 cleavage, JNK activity, Bcl-2 phosphorylation, and apoptosis. The samples were prepared from cells that were treated as in A. C, effect of Ac-YVAD-CHO on paclitaxel-induced MEKK1 cleavage, JNK activity, and Bcl-2 phosphorylation. HL-60 cells were preincubated 75 μM Ac-YVAD-CHO for 2 h and after 1 μM paclitaxel for 4 h. Cells were collected and cell lysates were assayed for MEKK1 cleavage and JNK kinase activity as described under *Materials and Methods*. Cells were labeled with [^{32}P]orthophosphoric acid and analyzed; the phosphorylation of Bcl-2 is described under *Materials and Methods*.

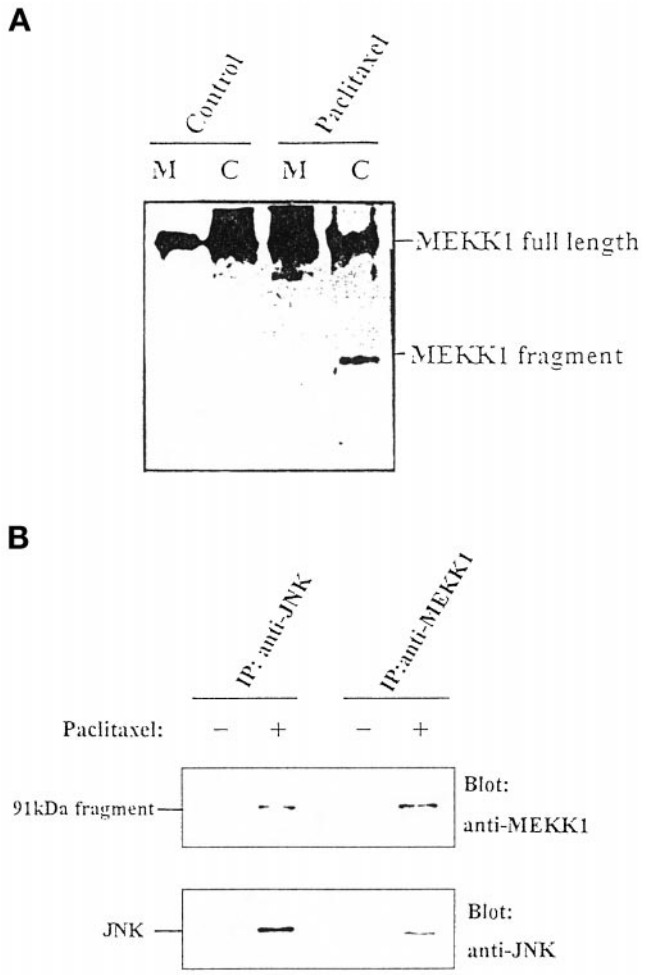

Fig. 6. A, immunoblotting analysis of the 91-kDa MEKK1 fragment in different subcellular fractions. Cells were treated with or without 1 μM paclitaxel for 4 h. The cells were then harvested and cytosolic (C) and membrane (M) fractions were isolated using a detergent lysis method (see *Materials and Methods*). The fractions were then analyzed by Western blotting for MEKK1 and its C-terminal fragment. B, JNK/91-kDa MEKK1 fragment complex formation during paclitaxel-induced apoptosis in HL-60 cells. Coimmunoprecipitation and immunoblotting were performed to investigate the possible JNK-MEKK1 fragment complex formation. JNK or MEKK1 immunoprecipitates collected from the cytosolic fraction of HL-60 cells were separated by SDS-PAGE and then immunoblotted with antibodies to JNK or MEKK1.

significant level of a C-terminal 91-kDa fragment was only observed in the cytosolic fraction for cells treated with paclitaxel. This result indicates that the full-length MEKK1 is predominantly membrane-associated, and its C-terminal kinase fragment is liberated to cytosol when cells are treated

with paclitaxel. We further asked whether the MEKK1 91-kDa fragment would be directly associated with cytosolic JNK. As depicted in Fig. 6B, JNK proteins were coimmunoprecipitated with the anti-MEKK1 antibody in paclitaxel-treated cells but not so for control cells. The 91-kDa MEKK1 fragment was consistently obtained from the immunoprecipitated complex with the anti-JNK antibody in paclitaxel-treated cells. Such findings suggest that the cleaved 91-kDa MEKK1 fragment acts as a proapoptotic mediator to transduce the death signal to the downstream JNK by cytosolic translocation.

Discussion

In this study, we report that the disruption of the cytoskeletal dynamics elicited a novel signal pathway wherein DEVDase initially cleaved MEKK1 protein, after which the elicited 91-kDa C-terminal MEKK1 fragment was released from the cellular membrane to associate with and activate the cytosolic SEK-JNK cascade. This JNK activation further phosphorylated the Bcl-2 protein, which in turn led to apoptosis. The activation of JNK in response to paclitaxel treatment has been found in various types of cells (Amato et al., 1998; Lee et al., 1998; Wang et al., 1999); however, the precise role of JNK in paclitaxel-induced apoptosis still seems to be controversial. Our current study clearly shows that JNK activity is dominantly elevated and remains so for up to 12 h after treatment with paclitaxel (Fig. 1A). Because the induction of JNK in T-cell activation and apoptosis may occur, respectively, in a transient and persistent fashion (Chen et al., 1996), the sustained activation of JNK after paclitaxel treatment may reflect the apoptosis-inducing characteristics of the drug. This persistent induction of JNK activity, however, did not occur during paclitaxel-induced apoptosis in human B lymphoblasts (Amato et al., 1998). In the present study, the experiment utilizing a dominant-negative MEKK1 and a JNK-specific antisense oligonucleotide (Figs. 2A and 3A) have demonstrated the direct involvement of JNK in paclitaxel-induced apoptosis. Our current results are consistent with those of a number of other studies (Amato et al., 1998; Lee et al., 1998; Wang et al., 1998) which have demonstrated that activated JNK is essential for paclitaxel-induced apoptosis. Recently, a direct evidence from the study of JNK knockout fibroblasts revealing that the absence of JNK in cells resulted in resistance to apoptosis induced by UV or other chemical drugs (Tournier et al., 2000). Together, the above results and ours suggest that JNK is preferentially required for stress-induced apoptosis in a wide variety of cells. Because MEKK1 may also affect ERK activity in a dominant-negative fashion (Yujiri et al., 1998), we have determined that the ERK and p38 kinase activity for HL-60 cells after treatment with paclitaxel. The data, however, reveal that paclitaxel treatment of such cells did not activate ERK or p38 kinase (data not shown).

Screening of a library of phage-displayed peptides showed that paclitaxel could directly bind with Bcl-2 protein (Rodi et al., 1999). The paclitaxel binding could inactivate Bcl-2 with concomitant phosphorylation of Bcl-2. Recently, JNK was reported to be capable of phosphorylating Bcl-2 (Maundrell et al., 1997). Furthermore, two-dimensional peptide-mapping and -sequencing experiments reveal that three residues (Ser70, Ser87, and Thr69) within the unstructured loop of Bcl-2 are phosphorylated via the ASK/JNK pathway in response to the activity of a number of microtubule-interfering agents, including paclitaxel (Yamamoto et al., 1999). However, it has recently been reported that JNK promoted cell death by affecting mitochondrial function and cytochrome *c* release but not by inducing Bcl-2 phosphorylation (Tournier et al., 2000). In contrast, our data suggest that JNK plays a crucial role in phosphorylating Bcl-2 in HL-60 cells in response to microtubule damage, because the phosphorylation of Bcl-2 may be completely blocked by JNK-specific antisense oligonucleotide (Fig. 3A). The conflicting observations on JNK and Bcl-2 phosphorylation are primarily caused by the cell type specificity and varied kinds of stimuli. Supportive of the concept, several candidate kinases, in addition to JNK, such as Raf (Blagosklonny et al., 1997), cyclin B1/cdc2 kinase (Ling et al., 1998), and cAMP-dependent protein kinase (Srivastava et al., 1998), have been found to be involved in Bcl-2 phosphorylation in different cell types by various stimuli.

Uncertainty prevails in the literature as to whether this phosphorylation activates or deactivates the antiapoptotic function of Bcl-2. Many investigators have pointed out that mutants in Ser70 or Ser87 of Bcl-2 were unable to be phosphorylated during such a process, becoming resistant to cell death induced by microtubule-damaging agents (Yamamoto et al., 1999). Another study revealed that Bcl-2 could be phosphorylated by Cdc2/cyclin B1 kinase, a marker of M-phase event but not a determinant of apoptosis (Ling et al., 1998). Our current and unpublished data indicate that blockage of JNK activity leads to the prevention of Bcl-2 phosphorylation and cell death but not to the G_2/M arrest of cells treated with paclitaxel (data not shown). This indicates that Bcl-2 phosphorylation is uncoupled from the G_2/M arrest for HL-60 cells in response to paclitaxel. Importantly, our findings are in agreement with the observations that the phosphorylation of Bcl-2 would be inactivating, this potentially augmenting the genesis of cell-death signals.

MEKK1 is a 196-kDa kinase, and acts upstream of the JNK pathway. It has recently been demonstrated that MEKK1 is a substrate for caspase-3-like proteases and that the kinase activity of MEKK1 stimulates caspase-3-like activity in cells (Widmann et al., 1998). A mutant of MEKK1 that is resistant to caspase cleavage is less capable of inducing apoptosis than the full-length, wild-type protein, demonstrating that caspase cleavage of MEKK1 accelerates its apoptotic activity. Another study has shown that MEKK1 knockout embryonic stem cells had a greater apoptotic response to microtubule damage; activated MEKK1 signaling resulted in protecting cells from death (Yujiri et al., 1998). They thus proposed that MEKK1 exhibited a dual role for regulating apoptosis via a mechanism of caspase-dependent cleavage (i.e., caspases act as switches to convert the MEKK1 survival signal to a proapoptotic response). Our data support this hypothesis and indicate that the cleavage of MEKK1 occurred consistently and contemporaneously with enhanced JNK activity as well as Bcl-2 phosphorylation and preceded DNA fragmentation in response to paclitaxel treatment. The caspase inhibitor DEVD-CHO almost completely blocked paclitaxel-induced MEKK1 cleavage, JNK activation, Bcl-2 phosphorylation, and subsequent apoptosis, suggesting that DEVDase plays an initial and important/critical role in triggering cell-death signals. A subcellular fractionation assay

demonstrated that a 91-kDa active C-terminal MEKK1 fragment is generated and translocates to the cytosol (Fig. 6A). Interestingly, the 91-kDa MEKK1 fragment was found to be associated with JNK protein in cytosol, as evidenced by the coimmunoprecipitation assay that we conducted (Fig. 6B). Because the C-terminal half of MEKK1 also binds to MEK4/SEK1 (Hibi et al., 1993), it is conceivable that the kinase complex of C-terminal MEKK1/SEK1/JNK exists in cytosol and facilitates the paclitaxel-induced death-signal transduction. Contrary to our studies, Gibson et al. (1999) suggest that MEKK1 is not significantly proteolyzed in human embryonic kidney 293 cells in response to microtubule-damaging agents. This discrepancy is predictable and may be caused by the different cellular context. It is generally believed that blood cells are more susceptible to exogenous stimuli than other cell types, such as epithelial or fibroblast cells. Indeed, we found that MEKK1 was not cleaved in human breast cancer MCF-7 cells after paclitaxel treatment (M.-L. Kuo, et al., unpublished observations).

It is now well documented that the 91-kDa C-terminal MEKK1 fragment is generated because of the cleavage occurring at position Asp874 by a DEVDase (Widmann et al., 1998). Purified recombinant caspase-3 exerts its proteolytic activity in vitro. The possibility that other DEVDases may be involved in the cleavage of MEKK1 in cells should not be excluded, however. For mammalian cells, procaspases typically form complexes with Apaf-1 and proapoptotic factors, namely apoptosome; this complex, however, is functionally inactive (Cain et al., 1999). When cells are exposed to cytotoxic insults, procaspases become active and are released from the complex to cleave the cellular-defense machinery. Thus it seems likely that disturbing the cellular cytoarchitecture by microtubule-damaging agents may facilitate the dissociation of caspases from the apoptosome. Such a hypothesis remains unconfirmed at this stage and warrants further investigation. In conclusion, we show that paclitaxel induces apoptosis through a novel signaling pathway in which a DEVDase initially cleaves MEKK1, resulting in a signaling cascade mechanism including JNK activation and Bcl-2 phosphorylation.

Acknowledgments

We thank Dr. Michael Karin for his kindness in providing dominant-negative MEKK1 and GST-c-jun constructs.

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