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Microtubule-interfering Agents Activate c-Jun N-terminal Kinase/Stress-activated Protein Kinase through Both Ras and Apoptosis Signal-regulating Kinase Pathways*

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The essential cellular functions associated with microtubules have led to a wide use of microtubule-interfering agents in cancer chemotherapy with promising results. Although the most well studied action of microtubule-interfering agents is an arrest of cells at the G_2/M phase of the cell cycle, other effects may also exist. We have observed that paclitaxel (Taxol), docetaxel (Taxotere), vinblastine, vincristine, nocodazole, and colchicine activate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signaling pathway in a variety of human cells. Activation of JNK/SAPK by microtubule-interfering agents is dose-dependent and time-dependent and requires interactions with microtubules. Functional activation of the JNKK/SEK1-JNK/SAPK-c-Jun cascade (where JNKK/SEK1 is JNK kinase/SAPK kinase) was demonstrated by activation of a 12-*O*-tetradecanoylphorbol-13-acetate response element (TRE) reporter construct in a c-Jun dependent fashion. Microtubule-interfering agents also activated both Ras and apoptosis signal-regulating kinase (ASK1) and coexpression of dominant negative Ras and dominant negative apoptosis signal-regulating kinase exerted individual and additive inhibition of JNK/SAPK activation by microtubule-interfering agents. These findings suggest that multiple signal transduction pathways are involved with cellular detection of microtubular disarray and subsequent activation of JNK/SAPK.

c-Jun N-terminal kinases (JNKs),[1] also known as stress-activated protein kinases (SAPKs), are involved in a signal transduction pathway parallel to that of mitogen-activated protein kinases (MAPKs) (1–6). This highly conserved cascade is responsive to stress-related stimuli such as UV irradiation, ionizing radiation, ischemia and reperfusion, and inflammatory cytokines, eliciting phosphorylation and activation of JNK/SAPKs (7–12). Activated JNK/SAPKs phosphorylate a variety of transcription factors including c-Jun, leading to transcriptional activation through interactions with c-Jun responsive DNA elements such as TPA response element (TRE). In addition to responding to extracellular stimuli (13), the JNK/SAPK pathway is also activated by intracellular stresses including inhibition of protein synthesis, treatment with antimetabolites, or DNA damage (8, 10, 14). No association has been shown, however, between microtubule disruption and JNK/SAPK activation.

Microtubule-interfering agents (MIAs) utilized in the present study include paclitaxel, docetaxel, vinblastine, vincristine, nocodazole, and colchicine. Through differential binding to microtubule polymers (paclitaxel, docetaxel) or tubulin monomer and dimers (vinblastine, vincristine, nocodazole, colchicine), MIAs interfere with the dynamic process of microtubule assembly (15). Effects of MIAs include an arrest of cells at the G_2/M phase of the cell cycle and initiation of apoptosis (16–21). It has been proposed, however, that G_2/M arrest may not be sufficient to induce apoptosis and that additional phosphoregulatory pathways may be required (17, 22, 23). On the other hand, evidence is also accumulating to indicate that JNK/SAPK activation may regulate the cell cycle (12, 24) and apoptosis (11, 25–27). In this report, we identify that treatment with MIAs activated JNK/SAPK in a variety of human cells, suggesting activation of JNK/SAPK to be a common cellular response to MIA-induced microtubular disarray.

Apoptosis signal-regulating kinase (ASK1) is a recently characterized MAPK kinase kinase (28). Overexpression of ASK1 induces apoptosis in mink lung epithelial cells, and ASK1 is activated in cells treated with tumor necrosis factor-α, suggesting a role of ASK1 in stress- and cytokine-induced apoptosis (28). Here we report that microtubule-interfering agents activate JNK/SAPK through signal transduction by both Ras and ASK1, indicating that multiple signal transduction pathways may be required for this type of cellular stress response. These results, for the first time, demonstrate involvement of Ras, ASK1, and JNK/SAPK in signal transduction pathways initiated by microtubular disarray.

EXPERIMENTAL PROCEDURES

Cell Culture—Human fibroblasts (CRL1502), breast cancer cells MCF-7 and T47D, choriocarcinoma JEG-3, and osteosarcoma SAOS-2

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[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; MIA, microtubule-interfering agent; SAPK, stress-activated protein kinase; ASK1, apoptosis signal-regulating kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; CAT, chloramphenicol acetyltransferase; dn, dominant-negative; JNKK/SEK1, JNK kinase/SAPK or ERK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; HA, hemagglutinin epitope of influenza virus; GST, glutathione *S*-transferase; RBD, Ras-binding domain of Raf-1; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MBP, myelin basic protein; ARIA, activated Ras interaction assay; PAGE, polyacrylamide gel electrophoresis.

were obtained from ATCC (Rockville, MD). Ovarian carcinoma cells BR (29), 67R (30), 1A9, and the tubulin mutant, paclitaxel-resistant derivatives of 1A9 cells, PTX10 and PTX22 (31, 32), were described previously, as was isolation of primary trophoblast from term placentae (33). All cell lines were cultured in DMEM/F-12 (Sigma) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin except that primary trophoblasts were cultured in DMEM-HG (Sigma) supplemented with 20% FBS. Both PTX10 and PTX22 cells were maintained in 15 ng/ml paclitaxel and 5 μg/ml verapamil continuously but were cultured in drug-free medium for 5 days prior to each experiment.

Chemicals and Cell Treatment—Unless noted, all chemicals were purchased from Sigma. Docetaxel (Taxotere) was kindly provided by S. A. Coughlin (Rhone-Poulenc Pharmaceutic Inc., Collegeville, PA), and lovastatin was a gift from W. L. Henckler (Merck and Co., Rahway, NJ). All stock solutions of MIAs were prepared with Me_2SO at a concentration of 10 mM except that colchicine (10 mM) was dissolved in absolute ethanol and bacterial lipopolysaccharide (2 mg/ml) was dissolved in water. Lovastatin (10 mM) was prepared with 10% ethanol (34). Cell treatments were performed in serum-containing culture medium when cells were approximately 80% confluent. As reviewed by Rowinsky (35), peak plasma concentrations of paclitaxel (Taxol) in patients are 0.21–13.0 μM. Thus, we treated cells with 1 μM paclitaxel in most experiments. For comparison, other MIAs were used at similar concentrations. UV irradiation was performed by exposing cells to a germicidal ultraviolet lamp (254 nm, 38 watts, 76-cm distance between plates and the UV lamp) in a tissue culture hood for 2 min. The UV dose was approximately 40 J/m^2 (36). Cells were then incubated at 37 °C in 5% CO_2 for 1 h before preparing cell lysates.

Immunocomplex Kinase Activity Assays—Anti-JNK1 or anti-MAPK (ERK2) antibodies, purified GST-c-Jun (amino acids 1–79), and protein A-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The procedure for the immunocomplex kinase assay of JNK was modified from Derijard *et al.* (2). Whole cell lysates were prepared with lysis buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 10 mM NaF), and 100 μg was immunoprecipitated with antibody in excess and protein A-agarose beads at 4 °C overnight. The precipitates were washed with lysis buffer and kinase buffer (25 mM HEPES, pH 7.5, 25 mM $MgCl_2$, and 25 mM β-glycerophosphate) and the kinase reactions for JNK/SAPK were performed by incubating immunoprecipitated proteins with kinase mixture (1 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 0.2 μg of GST-c-Jun in kinase buffer) at room temperature for 30 min. Laemmli's loading buffer was added to stop the reaction, and samples were resolved on SDS-PAGE. The procedure for the immunocomplex MAPK assay was identical to the JNK/SAPK assay except an anti-ERK2 antibody was used for immunoprecipitation and myelin basic protein (Upstate Biotechnology, Inc., Lake Placid, NY) was the substrate. GST-c-Jun or myelin basic protein (MBP) bands on autoradiograms were analyzed with a Lynx4000 video densitometer (Applied Imaging, Santa Clara, CA).

Western Blotting Analysis—Aliquots of cell lysates resolved on SDS-PAGE were transferred to nitrocellulose membranes and probed with antibodies as specified, followed by second antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). After washing, proteins were detected by enhanced chemiluminescence (Pierce).

Plasmid Constructs and Transfection—A reporter construct for the TPA response element, p(TRE)x5-TK-CAT (37), was from Z. Culig (University of Innsbruck, Austria). An expression vector for β-galactosidase (pCMV-lacZ), hemagglutinin (HA)-epitope tagged expression vectors pSRα-HA-JNK1 and pSRα-HA-ERK2 (38), and dominant-negative (dn) expression vectors pSRα-dn Ras (17N) and pSRα-dn Rac (17N) (39) were from M. Karin (University of California at San Diego). An expression vector for dn c-Jun (pCMV-TAM67) was from M. Birrer (NCI, NIH) (40). Dominant-negative expression vectors for JNK/SAPK (pSRα-APF) and for JNKK/SEK1 (pSRα-K116R) were from G. L. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) (41). Expression vectors for wild type ASK1 (pcDNA3-ASK1-HA) and dn ASK1 (pcDNA3-dn ASK1-HA) were described recently (28). Liposome-mediated transfections were performed by using LipofectAMINE (Life Technologies, Inc.) on MCF-7 cells, and using Tfx-50 (Promega, Madison, WI) on BR cells.

Chloramphenicol Acetyltransferase (CAT) Assay and Statistical Analyses—Cells in six-well plates were cotransfected with p(TRE)x5-TK-CAT (1.5 μg/well for MCF-7 and 2.5 μg/well for BR), 0.5 μg/well pCMV-lacZ, and 0.5 μg/well either control DNA or dn expression vectors. At 24 h after transfection, cells were treated with 1 μM MIA for 16 h. The liquid scintillation CAT assay was modified from a standard protocol (42). Expression of β-galactosidase was measured with a kit purchased from Promega (Madison, WI). Data for CAT activities were normalized with levels of β-galactosidase. Statistical analysis of CAT assay values was performed by analysis of variance and Student's *t*-test.

Activated Ras Interaction Assay (ARIA)—Activated Ras (Ras-GTP) was precipitated from whole cell lysates with the Ras-binding domain (RBD) of Raf-1 as a GST-RBD fusion protein immobilized on glutathione beads, followed by detection of precipitated Ras by Western blot with anti-Ras antibodies (43, 44). The bacterial expression vector for GST-RBD, pGEX-RBD, was provided by D. Shalloway (Cornell University) (43). BR cells growing to 90% confluence in 10-cm dishes were serum-starved (0.1% fetal calf serum) for 48 h, then treated with 1 μM paclitaxel or vinblastine for 30 or 120 min. Cells treated with 50 ng/ml epidermal growth factor for 10 min were used as positive controls. Treated cells were rinsed with ice-cold phosphate-buffered saline twice and lysed with 0.4 ml/dish of Mg-containing lysis buffer (MLB: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Activated Ras was precipitated by GST-RBD in excess. After three washes with MLB, activated Ras was eluted from beads by boiling in Laemmli's loading buffer, subjected to 14% SDS-PAGE, transferred to nitrocellulose membrane, and detected using anti-Ras antibody (Santa Cruz, SC-035).

Immunocomplex Kinase Assays of HA-JNK1, HA-ERK2, and ASK1-HA—Since efficiencies of transient transfection in both BR and MCF-7 cells were limited (20% and 15%, respectively), we were not able to accurately evaluate the effects of transfected expression vectors on regulation of JNK/SAPK by direct measurement of endogenous JNK/SAPK activities in the whole population of cells. Therefore, we cotransfected expression vectors for HA-JNK1 with vectors expressing its potential upstream regulators, then assayed activities of epitope-tagged HA-JNK1. Since we had determined in pilot experiments that transfected HA-JNK1 was optimally activated by 4-h treatment with MIAs in BR cells (data not shown), we treated with MIAs for 4 h before measuring HA-JNK1 activity.

BR cells in 60-mm Petri dishes were cotransfected with 4 μg of pSRα-HA-JNK1 and 2 μg of each expression vector. The total amount of DNA per dish was brought to 8 μg by adding control vectors (pSRα or pcDNA3). At 24 h after transfection, cells were treated with 1 μM MIAs, cell lysates were prepared and subjected to immunocomplex kinase assay using excess anti-HA monoclonal antibody, clone 12CA5 (Boehringer Mannheim), to immunoprecipitate expressed HA-JNK1. The same amounts of lysates were probed with either anti-HA or anti-JNK1 monoclonal antibodies (PharMingen, San Diego, CA). Since coexpression of some dominant negative upstream regulators inhibited HA-JNK1 expression, HA-JNK1 activities were normalized by the levels of HA-JNK1 measured in Western blot (38). Identical procedures were performed for HA-ERK2 except that MBP was the substrate and an anti-ERK antibody (Santa Cruz) was used in Western blots.

When pcDNA3-ASK1-HA was cotransfected with other expression vectors into cells, expressed ASK1-HA was immunoprecipitated with excess anti-HA antibody, extensively washed, and subjected to identical kinase reactions as for the JNK/SAPK assay. Equivalent increases in autophosphorylation of ASK1 were observed in parallel to increased phosphorylation of ATF2 in the coupled-kinase assay for ASK1 using GST-MKK6, GST-p38, and ATF2 as sequential substrates.[2] Therefore, in the present study, activities of ASK1 were measured by levels of *in vitro* autophosphorylation in ASK1-HA bands that migrated in SDS-PAGE at approximately 160 kDa.

RESULTS

MIAs Activate JNK/SAPK in a Variety of Human Cells—Activities of JNK/SAPK and MAPK in MIA-treated cells were measured by immunocomplex kinase assays using GST-c-Jun and MBP as substrates, respectively. Treatment with 1 μM paclitaxel, docetaxel, vinblastine, nocodazole, or colchicine for 2 h activated JNK/SAPK in BR ovarian cancer cells and in MCF-7 breast cancer cells. Activation of JNK/SAPK was not accompanied by alterations in JNK/SAPK protein levels as measured by Western blotting of whole cell extracts (Fig. 1*A*). In contrast, treatment with MIAs did not significantly activate MAPK/ERK activities. Since BR cells had higher basal MAPK

[2] H. Ichijo, unpublished data.

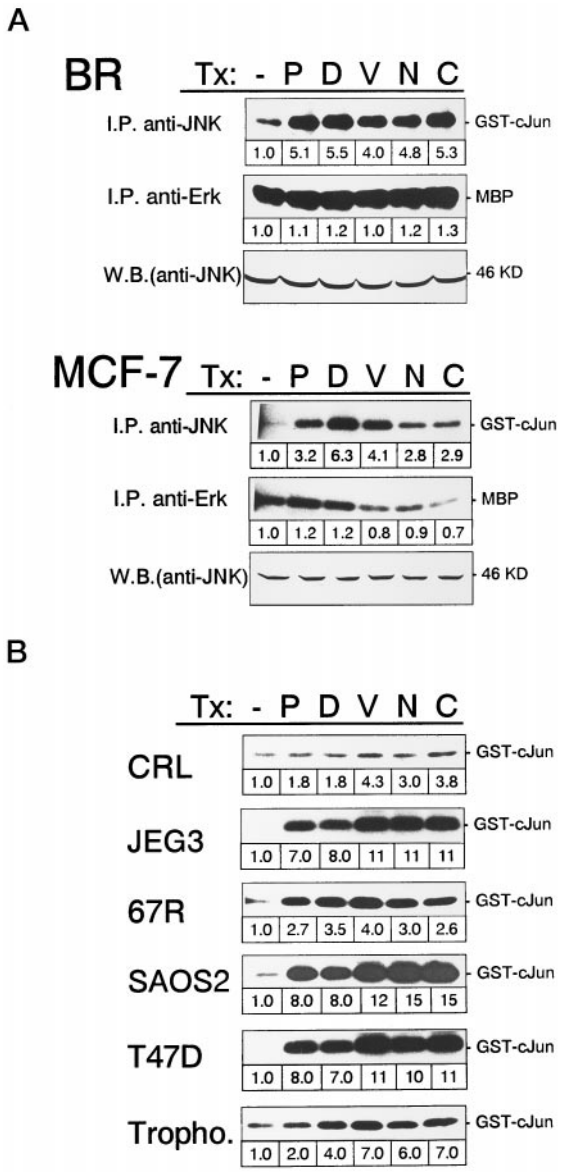

FIG. 1. **Microtubule-interfering agents activate JNK/SAPK in a variety of human cells.** *A*, ovarian cancer cells (*BR*) and breast cancer cells (*MCF-7*) were treated with 1 μM paclitaxel (*P*), docetaxel (*D*), vinblastine (*V*), nocodazole (*N*), colchicine (*C*), or Me_2SO alone (−) for 2 h before JNK/SAPK and MAPK (ERK2) activities were analyzed by immunocomplex kinase assays. GST-c-Jun and MBP were used as substrates for JNK/SAPK and MAPK, respectively. Equal amounts of whole cell lysates were also analyzed by Western blot (*W.B.*) with the same anti-JNK1 antibody. *B*, human fibroblasts (*CRL*), choriocarcinoma (*JEG-3*), ovarian cancer cells (*67R*), osteosarcoma (*SAOS-2*), breast cancer cells (*T47D*), and primary trophoblasts (*Tropho.*) were treated with 1 μM MIA (*P*, *D*, *V*, *N*, and *C*) or Me_2SO alone (−) for 2 h before JNK/SAPK activities were analyzed by immunocomplex kinase assay. *Numbers* under the corresponding bands indicate the -fold activation of JNK/SAPK or MAPK as based on video densitometry. Autoradiograms shown are from a representative experiment, which was repeated twice with comparable results.

activities which could obscure modest MAPK activation by MIAs, HA-ERK2 activity was measured following 1 μM paclitaxel or vinblastine, but no significant activation was detected (data not shown).

To test whether activation of JNK/SAPK by MIA is a general response, we measured JNK/SAPK activities in other cell lines and primary cells. MIAs activated JNK/SAPK in all tested cell types (Fig. 1*B*); the varying magnitude (1.8–15-fold) in different cell types suggests sensitivity to MIAs may be cell type-specific. It is noteworthy that MIAs activated JNK/SAPK in both proliferating cancer cell lines and non-proliferating trophoblasts (33), suggesting that activation is independent of cell cycle progression.

TABLE I
Dose-dependent activation of JNK/SAPK in BR and MCF-7 cells by microtubule-interfering agents

Cells were treated with MIAs at designated concentrations for 2 h. JNK/SAPK activities were assayed by immunocomplex kinase assays and quantitated by analyzing autoradiograms with a video densitometer. -Fold activation shown represent the mean ± S.E. from two or three independent experiments as indicated in parentheses.

Treatment	(no. of experiments)	-Fold activation			
		0.01 μM	0.1 μM	1 μM	10 μM
BR cells					
Paclitaxel	(3)	1.4 ± 0.3	3.2 ± 0.9	6.7 ± 0.7	7.8 ± 1.1
Docetaxel	(2)	1.5 ± 0.5	3.9 ± 1.9	4.4 ± 1.6	15.0 ± 4.9
Vinblastine	(3)	2.6 ± 1.1	2.6 ± 0.8	3.4 ± 0.8	5.2 ± 0.5
Vincristine	(2)	1.7 ± 0.6	2.7 ± 1.3	3.4 ± 1.6	5.5 ± 0.6
Nocodazole	(3)	2.3 ± 1.0	5.0 ± 1.7	6.9 ± 1.2	10.9 ± 1.4
Colchicine	(3)	1.2 ± 0.1	2.7 ± 0.6	3.9 ± 1.0	6.6 ± 1.0
MCF-7 cells					
Paclitaxel	(2)	1.3 ± 0.2	3.0 ± 1.0	3.4 ± 0.4	5.7 ± 2.3
Docetaxel	(2)	1.6 ± 0.5	5.2 ± 0.8	10.3 ± 4.2	12.0 ± 6.0
Vinblastine	(2)	2.0 ± 0.1	2.2 ± 0.2	4.0 ± 0.8	3.5 ± 0.3
Vincristine	(2)	1.8 ± 0.2	2.2 ± 0.2	3.3 ± 0.3	4.5 ± 0.5
Nocodazole	(2)	1.5 ± 0.3	2.7 ± 0.3	3.5 ± 1.5	5.4 ± 0.6
Colchicine	(2)	1.8 ± 0.2	1.8 ± 0.2	2.3 ± 1.3	4.6 ± 0.4

MIA-induced Activation of JNK/SAPK Is Dose-dependent and Time-dependent—In both BR and MCF-7 cells, MIAs activation of JNK/SAPK was dose-dependent over a range of 0.01–10 μM (Table I). MIAs activated JNK/SAPK within 30 min of treatment and the JNK/SAPK response peaked between 2 and 8 h, declining to basal levels by 12 h (Fig. 2). BR cells (Fig. 2*A*) appeared to respond more rapidly than MCF-7 cells (Fig. 2*B*), suggesting cell type-specific differences.

Interactions with Microtubules Are Required for Activation of JNK/SAPK by MIAs—To elucidate whether interactions between MIAs and tubulin/microtubules are required for activation of JNK/SAPK, we measured JNK/SAPK activities in BR and MCF-7 cells treated with an inactive precursor of paclitaxel, 10-deacetylbaccatin III, or an inactive form of colchicine, β-lumicolchicine. Both agents at concentrations up to 10 μM failed to activate JNK/SAPK (Fig. 3*A*). Since paclitaxel exerts lipopolysaccharide (LPS)-like effects (45) and activates JNK/SAPK in macrophages and monocytes (46), we assayed JNK/SAPK activities in BR and MCF-7 cells treated with purified bacterial LPS. No significant activation of JNK/SAPK was observed in BR cells, while in MCF-7 cells, JNK/SAPK activities fell 10–70% below basal activities in two independent experiments (Fig. 3*A*). These data do not suggest a role for LPS-like activity of paclitaxel in JNK/SAPK activation in cancer cells.

To further confirm the requirement for microtubular interactions with MIAs in the activation of JNK/SAPK, we compared JNK/SAPK activation by paclitaxel in two paclitaxel-resistant cell lines, PTX10 and PTX22 (which express mutant β-tubulins), with that in parental 1A9 cells (31). Both paclitaxel and vinblastine activated JNK/SAPK in parental 1A9 cells, but only vinblastine was able to activate JNK/SAPK in the paclitaxel-resistant cell lines (Fig. 3*B*). Furthermore, UV irradiation activated JNK/SAPK equally in all three cell lines, demonstrating a functional JNK/SAPK signaling cascade in these cells, and indicating the lack of JNK/SAPK activation in paclitaxel-treated PTX10 and PTX22 cells is a result of the failure of paclitaxel to bind tubulin (32, 47).

The MIA-activated, JNKK/SEK1-JNK/SAPK Signaling Cascade Activates Transcription through c-Jun—To identify possible downstream effectors of MIA-activated JNK/SAPK, we

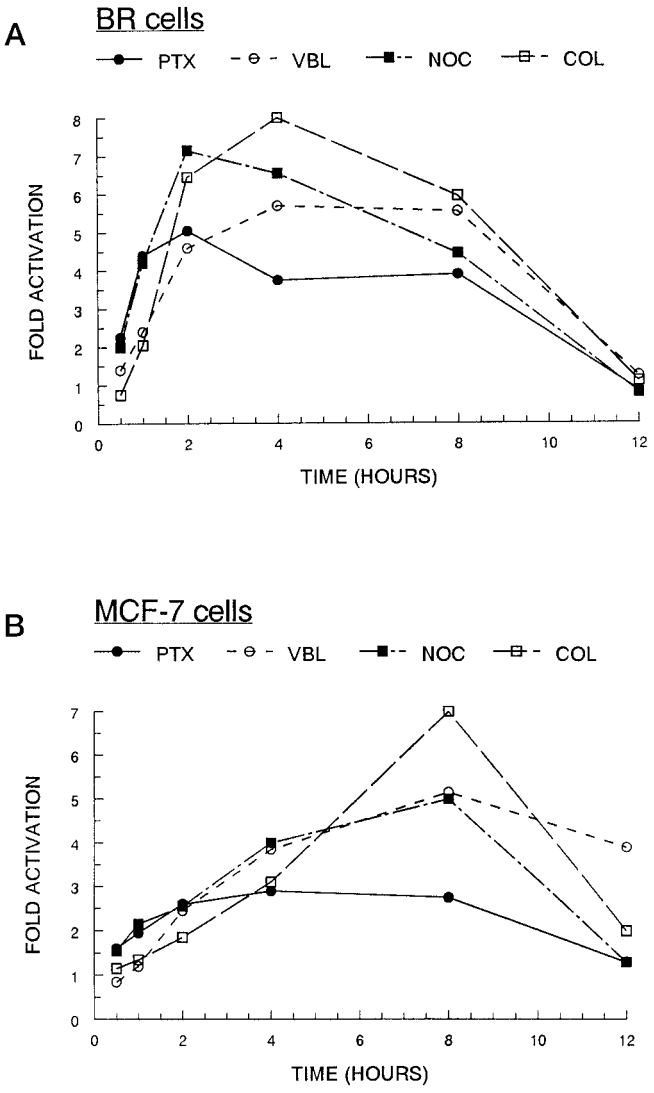

FIG. 2. **Time course of JNK/SAPK activation in BR and MCF-7 cells by microtubule-interfering agents.** BR cells (*A*) and MCF-7 cells (*B*) were treated with 1 μM paclitaxel (●, *PTX*), vinblastine (○, *VBL*), nocodazole (■, *NOC*), or colchicine (□, *COL*) for 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h, after which JNK/SAPK activities were analyzed by immunocomplex kinase assays. The -fold activation of JNK/SAPK was calculated by comparison to basal JNK/SAPK activities. Data shown represent the means from two independent experiments. Average S.E. for each time point were about 24% and 22% of mean values of BR and MCF-7, respectively (S.E. bars are not shown for clear presentation of all points).

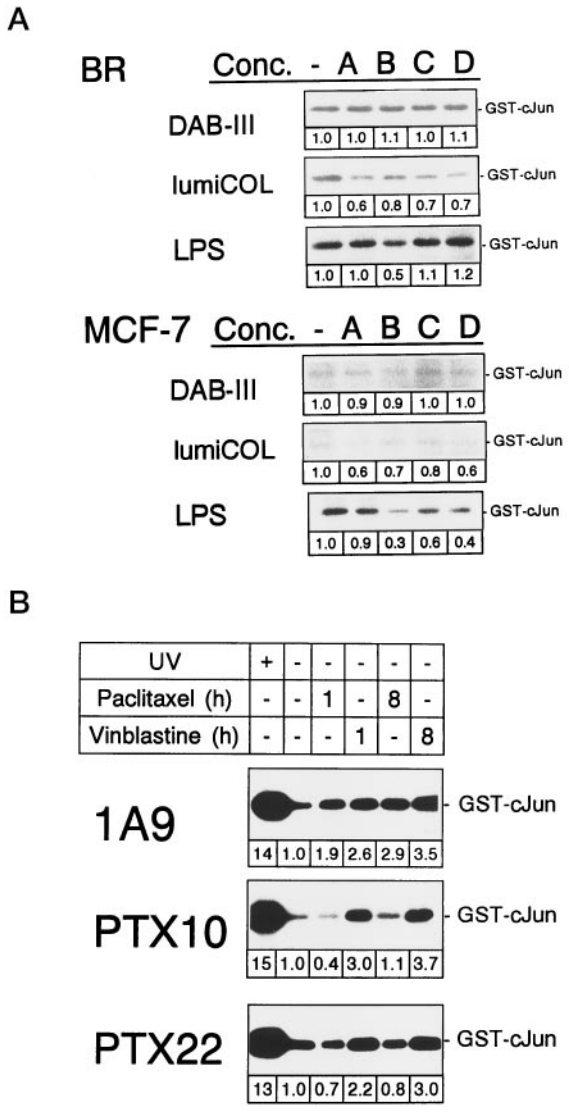

FIG. 3. **Interactions with microtubules are required for activation of JNK/SAPK by MIAs.** *A*, BR (*upper panel*) and MCF-7 cells (*lower panel*) were treated for 2 h with Me_2SO alone (−), 10-deacetylbaccatin-III (*DAB-III*), β-lumicolchicine (*lumiCOL*), or purified bacterial LPS at concentrations of 0.01, 0.1, 1, or 10 μM (*DAB-III* and *lumiCOL*) or μg/ml (*LPS*) (*concentrations A–D*, respectively), then JNK/SAPK activities were measured by immunocomplex kinase assays using GST-c-Jun as substrate. *B*, paclitaxel-sensitive ovarian cancer 1A9 cells and the paclitaxel-resistant derivatives PTX10 and PTX22 cells were treated with 40 J/m^2 UV, 1 μM paclitaxel, or 1 μM vinblastine for 1 or 8 h, after which JNK/SAPK activities were measured. The *numbers* under the corresponding bands indicate -fold activation of JNK/SAPK as determined by densitometry. Autoradiograms shown are from a representative experiment, which was repeated twice with comparable results.

measured AP-1 transcription factor activity by transfecting BR and MCF-7 cells with a p(TRE)x5-TK-CAT reporter construct for 24 h, followed by treatment with MIAs for 16 h and CAT assays. Fig. 4*A* shows statistically significant activation of the TRE reporter by MIAs ($p < 0.01$ when compared with basal TRE activities). Consistent with the immunocomplex JNK/SAPK assays in Fig. 3*A*, 10-deacetylbaccatin III, β-lumicolchicine, and LPS did not activate the TRE reporter (data not shown). In cells cotransfected with p(TRE)x5-TK-CAT and the dn c-Jun expression vector (pCMV-TAM67), both basal and MIA-activated TRE activity were lower than Me_2SO-treated controls ($p < 0.01$, Fig. 4*B*), confirming c-Jun was required for activation of the TRE reporter construct.

To verify that the JNKK/SEK1-JNK/SAPK signaling cascade is activated by MIAs, we cotransfected BR and MCF-7 cells with p(TRE)x5-TK-CAT and one of two dn expression vectors before treatment with MIAs. Coexpression of either dn JNKK/SEK1 or dn JNK/SAPK significantly ($p < 0.01$, Fig. 4*C*) reduced activation of the TRE reporter by vinblastine in both BR and MCF-7 cells. Similar inhibition of the TRE response by coexpressed dn JNKK/SEK1 or dn JNK/SAPK was found with other MIAs (data not shown). The inhibition of MIA-activated TRE reporter by dn JNKK/SEK1 suggests that MIAs activate JNK/SAPK upstream rather than by directly interacting with JNK/SAPK.

Multiple Signal Transduction Pathways, Including Ras and ASK1, Regulate JNK/SAPK Activation Induced by MIAs—Lovastatin inhibits lipidation of Ras (48), which is essential for anchorage to the inner cell membrane and for Ras activation of the Raf-MAPKK-MAPK signaling cascade. Pretreatment of BR

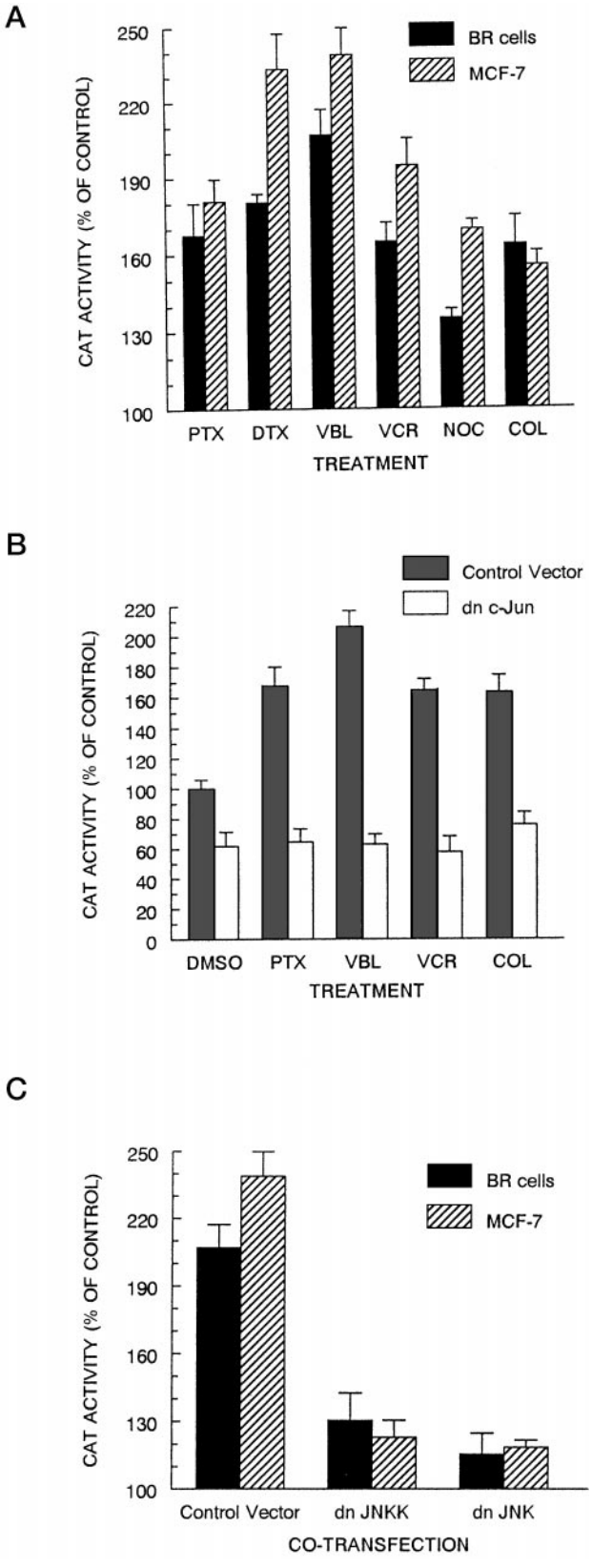

FIG. 4. **The MIA-activated, JNKK/SEK1-JNK/SAPK signaling cascade activates transcription through c-Jun.** *A*, BR cells (*solid bars*) and MCF-7 cells (*shaded bars*) were transiently cotransfected with the p(TRE)x5-TK-CAT reporter construct and pCMV-lacZ using liposomes. Twenty-four hours after transfection, cells were treated with 1 μM paclitaxel (*PTX*), docetaxel (*DTX*), vinblastine (*VBL*), vincristine (*VCR*), nocodazole (*NOC*), or colchicine (*COL*) for 16 h. CAT activity was measured by liquid scintillation counting and normalized by levels of β-galactosidase. The data given are percent activation of CAT activities compared with Me_2SO-treated controls (100 ± 6% for BR and 100 ± 5% for MCF-7, not shown in the graph). *B*, BR cells were cotransfected with p(TRE)x5-TK-CAT and control vector (*gray bars*) or dn c-Jun expression vector, pCMV-TAM67 (*empty bars*), then treated with 1 μM paclitaxel (*PTX*), vinblastine (*VBL*), vincristine (*VCR*), colchicine (*COL*), or Me_2SO only. *C*, activation of transiently transfected p(TRE)x5-TK-CAT by vinblastine was measured in BR (*solid bars*) and MCF-7 (*shaded bars*) cells that were cotransfected with control pSRα vectors (*Control Vector*), dn JNKK/SEK1 expression vector (*dn JNKK*), or dn JNK/SAPK expression vector (*dn JNK*). Data of BR cells shown are the means ± S.E. of four readings from duplicate samples in two independent experiments. Data of MCF-7 cells shown are the means ± S.E. of triplicate samples in a representative experiment that was repeated twice with comparable results.

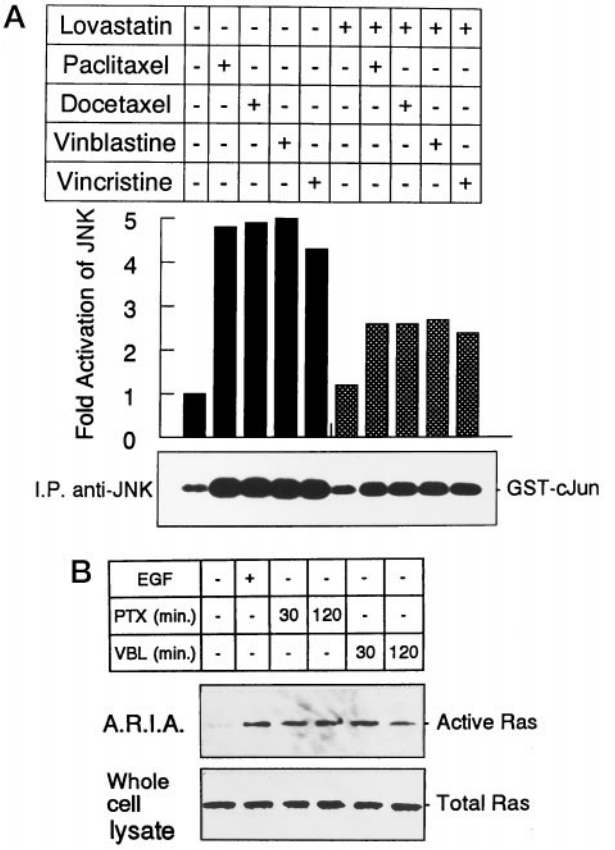

FIG. 5. **A role of Ras in activation of JNK/SAPK by MIAs.** *A*, lovastatin, an inhibitor of both farnesylation of Ras and geranylgeranylation of Rac, partially inhibits activation of JNK/SAPK by MIAs. BR cells were cultured in DMEM/F-12 medium containing 10% FBS without or with 100 μM lovastatin for 24 h, then treated with 1 μM paclitaxel, docetaxel, vinblastine, or vincristine for 2 h. JNK/SAPK activities were measured by immunocomplex kinase assay and densitometry. *B*, treatment with paclitaxel or vinblastine activates Ras in serum-starved BR cells. BR cells were serum-starved (0.1% fetal calf serum) for 48 h, then treated with 1 μM paclitaxel or vinblastine for 30 or 120 min, or treated with 50 ng/ml epidermal growth factor for 10 min as positive control. ARIA was described under "Experimental Procedures." Active Ras (*upper panel*) in each lane was affinity-purified by GST-RBD from 1200 μg of protein from BR cell lysates, while total Ras (*lower panel*) in each lane were detected from 84 μg of protein from the same whole cell lysates. Autoradiograms and Western blots shown are from a representative experiment, which was repeated twice with comparable results.

cells with 100 μM lovastatin for 24 h partially (about 50%) inhibited JNK/SAPK activation by MIAs (Fig. 5*A*). Using ARIA (43, 44), we identified activation of Ras in serum-starved BR cells treated with paclitaxel or vinblastine (Fig. 5*B*) and confirmed the inhibitory effect of lovastatin on Ras (data not shown). These results suggest a role for Ras in JNK/SAPK activation by MIAs and that additional signaling pathways independent of Ras may function as well.

To further elucidate upstream regulators of JNK/SAPK activities, BR cells cotransfected with expression vectors for HA-epitope-tagged JNK1 and with vectors expressing mutant, dominant-negative forms of potential upstream regulators, were treated with MIAs. Kinase assays for exogenously expressed HA-JNK1 were performed to evaluate JNK/SAPK activation. In these experiments, treatment of BR cells with 1 μM paclitaxel (Fig. 6*A*) or vinblastine (Fig. 6*B*) for 4 h resulted in 6.5–6.8-fold activation of HA-JNK1, respectively. Coexpression of either dn Ras (17N)[3] or dn ASK1 (K709R) inhibited HA-JNK1 activation, although neither inhibited as efficiently as dn JNKK/SEK1. However, coexpression of dn Ras and dn ASK1 exerted additive inhibition on HA-JNK1 activation elicited by either paclitaxel or vinblastine. In contrast, coexpression of dn ASK1 and dn JNKK/SEK1 did not inhibit HA-JNK1 activity more than dn JNKK/SEK1 alone. These results suggest that ASK1 and JNKK/SEK1 are in the same signal transduction pathway, in agreement with studies indicating JNKK/SEK1 is a downstream effector of ASK1 (28).

Treatment with MIAs also activated transfected ASK1-HA, as shown by induced autophosphorylation of ASK1-HA and activation of cotransfected HA-JNK1 (Fig. 7*A*). Similarly, in cells transfected with ASK1-HA alone, treatment with MIAs induced comparable levels of autophosphorylation of ASK1-HA without phosphorylation of GST-c-Jun, which was present in the kinase reaction mixture (data not shown). Furthermore, coexpressed HA-dn JNK did not phosphorylate GST-c-Jun and did not interfere with autophosphorylation of ASK1-HA (Fig. 7*A*). Taken together, these results indicate that: (i) ASK1-HA does not itself phosphorylate GST-c-Jun, and (ii) the co-immunoprecipitated HA-JNK1 is unlikely to phosphorylate ASK1-HA.

Overexpression of ASK1-HA enhanced paclitaxel activation of HA-JNK1 to 16-fold over control levels, comparable to the 15-fold activation induced by UV irradiation (Fig. 7*B*). The augmentation of paclitaxel-induced HA-JNK1 activation by ASK1-HA was inhibited by coexpression of dn Ras, dn Rac, or dn JNKK/SEK1. As shown in Fig. 7*B*, a 1:1 ratio of the expression vectors for dn JNKK/SEK1 and wild type ASK1 did not completely inhibit the ASK1-enhanced activation of HA-JNK1. However, increasing levels of dn JNKK/SEK1 completely blocked the enhanced HA-JNK1 activation by overexpressed ASK1 without decreasing the levels of MIA-induced activation of ASK1-HA (Fig. 7*C*). In agreement with the partial inhibition of HA-JNK1 by dn Ras of dn Rac (Fig. 6), overexpression of either dn Ras or dn Rac partially inhibited ASK1-augmented activation of HA-JNK1 (Fig. 7*C*).

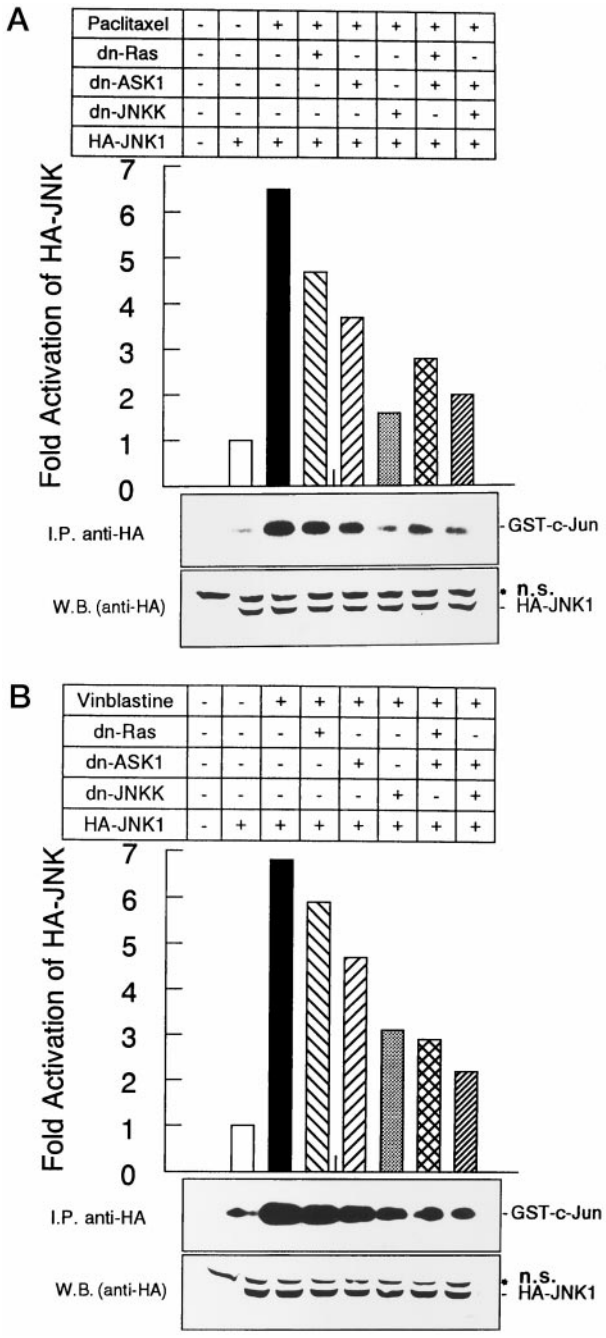

FIG. 6. **Activation of transiently transfected HA-JNK1 by paclitaxel or vinblastine was inhibited by coexpression of dn Ras, dn ASK1, or dn JNKK/SEK1.** BR cells in 60-mm dishes were transiently transfected with 4 μg of pSRα-HA-JNK1 and 2 μg of each dominant negative expression vector (or combinations as indicated) for 24 h, then treated with 1 μM paclitaxel (*A*) or vinblastine (*B*) for 4 h. HA-JNK1 activities were measured by immunocomplex kinase assay using an anti-HA antibody (12CA5) to immunoprecipitate HA-JNK1 from cell lysates and GST-c-Jun as substrate. Activities of HA-JNK1 were normalized to levels of HA-JNK1 protein. A nonspecific band recognized by the 12CA5 antibody is labeled with an *asterisk* (*, *n.s.*). Autoradiogram and Western blot shown are from a representative experiment, which was repeated three times with comparable results.

DISCUSSION

Microtubules serve as an intracellular scaffold, and their unique polymerization dynamics are critical for many cellular functions (15, 49, 50). It is conceivable that cytoskeletal dysfunction, manifested as either a disrupted microtubule network or a stabilized, "rigid" microtubule cytoskeleton, is an intracellular stress. In the present study, we report that disruption of the equilibrium between tubulin monomer/dimers and microtubule polymers with microtubule stabilizing (paclitaxel, docetaxel) or destabilizing (vinblastine, vincristine, nocodazole, colchicine) agents activated the stress-activated protein kinase (JNK/SAPK) signaling cascade. In both BR and MCF-7 cells, JNK/SAPK remained activated for up to 8 h after treatment with MIAs (Fig. 2). Since induction of JNK/SAPK in T-cell activation and apoptosis can occur in a transient or persistent pattern, respectively (51), the sustained activation of JNK/SAPK following MIA treatment may reflect the apoptosis-inducing nature of these drugs.

For the MIAs used in this study, no membrane-associated receptor or target has been identified (15, 45). Our data indi-

[3] The inhibitory efficacy of the expression vector for dn Ras used in this study was verified by cotransfection experiments with HA-tagged ERK2, where the dn Ras completely blocked activation of HA-ERK2 by treatment with epidermal growth factor (T.-H. Wang, unpublished data).

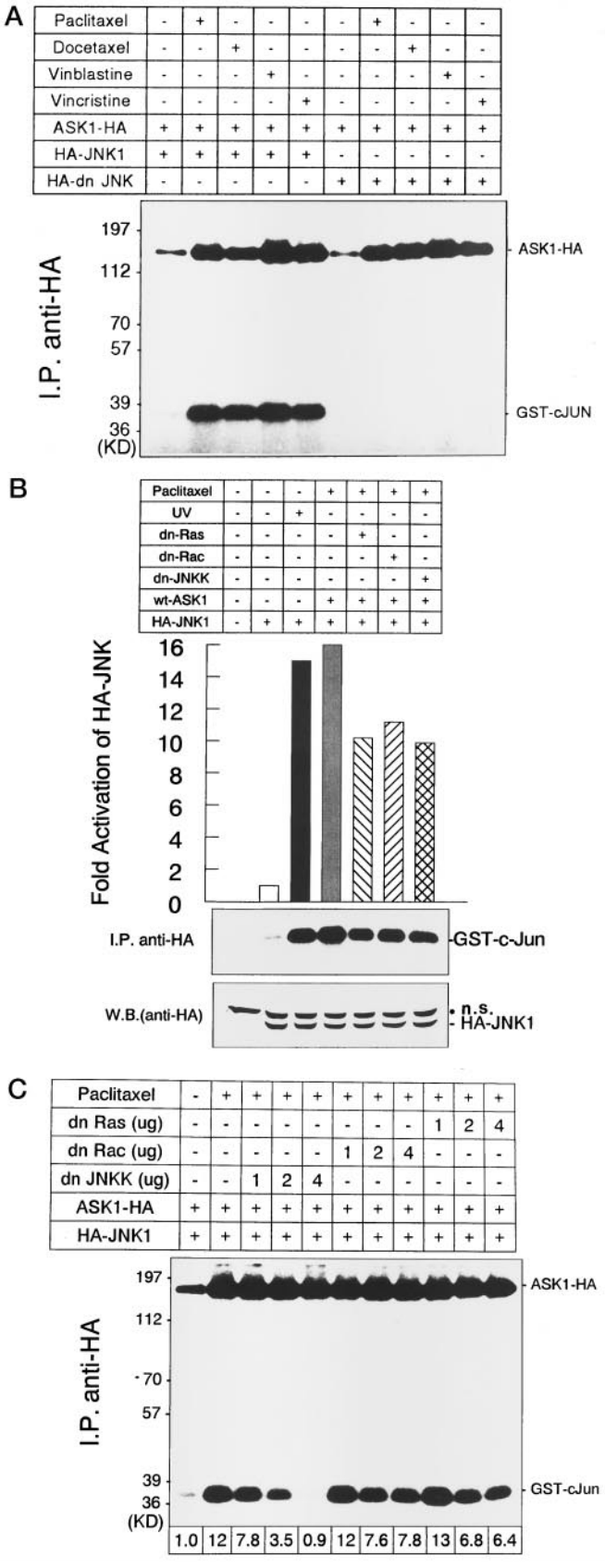

FIG. 7. **MIAs activate the ASK1-JNKK/SEK1-JNK/SAPK signaling cascade.** *A*, BR cells in 60-mm dishes were transiently cotransfected with 4 μg of pcDNA3-ASK1-HA and 4 μg of either pSRα-HA-JNK1 or pSRα-HA-dn JNK for 24 h, then treated with 1 μM paclitaxel, docetaxel, vinblastine, or vincristine for 4 h. ASK1-HA and HA-JNK1 or HA-dn JNK were coimmunoprecipitated by anti-HA antibody and used in a kinase reaction containing GST-c-Jun. Activities of ASK1-HA and HA-JNK1 were measured by levels of autophosphorylation of ASK1-HA and phosphorylation of GST-c-Jun, respectively. *B*, BR cells were cotransfected with 4 μg of pSRα-HA-JNK1, 2 μg of pcDNA3-ASK1-HA, and 2 μg of dominant negative expression vectors as indicated, then treated with 1 μM paclitaxel for 4 h. For comparison, BR cells that were cotransfected with 4 μg of pSRα-HA-JNK1 and 4 μg of control vectors were treated with UV irradiation. Activities of HA-JNK1 measured by immunocomplex kinase assay were normalized to levels of HA-JNK1. A nonspecific band recognized by the 12CA5 antibody is labeled with an *asterisk* (*, *n.s.*). *C*, BR cells were cotransfected for 24 h with 3 μg of pSRα-HA-JNK1, 1 μg of pcDNA3-ASK1-HA, and increasing amounts (1, 2, and 4 μg) of each dominant negative expression vector as indicated. Transfected cells were then treated with 1 μM paclitaxel for 4 h and assayed for activities of ASK1-HA and HA-JNK1. The *numbers* under the corresponding bands of GST-c-Jun in the autoradiogram indicate the -fold activation of HA-JNK1 as based on video densitometry. Data shown are from a representative experiment, which was repeated twice with comparable results.

cate that binding to tubulin and/or microtubules was required for MIA activation of JNK/SAPK. First, inactive structural derivatives of some MIAs (10-deacetylbaccatin III and β-lumicolchicine), which do not bind tubulin/microtubules did not activate JNK/SAPK (Fig. 3A). Second, paclitaxel did not activate JNK/SAPK in the paclitaxel-resistant cell lines, PTX10 (F270V) and PTX22 (A364T), where single amino acid mutations in β-tubulin abolish binding of paclitaxel to microtubule and result in paclitaxel resistance (32, 47). The notion that microtubular interactions are required for MIA-activated JNK/SAPK is strengthened by the observation that, although both paclitaxel and vinblastine activated JNK/SAPK in the parental 1A9 cells, only vinblastine, but not paclitaxel, activated JNK/SAPK in the paclitaxel-resistant PTX10 and PTX22 cell lines (Fig. 3*B*).

We have verified that the JNKK/SEK1-JNK/SAPK-c-Jun signaling cascade was activated by MIAs with the following evidence. First, MIA treatment activated transcription from a TRE-CAT reporter construct and this activation was inhibited by coexpressed dn c-Jun (Fig. 4, *A* and *B*), indicating c-Jun was a downstream effector responsive to treatment with MIAs. Second, coexpression of dn JNK/SAPK or dn JNKK/SEK1 inhibited MIA-induced TRE reporter activity (Fig. 4*C*). These results suggest that MIA-activated JNK/SAPK may regulate transcription by activation of c-Jun and formation of functional c-Jun/c-Fos heterodimers (AP-1).

Multiple signal transduction pathways are required for activation of the JNK/SAPK cascade when a cell is perturbed by physical stress (52) and activation of the JNK/SAPK pathway by environmental stress can occur via Ras-dependent or Ras-independent pathways (53). Farnesylation and geranylgeranylation, the major posttranslational modifications of Ras and Rac, respectively, are essential for membrane anchoring and physiological functions (54). Our observations that lovastatin at concentrations that block farnesylation and geranylgeranylation (48) was unable to completely block JNK/SAPK activation by MIAs suggest involvement of other pathways, independent of the Ras-Rac cascade.

Indeed, we demonstrated a requirement for both Ras and ASK1 signaling for full activation of JNK/SAPK by MIAs. First, treatment with paclitaxel or vinblastine activated Ras (Fig. 5*B*). Second, dn Ras and dn ASK1 exerted individual and additive inhibition of HA-JNK1 activation by MIAs (Fig. 6). Third, MIAs activated ASK1-HA with corresponding activation of HA-JNK1 (Fig. 7*A*). Finally, overexpression of ASK1-HA augmented MIA-induced activation of HA-JNK1 (Fig. 7*B*), and this augmentation could be completely blocked by high levels of dn JNKK/SEK1, but not by dn Ras or dn Rac (Fig. 7*C*). Collectively, these data suggest that both Ras and ASK1 are involved in optimal activation of JNK/SAPK after microtubular disrup-

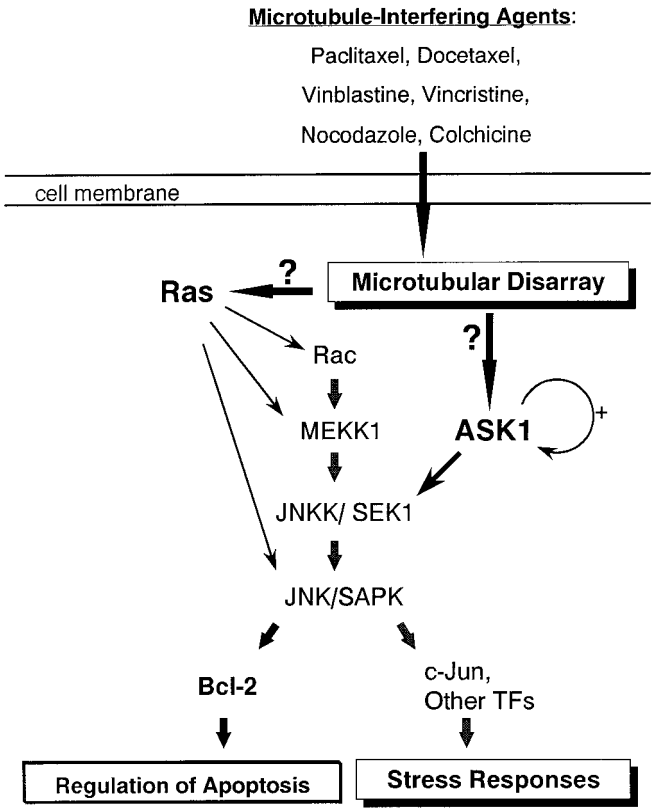

FIG. 8. **Interaction of signal transduction pathways activated by microtubule-interfering agents.** Intracellular stress caused by microtubule-interfering agents activates both Ras and ASK1 signaling cascades, resulting in activation of JNK/SAPK. Activated JNK/SAPK in turn activates transcription factors, including AP-1, to mediate cellular responses to the stress. Activated JNK/SAPK may also regulate apoptosis through phosphoregulation on Bcl-2.

tion and that both may regulate JNK/SAPK activity through the same downstream transducer, JNKK/SEK1.

Unlike treatment with epidermal growth factor, which activates Ras with an amplified activation of MAPK, treatment with MIAs induced a more sustained activation of Ras (Fig. 5*B*) with negligible activation of MAPK (Fig. 1*A*). Explanations for this discrepancy might be twofold. First, the effects of MIAs on the cell cycle might dissociate the sequential activation in the Ras-Raf1-MEK-MAPK cascade. This is supported by a report that, during progression of the cell cycle, there is a temporal dissociation between Ras and MAPK activation, suggesting Ras may target alternate effector pathways (43). Second, in addition to activation of Ras and ASK1, microtubular disarray might also activate phosphatase(s) that attenuate MAPK activation. Several phosphatases that might target MAPK have been identified (55–59).

Based on these results, we propose that activation of the JNK/SAPK pathway may be a stress response to the disruption of microtubule dynamics (Fig. 8). In this model, microtubule-interfering agents enter the cell and disrupt the dynamics of microtubule assembly. Through a yet-to-be defined mechanism, microtubular disarray activates both Ras and ASK1. Activated Ras may activate the JNK/SAPK through activation of Rac (60, 61), activation of MEKK1 (62), or through direct activation of JNK/SAPK by formation of the Ras-JNK complex (63). On the other hand, the signal from activated ASK1 may involve autophosphorylation followed by sequential activation of JNKK/SEK1 and JNK/SAPK. JNK/SAPK in turn activates downstream effectors, including c-Jun and other transcription factors, mediating cellular responses to this stress. Furthermore, disruption of microtubule integrity has been shown to result in phosphorylation of an anti-apoptosis regulator, Bcl-2 (31, 64), and Bcl-2 can be phosphorylated by JNK/SAPK in the presence of Rac1 (65). Since the protective effects of Bcl-2 may be regulated by its phosphorylation status (66), these studies collectively suggest a potential role of activated JNK/SAPK in apoptotic regulation of cancer cells after chemotherapy with MIAs.

It is intriguing that MIAs with stabilizing or destabilizing effects on microtubules elicit similar activation of JNK/SAPK. These observations suggest a surveillance mechanism exists that signals the functional integrity of microtubules to nuclear transcription factors. Interestingly, tubulin itself exhibits GTPase activity and acts as a nucleotide-binding protein (67), implying that tubulin may function in a fashion similar to Cdc42/Rac in the JNK/SAPK signal transduction pathway (10, 13). The mechanism(s) by which microtubular disarray activates both Ras and ASK1 remains to be elucidated.

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