

# I $\kappa$ B Kinase Activation Is Involved in Regulation of Paclitaxel-Induced Apoptosis in Human Tumor Cell Lines

YI HUANG and WEIMIN FAN

*Departments of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina*

Received June 26, 2001; accepted October 2, 2001

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

## ABSTRACT

Paclitaxel (Taxol), a naturally occurring antimetabolic agent, has shown significant cell-killing activity against human solid tumor cells through induction of apoptosis. The molecular mechanism underlying paclitaxel-induced apoptosis is not entirely clear. Using the unique inhibitory effect of glucocorticoids on paclitaxel-induced apoptosis, we recently discovered that paclitaxel-induced inhibitor  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) degradation and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation might contribute to the mediation of paclitaxel-induced apoptosis. In this study, using a novel I $\kappa$ B $\alpha$  phosphorylation inhibitor, we demonstrated that the blockage of paclitaxel-induced I $\kappa$ B $\alpha$  degradation inhibited apoptotic cell death in human breast cancer BCap37 and ovarian cancer OV2008 cell lines. Furthermore, *in vitro* kinase assays showed that the activity of I $\kappa$ B kinase (IKK), which is responsible for the

phosphorylation and degradation of I $\kappa$ B proteins, was significantly activated by paclitaxel in these paclitaxel-sensitive tumor cells. Stable transfection of a mutant I $\kappa$ B $\alpha$  lacking Ser<sup>32</sup> and Ser<sup>36</sup> that was insensitive to IKK-mediated phosphorylation and degradation resulted in reduced sensitivity of tumor cells to paclitaxel-induced apoptosis. Moreover, we also found that the expression of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1, an upstream regulator of IKK, was up-regulated by paclitaxel. These findings suggest that the activation of IKK might play a critical role in the regulation of paclitaxel-induced NF- $\kappa$ B activation that subsequently mediates paclitaxel-induced apoptotic cell death in solid tumor cells.

Paclitaxel (Taxol), a naturally occurring antineoplastic agent, has shown great promise in the therapeutic treatment of certain human solid tumors, particularly in drug-refractory ovarian cancer and metastatic breast cancer (Wani et al., 1971; Holmes et al., 1991; Tishler et al., 1992). However, the exact mechanism by which paclitaxel exerts its cytotoxic action remains unclear. Previous studies demonstrated that paclitaxel is a unique antimicrotubule agent that acts by inhibiting microtubule depolymerization and promoting the formation of unusually stable microtubules, thereby disrupting the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation (Schiff et al., 1979; Rowinsky et al., 1990; Williams and Smith, 1993; Willingham and Bhalla, 1994). Thus, it was generally believed that the antitumor effects of paclitaxel resulted mainly from interference with the normal function of microtubules and blockage of cell cycle progression in the late G<sub>2</sub>-M phase

via prevention of mitotic spindle formation (Fuchs and Johnson, 1978).

In recent years, several laboratories demonstrated that paclitaxel, at clinically relevant concentrations, was able to induce typical internucleosomal DNA fragmentation and other morphological features of apoptosis in a number of solid tumor cell lines (Bhalla et al., 1993; Fan et al., 1994; Cheng et al., 1995). These results clearly indicated that, in addition to its classical activity against microtubules and cell cycle arrest, paclitaxel also possesses cell-killing activity by induction of apoptosis. It is currently unclear whether this finding suggests a novel mechanism of action for paclitaxel against tumor cells or just represents an end product of the well known action of paclitaxel on microtubules and cell cycle arrest. Recent studies in this laboratory have revealed that glucocorticoids selectively inhibit paclitaxel-induced apoptotic cell death in a number of solid tumor cells but do not affect the ability of paclitaxel to induce microtubule bundling and mitotic arrest (Fan et al., 1994, 1996a,b). This selective inhibition of glucocorticoids on paclitaxel's cell-killing activity implies that paclitaxel-induced apoptosis may take place via a signaling pathway independent of cell cycle arrest. In other

This work was supported by United States Public Health Service grants CA82440 and CA71851 (to W.F.).

**ABBREVIATIONS:** NF- $\kappa$ B, nuclear factor- $\kappa$ B; I $\kappa$ B $\alpha$ , inhibitor  $\kappa$ B $\alpha$ ; IKK, I $\kappa$ B $\alpha$  kinase; MEKK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI, propidium iodide; EMSA, electrophoretic mobility shift assay; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; BAY 117821, (E)-3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile.

words, paclitaxel may cause cell death through a gene-directed process; i.e., paclitaxel may directly induce or activate apoptosis-associated genes or regulatory proteins, which in turn triggers the apoptotic process.

Although there is no solid evidence that paclitaxel-induced apoptosis occurs via a pathway independent of mitotic arrest, the possible existence of such a pathway has been proposed by many investigators (Jordan et al., 1996; Torres et al., 1997; Miller et al., 1999). In addition to the features of apoptotic cell death induced by low concentrations of paclitaxel and the selective inhibition by glucocorticoids, a number of apoptosis-associated genes or proteins have been reported to be activated or regulated by paclitaxel (Haldar et al., 1995; Strober et al., 1996; Moos and Fitzpatrick, 1998; Fan, 1999). One of these factors, NF- $\kappa$ B, a member of the *Rel* transcription factor family, and its specific intracellular inhibitor I $\kappa$ B $\alpha$ , participate in the regulation of many biological processes, including inflammation and immune response, cell proliferation, and apoptotic cell death (Brown et al., 1993; Baldwin, 1996). NF- $\kappa$ B normally resides in the cytoplasm as an inactivated form in a complex with I $\kappa$ B $\alpha$ . I $\kappa$ B $\alpha$  modulates the function or activity of NF- $\kappa$ B through its proteolytic degradation in response to different extracellular stimuli (Baeuerle, 1991; Sun et al., 1995). A key player in this cascade of events is I $\kappa$ B kinase complex (IKK $\alpha$  and  $\beta$ ) that is responsible for the phosphorylation and degradation of I $\kappa$ B $\alpha$  (Zandi et al., 1997; Delhase et al., 1999).

In recent years, increasing evidence indicates that activation of NF- $\kappa$ B plays an important role in coordinating the control of apoptotic cell death, which either promotes or inhibits apoptosis, depending on different apoptotic stimuli and cell types (Beg and Baltimore, 1996; Grimm et al., 1996; Wang et al., 1996; Qin et al., 1998; Ryan et al., 2000). By using the unique inhibitory action of glucocorticoids on paclitaxel-induced apoptosis, we recently discovered that paclitaxel significantly down-regulated I $\kappa$ B $\alpha$ , which in turn promoted the nuclear translocation of NF- $\kappa$ B and its DNA-binding activity. In contrast, we found that glucocorticoids could antagonize paclitaxel-mediated NF- $\kappa$ B nuclear translocation and activation through induction of I $\kappa$ B $\alpha$  protein synthesis (Huang et al., 2000). Further investigation demonstrated that tumor cells stably transfected with antisense I $\kappa$ B $\alpha$  expression vectors exhibited a marked increase in sensitivity to paclitaxel-induced apoptosis (Huang et al., 2000). These results suggest that the NF- $\kappa$ B/I $\kappa$ B $\alpha$  signaling pathway may contribute to the mediation of paclitaxel-induced cell death in solid tumor cells.

In the present study, we further investigated the molecular mechanism of paclitaxel-induced apoptosis via activation of NF- $\kappa$ B signaling pathway. Using an I $\kappa$ B $\alpha$  phosphorylation inhibitor and stable transfection of a mutant I $\kappa$ B $\alpha$ , we demonstrated that the prevention of I $\kappa$ B $\alpha$  phosphorylation and degradation could significantly inhibit NF- $\kappa$ B activation and apoptotic cell death induced by paclitaxel. Furthermore, we found that paclitaxel could activate IKK activity and up-regulate its upstream regulator, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1). Our results suggest that IKK might play a crucial role in the mediation or regulation of paclitaxel-induced apoptosis in solid tumor cells.

## Materials and Methods

**Drugs and Cell Culture.** Paclitaxel was purchased from Calbiochem (La Jolla, CA) and dissolved in 100% dimethyl sulfoxide to make a 1.0 mM stock solution, which was then diluted in culture medium to obtain the desired concentrations. Glucocorticoids (triamcinolone acetonide) were dissolved in 100% ethanol as  $10^{-2}$  to  $10^{-5}$  M stock solutions. The I $\kappa$ B $\alpha$  phosphorylation inhibitor compound Bay 117821 was purchased from Alexis Co. (San Diego, CA) and dissolved in 100% dimethyl sulfoxide to make a 10 mM stock solution. Human wild-type breast tumor BCap37 cells, BCap37 cell lines stably transfected with sense or antisense I $\kappa$ B $\alpha$  cDNA (Huang et al., 2000), and human ovary tumor OV2008 cells were cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Hyclone Laboratories, Logan, UT).

**Plasmids and Recombinant Proteins.** pGEX-4T-I $\kappa$ B $\alpha$  fusion protein expression vectors were constructed by subcloning I $\kappa$ B $\alpha$  cDNA restriction enzyme fragments from pCR2.1-I $\kappa$ B $\alpha$  vectors. The constructs were confirmed by DNA sequencing. Glutathione *S*-transferase (GST)-I $\kappa$ B $\alpha$  fusion proteins were purified from *Escherichia coli* cells transformed with pGEX-I $\kappa$ B $\alpha$  expression vectors by using glutathione-agarose affinity chromatography (Amersham Biosciences, Piscataway, NJ) and confirmed by Western blot. Mutated human I $\kappa$ B $\alpha$  gene (deletion of NH<sub>2</sub>-terminal 36 amino acids, including Ser<sup>32</sup> and Ser<sup>36</sup>) was obtained by using polymerase chain reaction with wild-type I $\kappa$ B $\alpha$  cDNA as template and the pair of primers MUTI $\kappa$ B-5' (5'-ATGAAAGACGAGGAGTACGAG-3') and MUTI $\kappa$ B-3' (5'-CTTTGCACTCATAACGT-CAGA-3'). The polymerase chain reaction products were inserted into pCR 2.1 vectors (Invitrogen, Carlsbad, CA) and sequenced. Subsequently, mutant I $\kappa$ B $\alpha$  expression vectors were constructed from unique restriction sites available within the pCR2.1 vector. Mutant I $\kappa$ B $\alpha$  cDNAs were excised from pCR2.1 vectors and inserted into the high-level pDNA3 mammalian expression vector system (Invitrogen). All constructs were confirmed by DNA sequencing.

**Stable Transfection and Selection of Mutant I $\kappa$ B $\alpha$  cDNA-Transfected Cells.** Transfections were performed by Lipofectin (Invitrogen) as recommended by the manufacturer. Briefly, BCap37 cells were washed twice with Opti-MEM reduced-serum medium, and 3 ml of the same medium was added to the cells. Plasmid DNA (2  $\mu$ g/6-cm plate) containing mutant I $\kappa$ B $\alpha$  inserts was mixed with Lipofectin before addition to the tumor cells. Stable transfectants were selected by incubating the cells in the medium containing 500  $\mu$ g/ml Geneticin (G418). Surviving colonies were picked approximately 2 weeks later. Single colonies were amplified and continually grew in medium containing G418. Cells from each individual colony were examined for mutant I $\kappa$ B $\alpha$  expression by Western blot assays. Positive colonies were maintained in culture medium with G418 for further experiments. All transfectants were routinely cultured in RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin/streptomycin.

**Western Blotting.** Cells treated with different agents were harvested by trypsinization and washed with phosphate-buffered saline (PBS). Cellular protein was isolated using the protein extraction buffer containing 150 mM NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, 2% SDS. Protein concentrations were determined using Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of proteins (50  $\mu$ g/lane) were fractionated on a 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to PVDF membranes. The membranes were incubated with anti-I $\kappa$ B $\alpha$ , IKK $\alpha$ , MEKK1 primary antibodies, respectively (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:4000; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) followed by enhanced chemiluminescent staining using the enhanced chemiluminescence system (Amersham Biosciences).  $\beta$ -Actin was used to normalize for protein loading.

**Determination of Internucleosomal DNA Cleavage.** After tumor cells were treated with various drug regimes as indicated, cells were harvested, counted, and washed with PBS at 4°C. Then cells were suspended in lysis solution [5 mM Tris-HCl, 20 mM EDTA, and 0.5% (v/v) Triton X-100] for 20 min on ice. Detection of DNA fragmentation was performed as described previously (Cheng et al., 1995). DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 µg/ml ethidium bromide, and visualized under UV illumination.

**MTT Assays.** Cells were harvested with trypsin and resuspended to a final concentration of  $4 \times 10^4$  cells/ml in fresh medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Aliquots of the cell suspension were evenly distributed into 96-well tissue culture plates (100 µl/well) with lids (Falcon, Oxnard, CA). Designated columns were treated with the various drug regimes. One column from each plate contained medium alone and another column contained cells in drug-free media. At the end of each time points, the 96-well plates were centrifuged to collect all the detached cells and the media were carefully removed. Then 100 µl of a 1 mg/ml MTT solution, diluted in culture media, was added to each well. The plates were incubated at 37°C in 5% CO<sub>2</sub> atmosphere for 3 h, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. At the end of the 3-h incubation, the MTT solution was removed and 100 µl of dimethyl sulfoxide (Sigma, St. Louis, MO) was added to each well to dissolve the formazan crystals. To ensure complete dissolution of the formazan crystals, the plates were vortexed gently at low speed for 10 min. The absorbance in individual wells was determined at 560 nm by a microplate reader (Molecular Devices, Sunnyvale, CA).

**Immunoprecipitation and Kinase Assays.** Cells treated with various drug regimes were harvested by trypsinization and washed with PBS buffer and the pellet was resuspended in 60 to 90 µl of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 100 mM NaF, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg each of aprotinin and leupeptin per milliliter) and stored on ice for 20 min before centrifugation (14,000g, 20 min, 4°C). IκB kinase complex was immunoprecipitated by incubation for 1 h at 4°C with IKKα rabbit polyclonal antibodies (Santa Cruz Biotechnology) bound to protein-A Sepharose (Amersham Biosciences). The immunoprecipitates were washed twice with immunoprecipitation buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 1 mg of GST-IκBα fusion protein as substrate and 10 Ci/mmol [ $\gamma$ -<sup>32</sup>P]ATP. Reaction mixtures were incubated for 30 min at 30°C and stopped by the addition of 2× SDS-PAGE sample buffer. The phosphorylation of the IκBα proteins was examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The portion containing IKK was analyzed by Western blotting for IKKα protein as control.

**Flow Cytometry Analysis.** Cell sample preparation and propidium iodide (PI) staining for flow cytometry analysis were performed according to the method described by Nicoletti et al. (1991). BCap37 cells transfected with empty expression vector pcDNA3 (Vector), IκBα sense cDNA (WT IκBα), and mutant IκBα cDNA (MUT IκBα) were treated with paclitaxel in different concentrations (10, 100, and 500 nM) for 48 h. Cells were then harvested by trypsinization and washed twice with PBS followed by fixation in 1% formaldehyde and dehydration in 70% ethanol diluted in PBS. Cells were then incubated in PBS containing 100 µg/ml RNase and 40 µg/ml PI at 37°C for 1 h before flow cytometry analysis. Cell cycle distribution was determined using a Coulter Epics V instrument (Beckman Coulter, Inc., Fullerton, CA) with an argon laser set to excite at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, CA). The percentage of cells at the sub-G<sub>1</sub> was taken as measure of the apoptotic rate of the cell population.

**Nuclear Extraction Preparation and Electrophoretic Mobility Shift Assays.** Nuclear extracts were prepared via procedures described previously (Huang et al., 2000). In brief, after BCap37 cells transfected with empty pcDNA3 vectors or mutant IκBα were treated with paclitaxel for different concentrations for 24 h, cells were harvested and resuspended in 800 µl of hypotonic lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). Then cells were incubated on ice for 15 min. After that, 50 µl of 10% Nonidet P-40 was added, and cells were vigorously mixed and centrifuged. The nuclear pellets were suspended in 50 µl of buffer containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol (v/v) and mixed for 20 min and centrifuged to produce supernatant containing nuclear proteins. Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad).

EMSAs were performed using <sup>32</sup>P-labeled double-stranded oligonucleotide probes, which contain a specific consensus sequence (5'-AGTTGAGGGGAGTTTCCCAGGC-3'; Santa Cruz Biotechnology) recognized by NF-κB. Probes were labeled with T4 polynucleotide kinase (Promega, Madison, WI) and [ $\gamma$ -<sup>32</sup>P]ATP and purified using G-50 spin columns (Eppendorf-5 Prime, Inc., Boulder, CO). EMSAs binding reaction mixture contained 1 µg of protein of nuclear extract, 2 µg of poly(dI-dC) (Sigma), and  $\gamma$ -<sup>32</sup>P-labeled probe (4000 cpm) in binding buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.2 mg/ml albumin). The binding reaction was incubated for 30 min at room temperature. After the binding reactions, bound and free probes were separated by electrophoresis on 6% native polyacrylamide gels. The gels were dried and exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY).

**Northern Blotting.** BCap37 cells were treated with different concentrations of paclitaxel for 24 h. Total RNA was isolated and 20 µg was fractionated in 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane, and UV cross-linked. The membrane was probed with [<sup>32</sup>P]UTP-labeled antisense MEKK1 RNA probes generated from the subcloned MEKK1 cDNA fragments in pcDNA3 vectors. The membrane was then washed and autoradiographed. The same membrane was stripped and reprobed with human antisense β-actin RNA probes to normalize RNA loading.

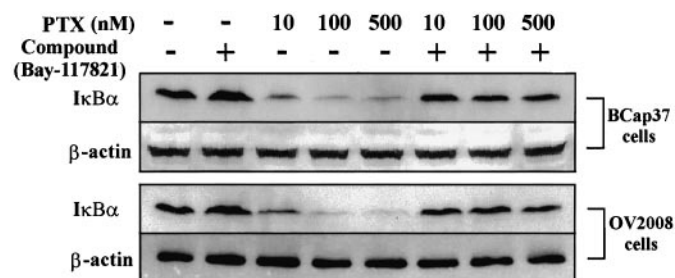
## Results

**IκBα Phosphorylation Inhibitor Prevents Paclitaxel-Induced IκBα Degradation and Inhibits Paclitaxel-Induced Apoptosis.** Our previous studies revealed that paclitaxel induced IκBα protein degradation in BCap37, OV2008, and other solid tumor cells (Huang et al., 2000). Because IκBα degradation was mainly caused by its phosphorylation and ubiquitination (Baeuerle, 1991), we used a novel IκBα phosphorylation inhibitor, Bay 117821, that was recently identified to selectively inhibit cytokine-induced IκBα phosphorylation and degradation in human endothelial cells (Pierce et al., 1997), to examine whether the inhibition of IκBα degradation could affect paclitaxel-induced apoptotic cell death. By Western blot, we determined that cotreatment with Bay 117821 (10 µM) significantly blocked the degradation of IκBα induced by paclitaxel in both BCap37 and OV2008 cells (Fig. 1). Subsequently, we performed DNA fragmentation and MTT assays to evaluate the influence of the IκBα phosphorylation inhibitor on paclitaxel-induced apoptotic cell death and overall cytotoxicity. The results shown in Fig. 2A indicate that paclitaxel alone was able to induce characteristic DNA fragmentation at 10 nM or greater concentrations within 48 h. However, concurrent treatment of Bay 117821 (10 µM) with paclitaxel significantly inhibited

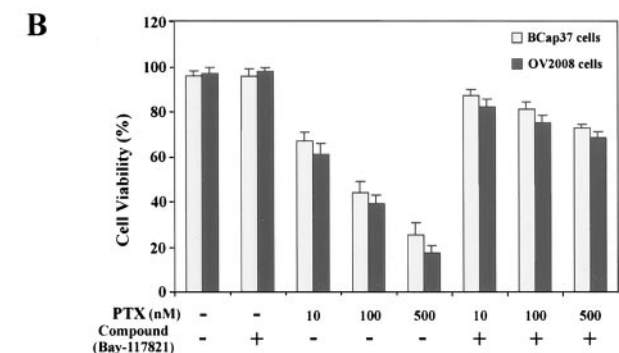
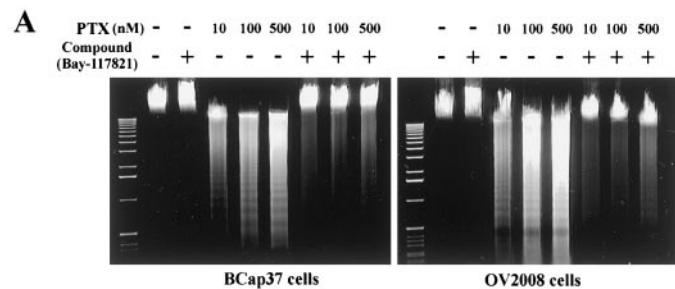
paclitaxel-induced apoptosis. Furthermore, MTT assays also showed that the specific inhibitor of I $\kappa$ B $\alpha$  phosphorylation interfered with the cytotoxicity of paclitaxel in both BCap37 cells and OV2008 cells (Fig. 2B). These results suggest that the phosphorylation and degradation of I $\kappa$ B $\alpha$  might be a critical step for the activation of NF- $\kappa$ B and the mediation of paclitaxel-induced apoptosis.

**Paclitaxel Activates I $\kappa$ B Kinase Activity.** To study the intrinsic mechanisms by which paclitaxel down-regulates I $\kappa$ B $\alpha$  protein and leads to the activation of NF- $\kappa$ B, we next examined the effect of paclitaxel on endogenous IKK activity. As described under *Materials and Methods*, GST-I $\kappa$ B $\alpha$  fusion proteins were purified from isopropyl  $\beta$ -D-thiogalactoside-induced *E. coli* cells that were transformed with pGEX-I $\kappa$ B $\alpha$  fusion vectors and used as substrates for kinase assays, whereas the IKK complexes were prepared by immunoprecipitation of cell extracts harvested from BCap37 and

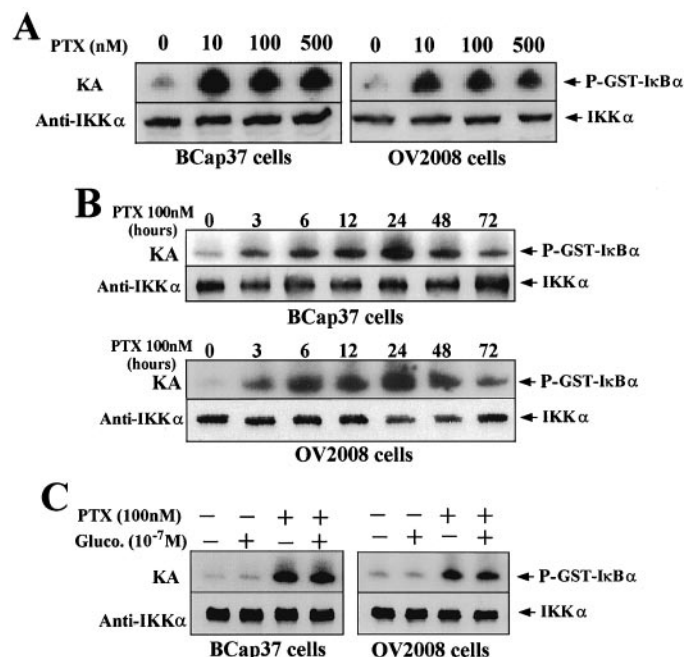
OV2008 cells treated with a variety of concentrations or different time points of paclitaxel. The results of kinase assays depicted in Fig. 3 indicate that the phosphorylation of substrate GST-I $\kappa$ B $\alpha$  protein (P-GST-I $\kappa$ B $\alpha$ ) was remarkably stimulated by IKK complexes obtained from BCap37 or OV2008 cells treated with 10 nM or greater concentrations of paclitaxel for 24 h (Fig. 3A). When tumor cells were treated with 100 nM paclitaxel, the IKK activation was observed as early as 3 h (Fig. 3B). These results indicate that paclitaxel activated I $\kappa$ B kinase activity, which in turn led to I $\kappa$ B $\alpha$  phosphorylation and degradation. In addition, we also exam-



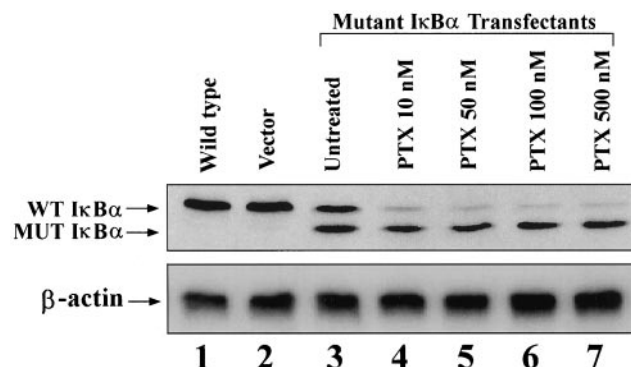
**Fig. 1.** I $\kappa$ B $\alpha$  phosphorylation inhibitor prevents paclitaxel-induced I $\kappa$ B $\alpha$  degradation. BCap37 and OV2008 cells were exposed to the indicated concentrations of paclitaxel (PTX) for 24 h with or without the simultaneous treatment of compound Bay 117821 (10  $\mu$ M). Equal amounts (50  $\mu$ g/lane) of cellular protein were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes followed by immunoblotting with an anti-I $\kappa$ B $\alpha$  polyclonal antibody.



**Fig. 2.** I $\kappa$ B $\alpha$  phosphorylation inhibitor represses paclitaxel-induced apoptosis. BCap37 and OV2008 cells were treated with different concentrations of paclitaxel (PTX) with or without the cotreatment of 10  $\mu$ M compound Bay 117821 for 48 h. Cells were then harvested for DNA fragmentation assays (A) or MTT assays (B). The results of the MTT assay were presented as means  $\pm$  S.D. based on three independent experiments.



**Fig. 3.** Paclitaxel activates I $\kappa$ B kinase activity. BCap37 cells and OV2008 cells were treated with different concentrations of paclitaxel (PTX) for 24 h (A); or 100 nM paclitaxel in a time course as indicated (B); or 100 nM paclitaxel for 24 h with/without pretreatment of glucocorticoids (10<sup>-7</sup> M) (C). IKK complex was immunoprecipitated with an anti-IKK $\alpha$  antibody and then was subjected to the in vitro kinase assay (KA) by using GST-I $\kappa$ B $\alpha$  as the substrate. After SDS-PAGE, the gel containing the substrate was dried and processed for autoradiography. The portion containing IKK was analyzed by Western blotting for IKK $\alpha$  protein.

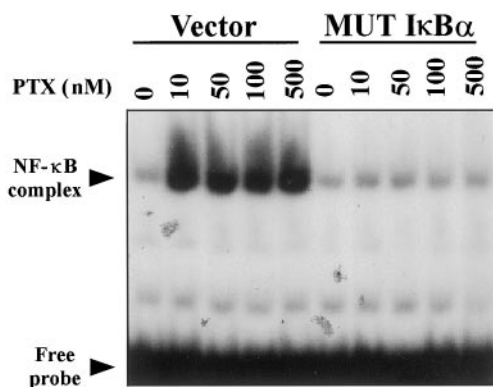


**Fig. 4.** Paclitaxel does not degrade mutant I $\kappa$ B $\alpha$ . Equal amounts (50  $\mu$ g/lane) of cellular proteins from wild-type BCap37 cells (lane 1), pcDNA 3 vector transfectants (lane 2), and mutant I $\kappa$ B $\alpha$  transfectants (lanes 3–7) were fractionated on 12.5% SDS-PAGE gel and transferred to PVDF membranes followed by immunoblot with anti-I $\kappa$ B $\alpha$  polyclonal antibody.  $\beta$ -Actin protein was used as a control.

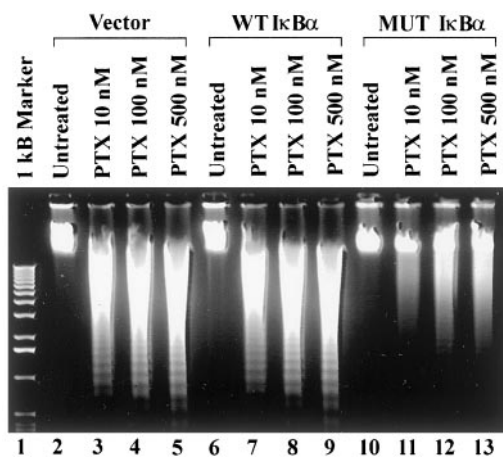
ined the possible effect of glucocorticoids on IKK activity. The result showed that glucocorticoids did not interfere with IKK activity induced by paclitaxel in both BCap37 and OV2008 cells (Fig. 3C). These data provide another piece of evidence that glucocorticoids antagonize paclitaxel-induced NF- $\kappa$ B activation through induction of I $\kappa$ B $\alpha$  protein expression rather than inhibition of I $\kappa$ B $\alpha$  degradation.

**Mutant I $\kappa$ B $\alpha$  Lacking Ser<sup>32</sup> and Ser<sup>36</sup> Suppresses Paclitaxel-Induced NF- $\kappa$ B Activation.** Proteolytic degradation of I $\kappa$ B $\alpha$  is essential for activation of NF- $\kappa$ B (Baeuerle, 1991; Sun et al., 1995). Previous studies have revealed that the degradation of I $\kappa$ B $\alpha$  protein is mainly due to the inducible phosphorylation of I $\kappa$ B $\alpha$  at Ser<sup>32</sup> and Ser<sup>36</sup> by I $\kappa$ B kinase complex (Brown et al., 1995; Traenckner et al., 1995; DiDonato et al., 1997). To further confirm that paclitaxel down-

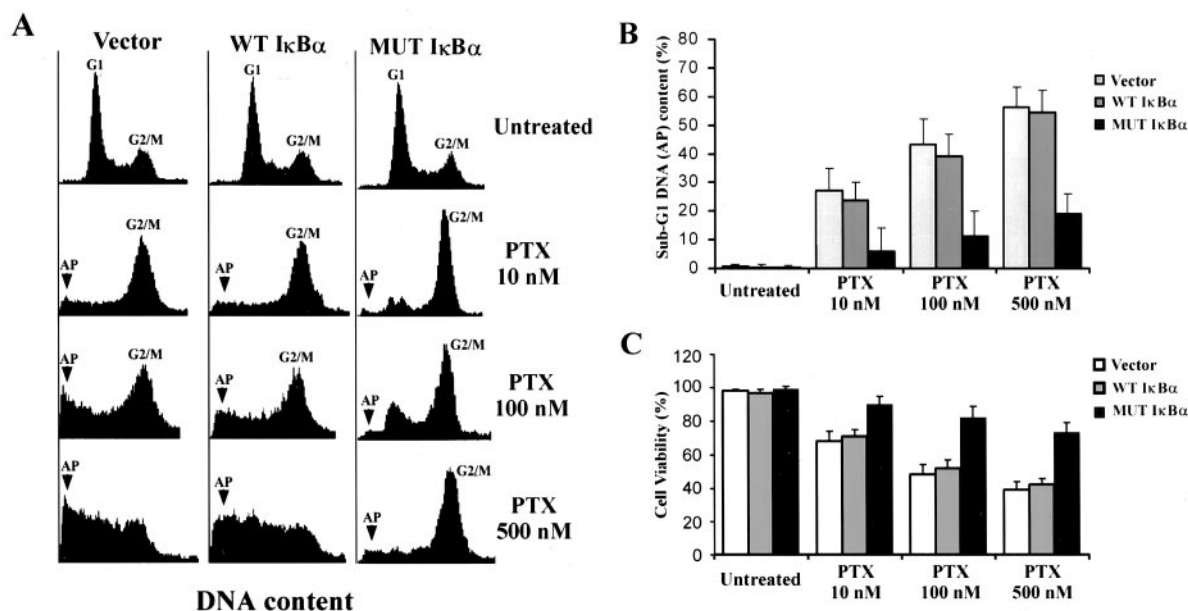
regulates I $\kappa$ B $\alpha$  through induction of I $\kappa$ B $\alpha$  phosphorylation and degradation, we constructed a mutant I $\kappa$ B $\alpha$  expression vector by deleting 36 amino acids, including Ser<sup>32</sup> and Ser<sup>36</sup> from the NH<sub>2</sub> terminus of I $\kappa$ B $\alpha$  gene. Such a mutant I $\kappa$ B $\alpha$  protein cannot be degraded by the I $\kappa$ B kinase complex but still possesses the ability to bind to NF- $\kappa$ B through the interior ankyrin motif domain and functions as a super suppressor of NF- $\kappa$ B molecules (Brown et al., 1995; Shinohara et al., 2001). As shown in Fig. 4, BCap37 cells with stable transfection of this mutant I $\kappa$ B $\alpha$  expressed a smaller size of I $\kappa$ B $\alpha$  protein, which was not degraded in the presence of paclitaxel. Furthermore, we examined the effect of mutant I $\kappa$ B $\alpha$  on paclitaxel-induced DNA-binding activity of NF- $\kappa$ B. By EM-



**Fig. 5.** Expression of mutant I $\kappa$ B $\alpha$  blocks paclitaxel-induced NF- $\kappa$ B activation. BCap37 cells transfected with mutant I $\kappa$ B $\alpha$  (MUT I $\kappa$ B $\alpha$ ) or pCDNA3 vector only (Vector) were treated with different concentrations of paclitaxel for 24 h. Equal amounts of nuclear cell extracts were subjected to EMSAs with a  $\gamma$ -<sup>32</sup>P-labeled oligonucleotide encompassing the NF- $\kappa$ B binding site.



**Fig. 6.** Transfection of mutant I $\kappa$ B $\alpha$  inhibits paclitaxel-induced DNA fragmentation. BCap37 cells transfected with empty vectors (lanes 2–5), wild-type sense I $\kappa$ B $\alpha$  cDNA (lanes 6–9), and mutant I $\kappa$ B $\alpha$  cDNA (lanes 10–13) were treated with 10, 100, and 500 nM paclitaxel for 48 h. Fragmented DNA was analyzed by electrophoresis in 1.2% agarose gel containing 0.1% ethidium bromide. Lane 1 was 1-kilobase DNA marker.



**Fig. 7.** Transfection of mutant I $\kappa$ B $\alpha$  suppresses paclitaxel-induced apoptosis. BCap37 cells transfected with empty vectors (Vectors), wild-type sense I $\kappa$ B $\alpha$  cDNA (WT I $\kappa$ B $\alpha$ ), and mutant I $\kappa$ B $\alpha$  cDNA (MUT I $\kappa$ B $\alpha$ ) were treated with 10, 100, and 500 nM paclitaxel for 48 h. Then cells were harvested and stained for DNA with propidium iodide (PI) for flow cytometric analysis. The peaks corresponding to G<sub>1</sub> and G<sub>2</sub>/M phases of the cell cycle are indicated. The sub-G<sub>1</sub> peaks labeled as AP represent apoptotic cells (A). The percentage of cells with a sub-G<sub>1</sub> DNA content was taken as a measure of apoptotic rate of the cell population (B). The cell viability was measured by MTT assays (C). Data in the bar graph are means  $\pm$  S.D. of three independent experiments.

SAs, an increased level of DNA-binding activity was clearly detected in empty vector-transfected BCap37 cells exposed to paclitaxel, but this elevated DNA-binding activity of NF- $\kappa$ B by paclitaxel was markedly inhibited in the cells transfected with the mutant I $\kappa$ B $\alpha$  (Fig. 5). These findings demonstrated that the mutant I $\kappa$ B $\alpha$  could interfere with paclitaxel-induced NF- $\kappa$ B activation.

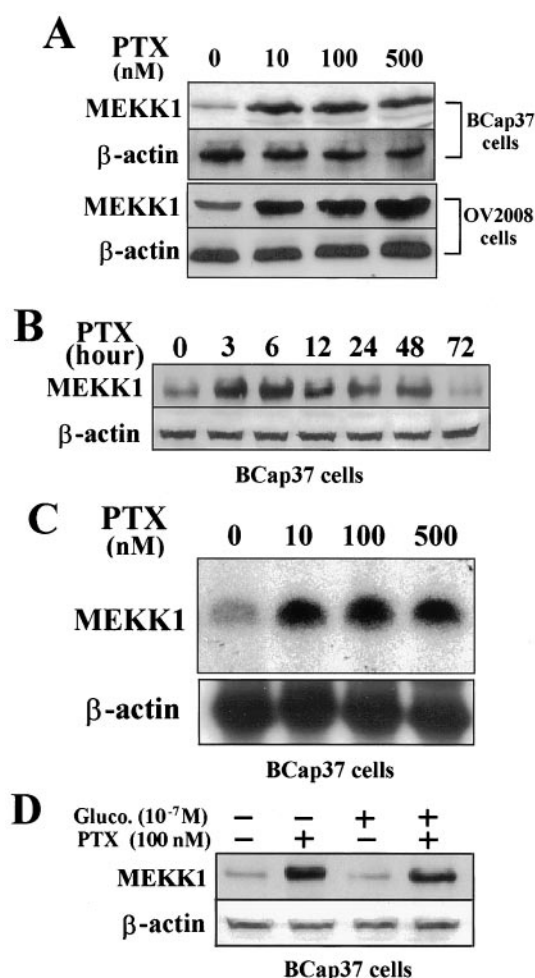
**Transfection of Mutant I $\kappa$ B $\alpha$  Reduces Sensitivity of Tumor Cells to Paclitaxel-Induced Apoptosis.** Next, the tumor cells with stable transfection of the mutant I $\kappa$ B $\alpha$  were compared with their parental cells to determine whether the expression of the mutant I $\kappa$ B $\alpha$  altered the sensitivity of tumor cells to paclitaxel-induced apoptosis. As depicted in Fig. 6, BCap37 cells transfected with empty pcDNA3 expression vectors (Vector), wild-type I $\kappa$ B $\alpha$  cDNA (WTI $\kappa$ B $\alpha$ ) (Huang et al., 2000), and mutant I $\kappa$ B $\alpha$  cDNA (MUTI $\kappa$ B $\alpha$ ) were treated with a series of increasing concentrations of paclitaxel (10–500 nM) for 48 h followed by the DNA fragmentation assay. We observed that the cells transfected with the mutant I $\kappa$ B $\alpha$  exhibited more resistance to paclitaxel-induced apoptosis. By flow cytometric analyses, we also observed that the percentage of cells at sub-G<sub>1</sub> DNA, which is believed to represent apoptotic cell populations, was dramatically decreased in mutant I $\kappa$ B $\alpha$  transfectants in comparison with those transfected with the empty vector or wild-type I $\kappa$ B $\alpha$  (Fig. 7, A and B). MTT assays showed that mutant I $\kappa$ B $\alpha$  significantly increased the cell viability in presence of paclitaxel (Fig. 7C). These results indicate that the introduction of the mutant I $\kappa$ B $\alpha$  resulted in the decreased sensitivity of tumor cells to paclitaxel-induced apoptosis.

**Paclitaxel Up-Regulates MEKK1 Expressions.** Latest studies revealed that MEKK1 phosphorylates the IKK subunit, preferentially IKK $\beta$ , resulting in the activation of NF- $\kappa$ B in response to some cytokine stimuli [such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1] (Karin and Delhase, 1998; May and Ghosh, 1999). To investigate whether paclitaxel activates IKK through regulation of MEKK1 activity, we examined the possible alteration of MEKK1 protein in the tumor cells treated with different concentrations of paclitaxel. By Western blot, we found that paclitaxel enhanced the protein levels of MEKK1 in both BCap37 and OV2008 cells (Fig. 8A). The increase of MEKK1 protein level was observed as early as 3 h after paclitaxel treatment (Fig. 8B). Furthermore, Northern blot analysis showed that the mRNA expressions of MEKK1 were stimulated by paclitaxel treatment (Fig. 8C), suggesting that MEKK1 might be the primary target of paclitaxel in the NF- $\kappa$ B/I $\kappa$ B signaling pathway. In addition, we examined whether glucocorticoids exposure affects paclitaxel-induced MEKK1 activity. As expected, glucocorticoids did not change paclitaxel-enhanced MEKK1 protein expressions (Fig. 8D), implying that glucocorticoids do not interfere with paclitaxel-mediated activities of upstream regulators of NF- $\kappa$ B/I $\kappa$ B signaling pathway.

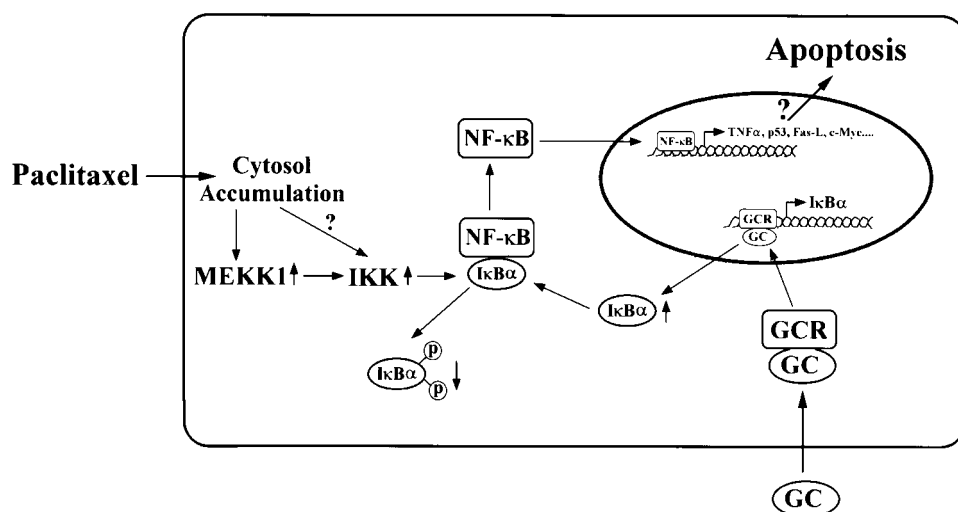
## Discussion

This study investigates the molecular mechanisms of paclitaxel-induced apoptosis via the activation of the NF- $\kappa$ B/I $\kappa$ B signaling pathway. In previous studies, we have demonstrated that paclitaxel could degrade I $\kappa$ B $\alpha$  protein and promote the nuclear translocation and DNA-binding activity of transcription factor NF- $\kappa$ B (Huang et al., 2000). Activation

of NF- $\kappa$ B has been believed to play an important role in coordinating the control of apoptotic cell death, either as a promoter or, perhaps more commonly, as a blocker of apoptosis (Beg and Baltimore, 1996; Grimm et al., 1996; Qin et al., 1998; Ryan et al., 2000). The purpose of this study was to determine how activation of NF- $\kappa$ B regulates paclitaxel-induced apoptotic cell death and whether I $\kappa$ B $\alpha$  phosphorylation and degradation are essential for paclitaxel-induced NF- $\kappa$ B activation. BCap37 and OV2008 cells were first tested by cotreatment with paclitaxel and a recently identified I $\kappa$ B $\alpha$  phosphorylation inhibitor, Bay 117821, which has been shown to specifically inhibit the phosphorylation of I $\kappa$ B $\alpha$  induced by some cytokines such as TNF- $\alpha$  (Pierce et al., 1997). The results indicate that Bay 117821 can prevent paclitaxel-induced I $\kappa$ B $\alpha$  degradation in both BCap37 and OV2008 cells (Fig. 1). Meanwhile, we determined that the tumor cells cotreated with Bay 117821 exhibited reduced sensitivity to paclitaxel-induced apoptosis (Fig. 2). These re-



**Fig. 8.** Paclitaxel induces MEKK1 expression. BCap37 cells or OV2008 cells treated with increasing concentrations of paclitaxel (PTX) as indicated for 24 h (A) or 100 nM paclitaxel in a time course as indicated (B) were immunoblotted with anti-MEKK1 polyclonal antibody; BCap37 cells were treated with different concentrations of paclitaxel (PTX) for 24 h (C). RNA (20  $\mu$ g/lane) was size fractionated by formaldehyde/agarose gel electrophoresis. After transfer to the nitrocellulose membrane, RNA was hybridized with [<sup>32</sup>P]UTP-labeled antisense riboprobes synthesized from MEKK1 pCDNA3 vectors; BCap37 cells were exposed 100 nM paclitaxel with or without the preincubation of glucocorticoids (10<sup>-7</sup> M) for 24 h followed by immunoblotting with anti-MEKK1 polyclonal antibody (D).



**Fig. 9.** Hypothesized mechanism of paclitaxel-induced apoptosis mediated by the I $\kappa$ B $\alpha$ -F- $\kappa$ B signaling pathway and the inhibitory action of glucocorticoids. GC, glucocorticoids; GCR, glucocorticoids receptor.

sults suggest that the proteolytic degradation of I $\kappa$ B $\alpha$  might be an important step for the activation of NF- $\kappa$ B, which in turn mediates paclitaxel-induced apoptosis in these solid tumor cells. The precise molecular target for I $\kappa$ B $\alpha$  phosphorylation inhibitor Bay 117821 is not yet clear. Although Bay 117821 was shown to inhibit I $\kappa$ B $\alpha$  phosphorylation and degradation, this may be the result of direct inhibition of a paclitaxel-inducible I $\kappa$ B $\alpha$  kinase or due to inhibition of a signaling event upstream of the I $\kappa$ B $\alpha$  kinase. The exact mechanism of the inhibitory action of Bay 117821 on paclitaxel-induced I $\kappa$ B $\alpha$  degradation needs to be investigated further.

Recent studies have identified a high molecular mass complex of I $\kappa$ B kinases (IKK $\alpha$  and IKK $\beta$ ) that plays a key role in I $\kappa$ B $\alpha$  protein phosphorylation and degradation (DiDonato et al., 1997; Delhase et al., 1999; May and Ghosh, 1999). We therefore examined the potential effect of paclitaxel on IKK activity. By *in vitro* I $\kappa$ B kinase assay, we demonstrated that IKK activities were significantly stimulated by paclitaxel in both BCap37 and OV2008 cells (Fig. 3). Next, we constructed a mutant I $\kappa$ B $\alpha$  expression vector in which an N-terminal fragment containing Ser<sup>32</sup> and Ser<sup>36</sup> was deleted. Based on current knowledge, the degradation of I $\kappa$ B $\alpha$  is mainly due to the inducible phosphorylation of Ser<sup>32</sup> and Ser<sup>36</sup>. Deletion or substitution of these two amino acids with other residues has been reported to prevent I $\kappa$ B $\alpha$  from signal-induced phosphorylation (Brown et al., 1995; Shinohara et al., 2001). Through stable transfection of this mutant I $\kappa$ B $\alpha$  into wild-type BCap37 cells, we demonstrated that the mutant I $\kappa$ B $\alpha$  protein was insensitive to IKK-mediated phosphorylation and degradation but still possessed the ability to interact with cytoplasmic NF- $\kappa$ B and inhibit paclitaxel-induced NF- $\kappa$ B activation. (Figs. 4 and 5). Meanwhile, the results from DNA fragmentation and flow cytometric assays revealed that the expression of the mutant I $\kappa$ B $\alpha$  significantly inhibited paclitaxel-induced apoptotic cell death (Figs. 6 and 7). These findings further indicate that paclitaxel-stimulated IKK is critical for I $\kappa$ B $\alpha$  degradation and consequent activation of NF- $\kappa$ B. Blockage of NF- $\kappa$ B activation by the mutant I $\kappa$ B $\alpha$  disrupts the signaling pathway leading to paclitaxel-induced apoptotic cell death.

In light of these experimental results and our previous studies, the activation of NF- $\kappa$ B seems to act as a promoter in

paclitaxel-induced apoptosis. However, it is currently unclear how the activated NF- $\kappa$ B triggers the downstream apoptotic machinery. NF- $\kappa$ B is a nuclear transcriptional factor. Theoretically, it is assumed to mediate paclitaxel-induced apoptosis through the regulation of gene expressions, particularly for those genes whose expressions are associated with apoptotic cell death. To date, NF- $\kappa$ B has been reported to participate in the transcription of more than 150 target genes (Pahl, 1999). Many of these NF- $\kappa$ B target genes are considered as proapoptotic genes, such as FAS/APO-1 ligand (FasL), *c-myc*, ICE, and p53 (Suda et al., 1993; Wu and Lozano, 1994; Brown et al., 1995). Moreover, some of these genes, including p53, *c-myc*, and FasL were even found to respond to paclitaxel in certain normal and tumor cells (La Rosa et al., 1994; Blagosklonny et al., 1995; el Khyari et al., 1997; Srivastava et al., 1999). Therefore, although the effector gene(s) that potentially contributes to paclitaxel-induced apoptosis remains unidentified, it should be reasonable to hypothesize that activated NF- $\kappa$ B might stimulate the expression of a specific proapoptotic gene that eventually triggers the downstream signaling pathway, leading to the paclitaxel-induced apoptotic cell death.

MEKK1 is a 196-kDa enzyme that is involved in the regulation of the c-Jun NH<sub>2</sub>-terminal kinase pathway and apoptosis (Lange-Carter et al., 1993). Latest evidence shows that MEKK1 can phosphorylate and activate IKK (preferentially IKK $\beta$ ) in response to a variety of cytokine stimuli (May and Ghosh, 1999). It was also reported that under different circumstances overexpression of MEKK1 was found to stimulate NF- $\kappa$ B activities (Hirano et al., 1996; Meyer et al., 1996), and MEKK1-induced NF- $\kappa$ B activation can be inhibited by the dominant negative IKK $\alpha$  and IKK $\beta$  mutation (Lee et al., 1998). These findings suggest that the IKK complex may be the major substrate of MEKK1 and that IKK activation depends on MEKK1 activity. In this study, we analyzed the expression of MEKK1 in the cells treated with paclitaxel and found that paclitaxel was able to up-regulate both protein and mRNA levels of MEKK1 in BCap37 and OV2008 cells (Fig. 8). Based on this finding, we suspect that MEKK1 may be the primary target of paclitaxel. The up-regulated MEKK1 then, in turn, activates the IKK activity and the NF- $\kappa$ B signaling pathway.

Glucocorticoids are routinely used in the clinical applica-

tion of paclitaxel to prevent hypersensitivity reactions (McEvoy, 1995). Glucocorticoids have been previously demonstrated to inhibit paclitaxel-induced apoptosis and NF- $\kappa$ B activation through induction of I $\kappa$ B $\alpha$  synthesis (Fan, et al, 1996a,b; Huang et al., 2000). To exclude the possibility that glucocorticoids may directly affect IKK and MEKK1, we also examined IKK activity and MEKK1 expression in the cells exposed to glucocorticoids. Our results indicate that glucocorticoids do not interfere with either IKK activity or MEKK1 expression in the presence or absence of paclitaxel (Figs. 3D and 8D). These expected results support our previous hypothesis that glucocorticoids antagonize paclitaxel-induced I $\kappa$ B $\alpha$  degradation by stimulating I $\kappa$ B $\alpha$  synthesis rather than by interfering with the I $\kappa$ B $\alpha$  degradation or its upstream events.

On the basis of these observations and our previous data on the opposite regulation of NF- $\kappa$ B activation by paclitaxel and glucocorticoids, we would hypothesize the following pathway to explain paclitaxel-induced apoptosis and the inhibitory action of glucocorticoids (Fig. 9). Briefly, exposure of tumor cells to paclitaxel leads to the increased expression of MEKK1, which in turn activates IKK. The activated IKK then causes the degradation of I $\kappa$ B $\alpha$  and the disassociation of the NF- $\kappa$ B/I $\kappa$ B- $\alpha$  complex. Subsequently, the released cytoplasmic NF- $\kappa$ B translocates into the nucleus, where it functions as a transcription factor to regulate apoptosis-associated gene expression. Conversely, glucocorticoids inhibit paclitaxel-induced apoptosis through induction of I $\kappa$ B- $\alpha$  protein synthesis, which antagonizes paclitaxel-mediated NF- $\kappa$ B nuclear translocation and activation. Given this hypothesized pathway, MEKK1 might be the primary target of paclitaxel, whereas the activation of IKK plays a critical role in the subsequent activation of NF- $\kappa$ B and the regulation of paclitaxel-induced apoptotic cell death in solid tumor cells.

#### Acknowledgments

We thank Dr. Debra Hazen-Martin for critical review of this manuscript.

#### References

- Bauerle PA (1991) The inducible transcription factor NF- $\kappa$ B: regulation by distinct protein subunits. *Biochim Biophys Acta* **12**:141–179.
- Baldwin AS Jr (1996) The NF- $\kappa$ B and I $\kappa$ B proteins: new discoveries and insights. *Annu Rev Immunol* **14**:649–683.
- Beg AS and Baltimore D (1996) An essential role of NF- $\kappa$ B in preventing TNF- $\alpha$ -induced cell death. *Science (Wash DC)* **274**:782–784.
- Bhalla K, Ibrado AM, Tourkina E, Tang C, Mahoney ME, and Huang Y (1993) Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia* **7**:563–568.
- Blagosklonny MV, Schulte TW, Nguyen P, Mimnaugh EG, Trepel J, and Neckers L (1995) Taxol induction of p21WAF1 and p53 requires c-raf-1. *Cancer Res* **55**:4623–4626.
- Brown K, Gerstberg S, Carlson L, Franzoso G, and Siebenlist U (1995) Control of I $\kappa$ B- $\alpha$  proteolysis by site-specific, signal-induced phosphorylation. *Science (Wash DC)* **267**:1485–1488.
- Brown K, Park S, Kanno T, Franzoso G, and Siebenlist U (1993) Mutual regulation of the transcriptional activator NF- $\kappa$ B and its inhibitor, I $\kappa$ B- $\alpha$ . *Proc Natl Acad Sci USA* **90**:2532–2536.
- Cheng L, Zheng S, Raghunathan K, Priest DG, Willingham MC, Norris JS, and Fan W (1995) Characterizations of paclitaxel-induced apoptosis and altered gene expression in human breast cancer cells. *Cell Pharmacol* **2**:249–257.
- Delhase M, Hayakawa M, Chen Y, and Karin M (1999) Positive and negative regulation of I $\kappa$ B kinase activity through IKK $\beta$  subunit phosphorylation. *Science (Wash DC)* **284**:309–313.
- DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, and Karin M (1997) A cytokine-responsive I $\kappa$ B kinase that activates the transcription factor NF- $\kappa$ B. *Nature (Lond)* **388**:548–554.
- el Khyari S, Bourgaire V, Barra Y, Braguer D, and Briand C (1997) Pretreatment by tubulin agents decreases C-MYC induction in human colon carcinoma cell line HT29–D4. *Biochem Biophys Res Commun* **231**:751–754.
- Fan W (1999) Possible mechanism of paclitaxel-induced apoptosis. *Biochem Pharmacol* **57**:1215–1221.
- Fan W, Cheng LR, Norris JS, and Willingham MC (1996a) Glucocorticoids selectively inhibit paclitaxel-induced apoptosis in a human breast cancer cell line. *Cell Pharmacol* **3**:435–440.
- Fan W, Everett ET, Tang C, Cooper T, Fang Q, Bhalla K, and Norris JS (1994) Glucocorticoid-mediated inhibition of paclitaxel-induced apoptosis in leiomyosarcoma cells. *Cell Pharmacol* **1**:205–212.
- Fan W, Schandl CA, Cheng L, Norris JS, and Willingham MC (1996b) Glucocorticoids modulate Taxol cytotoxicity in human solid tumor cells. *Cell Pharmacol* **3**:343–348.
- Fuchs DA and Johnson RK (1978) Cytologic evidence that Taxol, an antineoplastic agent from *Taxus brevifolia*, acts as a mitotic spindle poison. *Cancer Treat Rep* **62**:1219–1222.
- Grimm S, Bauer MKA, Baeuerle PA, and Schulze-Osthoff K (1996) Bcl-2 downregulates the activity of transcription factor NF- $\kappa$ B induced upon apoptosis. *J Cell Biol* **134**:13–23.
- Haldar S, Jena N, and Croce CM (1995) Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* **92**:4507–4511.
- Hirano M, Osada S, Aoki T, Hirai S, Hosaka M, Inoue J, and Ohno S (1996) MEK kinase is involved in tumor necrosis factor alpha-induced NF-kappa B activation and degradation of I kappa B-alpha. *J Biol Chem* **271**:8971–8976.
- Holmes FA, Walters RS, Theriault RL, Forman AD, Newton LK, Raber MN, Buzdar AU, Frey DK, and Horrtobagyi GN (1991) Phase II trial of Taxol, an active drug in the treatment of metastatic breast cancer. *J Natl Cancer Inst* **83**:1797–1805.
- Huang Y, Johnson KR, Norris JS, and Fan W (2000) NF- $\kappa$ B/I $\kappa$ B signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. *Cancer Res* **60**:4426–4432.
- Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, and Wilson L (1996) Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* **56**:816–825.
- Karin M and Delhase M (1998) JNK or IKK, AP-1 or NF- $\kappa$ B, which are the targets for MEK kinase 1 action? *Proc Natl Acad Sci USA* **95**:9067–9069.
- La Rosa FA, Pierce JW, and Sonenshein GE (1994) Differential regulation of c-myc oncogene promoter by NF- $\kappa$ B Rel family of transcription factors. *Mol Cell Biol* **14**:1039–1044.
- Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, and Johnson GL (1993) A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science (Wash DC)* **260**:315–319.
- Lee FS, Peters RT, Dang LC, and Maniatis T (1998) MEKK1 activates both I $\kappa$ B kinase  $\alpha$  and I $\kappa$ B kinase  $\beta$ . *Proc Natl Acad Sci USA* **95**:9319–9324.
- May MJ and Ghosh S (1999) I $\kappa$ B kinase: kinsmen with different crafts. *Science (Wash DC)* **284**:271–273.
- McEvoy GK (1995) *Antineoplastic Agents, AHFS Drug Information*, pp 715–718, American Society of Health-System Pharmacists Inc., Bethesda, MD.
- Meyer CF, Wang X, Chang C, Templeton D, and Tan TH (1996) Interaction between c-Rel and the mitogen-activated protein kinase kinase 1 signaling cascade in mediating kappaB enhancer activation. *J Biol Chem* **271**:13234–13248.
- Miller MC, Johnson KR, Willingham MC, and Fan W (1999) Apoptotic cell death induced by baccatin III, a precursor of paclitaxel, may occur without G<sub>2</sub>/M arrest. *Cancer Chemother Pharmacol* **57**:1215–1221.
- Moos PJ and Fitzpatrick FA (1998) Taxane-mediated gene induction is independent of microtubule stabilization: induction of transcriptional regulators and enzymes that modulate inflammation and apoptosis. *Proc Natl Acad Sci USA* **95**:3896–3901.
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, and Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139**:271–279.
- Pahl H (1999) Activators and target genes of Rel/NF- $\kappa$ B transcription factors. *Oncogene* **18**:6853–6866.
- Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, and Gerritsen ME (1997) Novel inhibitors of cytokine-induced I $\kappa$ B $\alpha$  phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. *J Biol Chem* **272**:21096–21103.
- Qin ZH, Wang Y, Nakai M, and Chase TN (1998) Nuclear factor- $\kappa$ B contributes to excitotoxin-induced apoptosis in rat striatum. *Mol Pharmacol* **53**:33–42.
- Rowinsky EK, Cazenave LA, and Donehower RC (1990) Taxol: a novel investigational antimicrotubule agent. *J Natl Cancer Inst* **82**:1247–1259.
- Ryan KM, Ernst MK, Rice NR, and Vousden KH (2000) Role of NF- $\kappa$ B in p53-mediated programmed cell death. *Nature (Lond)* **404**:892–896.
- Schiff PB, Frant J, and Horwitz SB (1979) Promotion of microtubule assembly *in vitro* by Taxol. *Nature (Lond)* **22**:665–667.
- Shinohara T, Miki T, Nishimura N, Nohkura H, Hamada H, Mukaida N, and Sone S (2001) Nuclear factor- $\kappa$ B-dependent expression of metastasis suppressor KA11/CD82 gene in lung cancer cell lines expressing mutant p53. *Cancer Res* **61**:673–678.
- Srivastava RK, Sasaki CY, Hardwick JM, and Longo DL (1999) Bcl-2-mediate drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. *J Exp Med* **190**:253–265.
- Strober T, Swanson L, Korsmeyer S, and Cannistra SA (1996) BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway. *Proc Natl Acad Sci USA* **90**:14094–14099.
- Suda T, Takahashi T, Golstein P, and Nagata S (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75**:1169–1178.
- Sun S, Elwood J, and Greene WC (1995) Both amino- and carboxyl-terminal sequences within I $\kappa$ B $\alpha$  regulate its inducible degradation. *Mol Cell Biol* **16**:1058–1065.
- Tishler RB, Geard CR, Hall EJ, and Schiff PB (1992) Taxol sensitizes human astrocytoma cells to radiation. *Cancer Res* **52**:3495–3497.
- Torres KE, Castillo G, and Horwitz SB (1997) Induction of apoptosis by low concen-

- trations of taxol is not dependent on a G<sub>2</sub>/M block. *Proc Am Assoc Cancer Res* **38**:530.
- Traenckner EBM, Pahl HL, Henkel T, Schmidt KN, Wilk S, and Baeuerle PA (1995) Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO (Eur Mol Biol Organ) J* **14**:2876–2833.
- Wang CY, Mayo MW, and Baldwin AS (1996) TNF-and cancer therapy-induced apoptosis potentiation by inhibition of NF-κB. *Science (Wash DC)* **274**:784–787.
- Wani MC, Talor HL, Wall ME, Coggon P, and McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of paclitaxel, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* **93**:2325–2327.
- Williams GT and Smith CA (1993) Molecular regulation of apoptosis: genetic control on cell death. *Cell* **74**:777–779.

- Willingham MC and Bhalla K (1994) Transient mitotic phase localization of bcl-2 oncoprotein in human carcinoma cells and its possible role in prevention of apoptosis. *J Histochem Cytochem* **42**:441–450.
- Wu H and Lozano G (1994) NF-κB activation of p53: a potential mechanism for suppressing cell growth in response to stress. *J Biol Chem* **269**:20067–20074.
- Zandi E, Rothwarf DM, Delhase MH, and Karin M (1997) I-κB Kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell* **91**:243–252.

---

**Address correspondence to:** Weimin Fan, M.D., Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. E-mail: fanw@musc.edu

---