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**13. ABSTRACT (Maximum 200 Words)**

Accumulated evidences showed that Akt is a major cell survival pathway and plays an important role in malignant transformation and chemoresistance. However, the underlying mechanisms have not been well documented. We demonstrated that Akt protects stress-induced programmed cell death by inhibition of stress kinase JNK/p38 through activation of NFκB pathway. Further, we have shown that human cancer cells expressing constitutively active AKT2 resist to cisplatin through inhibition of cisplatin-induced ASK1 (apoptosis signal-regulating kinase 1) activation. AKT2 phosphorylation of ASK1 led to abrogation of cisplatin-induced JNK/p38 activation and Bax conformational changes. MST1, one member of STE20 like kinase, has been shown to induce apoptosis through its cleavage, activation and nuclear translocation. We have recently observed that AKT2 inhibits MST1-induced apoptosis by phosphorylatiion of MST1 at Thr-120.

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## Introduction

The purpose of this project is to: 1) Examine the incidence and clinical significance of AKT2 alterations in breast cancer, 2) Define the functional interaction between AKT2 and APBP and role of AKT2/APBP in mammary epithelial cell transformation and 3) Determine the FTIs as an inhibitor of AKT2 pathway for breast cancer intervention.

## Body:

During the last budget year, we have mainly focused on the involvement and mechanism of AKT2 in the development of drug resistance in human cancer.

1. AKT2 is activated by cellular stress- and TNFα and the activated AKT2 inhibits JNK and p38 activities through activation of the NFκB pathway in human epithelial cells. Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase (PI3K)-dependent and -independent pathways (1). However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity, as well as by tumor necrosis factor α (TNFα) through PI3K-dependent pathway. The activation of AKT2 inhibits UV- and TNFα–induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death (2, 3). Moreover, AKT2 interacts with and phosphorylates IKKα. The phosphorylation of IKKα and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, PI3K inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that Akt inhibits stress kinase JNK through activation of NFκB pathway. This work has been published in *J. Biol. Chem.* [2002 Aug 16; 277(33): 29973-29982].

2. AKT2 inhibition of cisplatin-induced JNK/p38 activation and Bax conformational change by phosphorylation of ASK1. Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers (4, 5). Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling down-stream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2

provides a new mechanism contributing to its antiapoptotic effects. This work has been published in *J. Biol. Chem.* [2003 Jun 27; 278(26):23432-23440].

3. Akt negatively regulates MST1 and protects cells from MST1-induced apoptosis. The serine/threonine kinase MST1 (mammalian STE20-like kinase 1) can be activated after cleavage by caspases and promotes apoptosis, however, the regulation of MST1 has not been well documented. In this study, we demonstrated that MST1 is phosphorylated by Akt at Thr 120 (Fig 1). The phosphorylation leads to the inhibition of cleavage and kinase activity of MST1 at the basal level or under treatment of Stauroporine (STS; Fig 2). Ectopic expression of constitutively active Akt inhibits the cleavage and kinase activity of MST1 induced by STS, however, nonphosphorylatable MST1-T120A (mutation of threonine-120 to alanine) block Akt inhibitory function whereas phosphomimic MST1-T120D (mutation of threonine-120 to aspartic acid) becomes resistant to caspases cleavage and exhibnits lower kinase activity. The activation of JNK, a downstream target of MST1, is inhibited in the cells expressing MST1-T120D, but not MST1-T120A. Moreover, the phosphorylated MST1 has the lowest caspase activity and JNK activation (Fig 3). Finally, constitutively active Akt block the nuclear translocation of MST1 (Fig 2C).

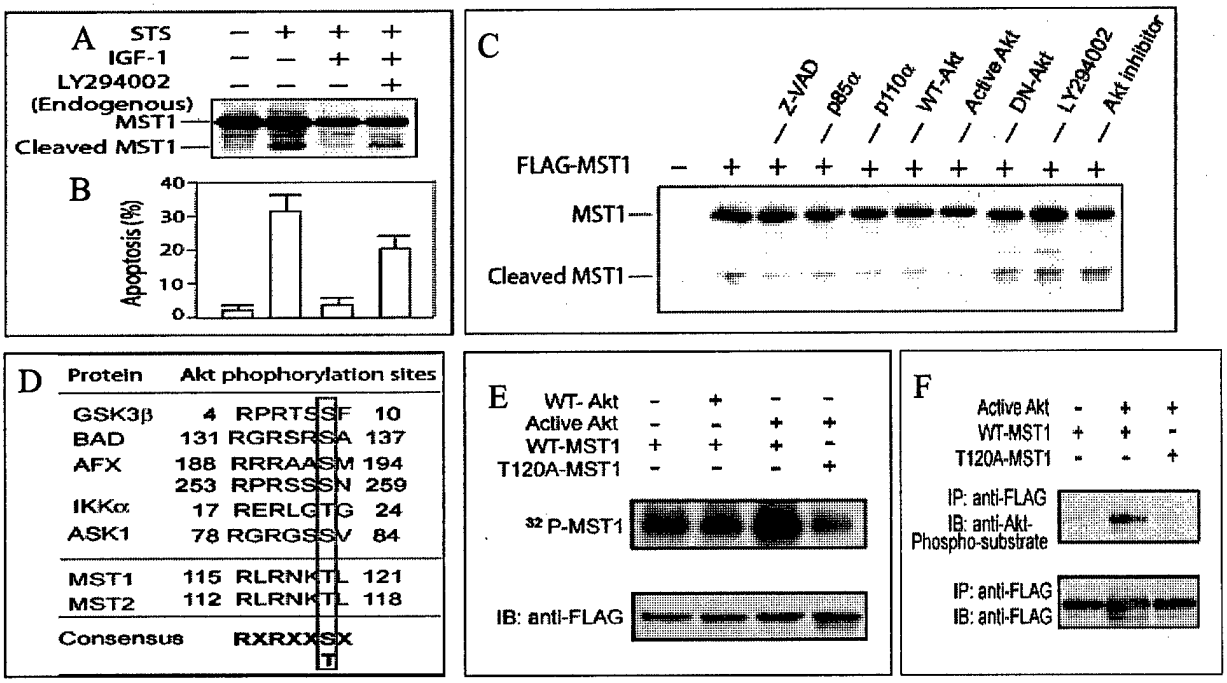


**Fig. 1. MST1 is a substrate of Akt.** **(A** and **B)** IGF-1 inhibits MST1 cleavage STS-induced cell death.. **(C)** PI3K and constitutively active Akt inhibit MST1 cleavage, whereas dominant negative Akt, LY294002 and Akt inhibitor enhance MST1 cleavage. **(D)** Comparison of the putative Akt phosphorylation sites in MST1 with the sequences of phosphorylation sites of known Akt substrates. **(E)** *In vivo* labeling (top). Akt phosphorylates MTS1 *in vivo*. Expression of Flag-MST1 was shown in bottom panel. **(F)** Immunoblotting analysis of the Flag-MST1 immunoprecipitates with anti-Akt-phopsho-substrate antibody (top). Bottom panel shows expression of Flag-MST1.

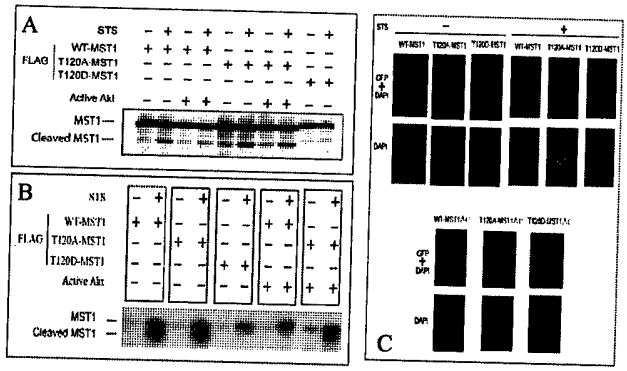


FIG 2

**Fig. 2. Akt phosphorylation of MST1 inhibits cleavage, kinase activity and nuclear localization of MST1.** **(A)** Akt inhibits STS-induced cleavage and kinase activity of MST1. **(B).** Phosphomimic MST1-T120D exhibits low levels of kinase activity. **(C)** GFP-tagged WT and nonphosphorylatable MST1 are localized in the nucleus, whereas phosphomimic MST1 stays in cytosol even following treatment with STS.

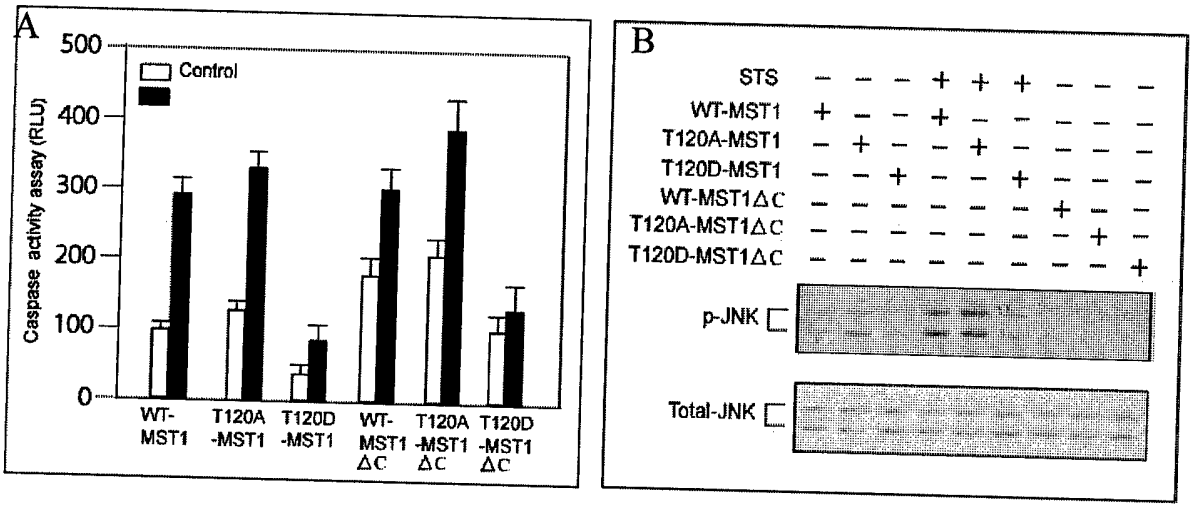


**Fig. 3.** Akt phosphorylation of MST1 inhibits MST1-induced caspase activity **(A)** and JNK activation **(B)**.

## Key Research Accomplishment

1 AKT2 inhibits JNK and p38 activation and prevents stress-induced apoptosis through activating NFκB Pathway. As JNK and p38 activation is required for chemotherapeutic drug-induced programmed cell death, activation and overexpression of AKT2 in human breast cancer will contribute to chemoresistance. Therefore, development reagent(s) to target AKT2 will greatly benefit to breast cancer intervention.

2 AKT2 inhibits of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1, suggesting AKT2 contributes to the development of drug resistance in ovarian cancer. Therefore, development reagent(s) to target AKT2 will greatly benefit to breast cancer intervention.

3 AKT2 inhibits MST1-induced cell death by phosphorylating MST1 at threonine 120. Akt phosphorylation of MST1 decreases its cleavage, kinase activity and nuclear localization..

## Reportable Outcomes

1. Inhibition of JNK by cellular stress- and TNFα-induced AKT2 through activation of NFκB pathway in human epithelial cells. *J. Biol. Chem.* 2002 Aug 16; 277(33): 29973-29982. See Appendix.
2. AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1: IMPLICATION OF AKT2 IN CHEMORESISTANCE. *J. Biol. Chem.* 2003 Jun 27; 278(26): 23432-23440. See Appendix.
3. Akt inhibits MST1-induced apoptosis through phosphorylation-dependent manner. (Manuscript is in preparation.)

## Conclusion

1. AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects.
2. Akt inhibits MST1 cleavage, kinase activity and nuclear translocation and protects cells from MST1-induced apoptosis.

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## Appendix:

1. Yuan ZQ, Feldman RI, Sun M, Olashaw NE, Coppola D, Sussman GE, Shelley SA, Nicosia SV, Cheng JQ. Inhibition of JNK by cellular stress- and TNFα-induced AKT2 through activation of the NFκB pathway in human epithelial cells. *J. Biol. Chem.* 2002 Aug 16; 277(33): 29973-29982.

2. AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1: IMPLICATION OF AKT2 IN CHEMORESISTANCE. *J. Biol. Chem.* 2003 Jun 27; 278(26):23432-23440.

3. Curriculum Vitae

4. Personnel information

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# Inhibition of JNK by Cellular Stress- and Tumor Necrosis Factor α-induced AKT2 through Activation of the NFκB Pathway in Human Epithelial Cells*

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**Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase-dependent and -independent pathways. However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity as well as by tumor necrosis factor α (TNFα) through a phosphatidylinositol 3-kinase-dependent pathway. The activation of AKT2 inhibits UV- and TNFα-induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death. Moreover, AKT2 interacts with and phosphorylates IκB kinase α. The phosphorylation of IκB kinase α and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with an accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that AKT inhibits stress kinase JNK through activation of the NFκB pathway.**

Exposure of cells to environmental stress results in the activation of several signal transduction pathways including the MEKK4/MKK7/JNK,[1] MKK3/MKK6/p38, and IκB kinase (IKK)/IκB/NFκB cascades. Stress-induced clustering and internalization of cell surface receptors, such as those for platelet-derived growth factor, tumor necrosis factor α (TNFα), epidermal growth factor, and insulin-like growth factor 1 (IGF1), mediate stress-kinase activation (1–3). Recent studies suggest that nearly all stress stimuli activate phosphatidylinositol 3-kinase (PI3K) (1), and of the downstream targets of PI3K, AKT is thought to play an essential role in the cellular response to stress.

AKT, also termed protein kinase B or RAC kinase, represents a family of PI3K-regulated serine/threonine kinases (4, 5). Three different isoforms of AKT have been identified, AKT1/protein kinase Bα (AKT1), AKT2/protein kinase Bβ (AKT2), and AKT3/protein kinase Bγ (AKT3), all of which are activated by growth factors in a PI3K-dependent manner (4–9). Full activation of the AKTs requires their phosphorylation at $Thr^{308}$ (AKT1), $Thr^{309}$ (AKT2), or $Thr^{305}$ (AKT3) in the activation loop and $Ser^{473}$ (AKT1), $Ser^{474}$ (AKT2), or $Ser^{472}$ (AKT3) in the C-terminal activation domain (9). AKT1, the most studied isoform, which was originally designated as AKT, suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. Possible mechanisms by which AKT1 promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (10, 11). AKT1 also phosphorylates and inactivates the Forkhead transcription factors, an event that results in the reduced expression of the cell cycle inhibitor, $p27^{Kip1}$, and the Fas ligand (12–14). Via phosphorylation of IKK, AKT1 also activates NFκB, a transcription factor that has been implicated in cell survival (15, 16).

Two separate studies demonstrated that AKT1 is activated when NIH 3T3 fibroblasts are stressed in a variety of ways (17, 18). Based on data showing that PI3K inhibitors do not prevent AKT1 activation by stress, these studies concluded that stress-induced AKT1 activation was PI3K-independent. Other studies, however, found that PI3K activity was required for AKT1 activation by heat shock or oxidative stress in Swiss 3T3 cells (19, 20). It has been suggested that certain cellular stresses activate AKT1 and AKT3 but not AKT2 (19), a finding that is consistent with the different functions of the AKTs as revealed by studies of mice lacking AKT1 or AKT2 (21–23). Nevertheless, activation of AKT2 by stress and the role of AKT2 in the stress response have yet to be fully explored. The data presented here show that AKT2 is significantly activated by stress stimuli (*e.g.* UV irradiation, heat shock, and hyperosmolarity) and by TNFα in human epithelial cells but not in fibroblasts. Stress-induced AKT2 activation in epithelial cells is completely blocked by inhibitors of PI3K. When activated by stress, AKT2 inhibits JNK and p38 activities that are required for stress-induced apoptosis. In addition, AKT2 binds to and phosphoryl-

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[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor α; IGF1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IKK, IκB kinase; NIK, NFκB-inducing kinase; GST, glutathione *S*-transferase; HEK, human embryonic kidney; TUNEL assay, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay.

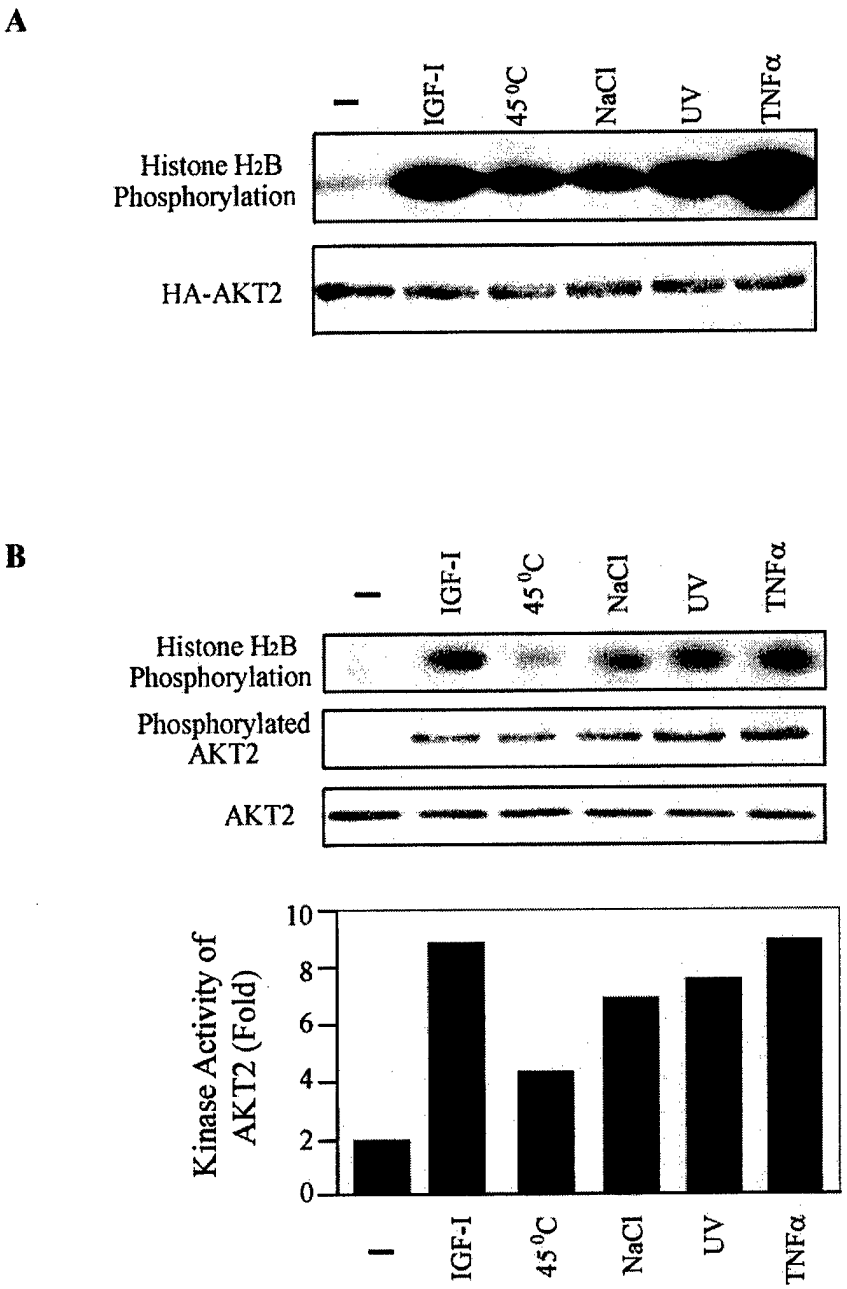



FIG. 1. **AKT2 is activated by cellular stress and TNFα.** *A*, *in vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transiently transfected with HA-AKT2. Cells were exposed to 100 ng/ml IGF-1 (15 min), heat shock (45 °C for 20 min), 0.4 M NaCl (15 min), 40 J/m$^2$ UV-C (254 nm), or TNFα 20 ng/ml (15 min), and AKT2 activity was determined by *in vitro* kinase assay using histone H2B as substrate. *B*, OVCAR3 cells were treated with the indicated stimuli and immunoprecipitated with anti-AKT2 antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (*upper*) and Western blotting analyses with anti-phospho-Ser473 AKT (*middle*), or anti-AKT2 (*lower*) antibody. The *bottom panel* shows relative AKT2 kinase activity quantified by phosphorimaging. Each experiment was repeated three times.

ates IKKα and, consequently, activates NFκB, resulting in inhibition of programmed cell death in response to stress stimuli. Moreover, AKT2-induced NFκB activation is required for the inhibition of JNK, but not p38, activity.

## EXPERIMENTAL PROCEDURES

*Cell Lines, Transfection, and Stimulation*—The human epithelial cancer cell lines, A2780, OVCAR3, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5 × 10$^6$ cells/dish. After incubation overnight, the cells were transfected with 2 μg of DNA/dish using LipofectAMINE Plus (Invitrogen). After 36 h of the transfection, the cells were serum-starved overnight and stimulated with UV-C irradiation, heat (45 °C), 0.4 M NaCl, or 20–50 ng/ml TNFα.

*Expression Constructs*—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2, constitutively active HA-Myr-AKT2, and dominant negative HA-E299K-AKT2 have been described (24). The HA-JNK1 construct was kindly provided by Michael Karin (School of Medicine, University of California at San Diego). GST-c-Jun-(1–79) and pCMV-FLAG-p38 were gifts from Roger J. Davis (School of Medicine, University of Massachusetts). The constructs used in the study of the NFκB pathway were prepared as previously described (25).

*Immunoprecipitation and Immunoblotting*—Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C before immunoprecipitation or Western blotting. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. After the removal of the beads by centrifugation, lysates were incubated with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals), anti-FLAG antibody (Sigma), or anti-AKT2 antibody (Santa Cruz Biotechnology) in the presence of 30 μl of protein A-protein G (2:1)-agarose beads for 2 h at 4 °C. The beads were washed once with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10, twice with phosphate-buffered saline, and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10

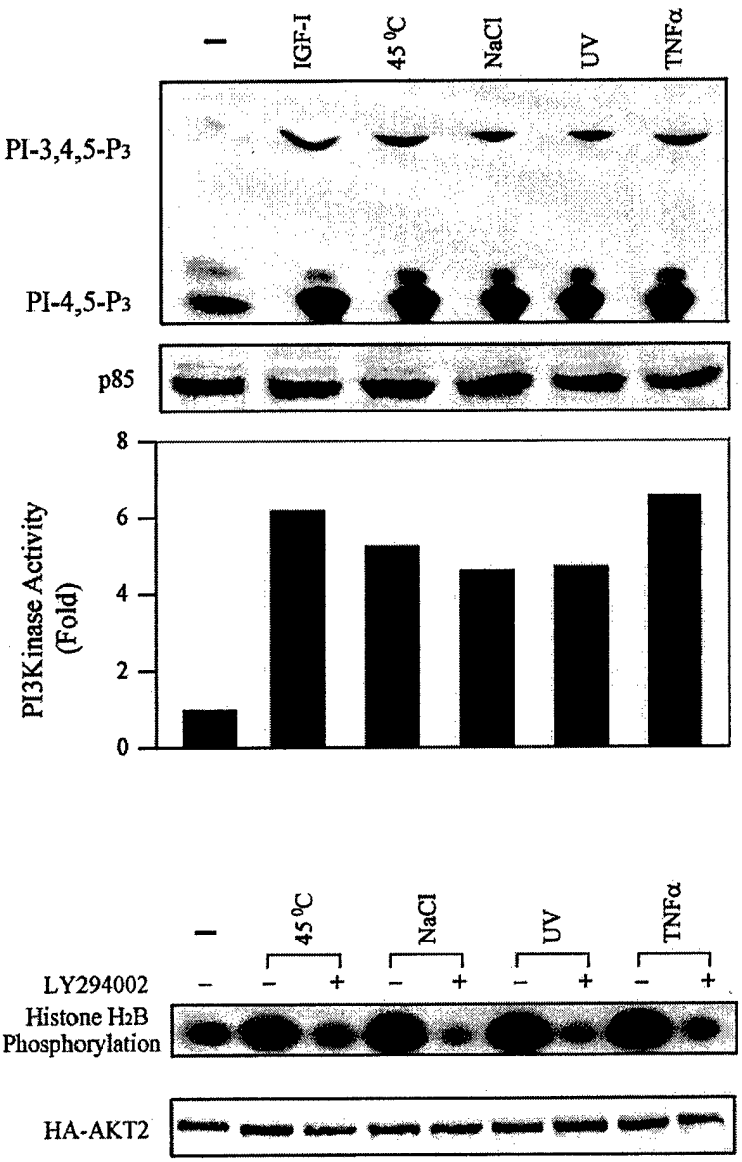



FIG. 2. **Activation of AKT2 by cellular stress and TNFα is PI3K-dependent.** *A*, *in vitro* PI3K assay. HA-AKT2-transfected HEK293 cells were exposed to the indicated stimuli. *Upper panel*, PI3K immunoprecipitates were prepared with anti-pan-p85 antibody and assayed for PI3K activity. The *middle panel* shows the p85 protein level using anti-p85 antibody, and the *bottom panel* represents the relative PI3K activity quantified by phosphorimaging. *B*, HA-AKT2-transfected A2780 cells were treated with LY294002 for 30 min before exposure to indicated stimuli. HA-AKT2 immunoprecipitates were subjected to *in vitro* kinase assay. Results were confirmed by four independent experiments. *PI-3,4,5-$P_3$*, phosphatidylinositol 3,4,5-trisphosphate; *PI-4,5-$P_3$*, phosphatidylinositol 4,5-trisphosphate.

mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the antibodies described above or with the appropriate antibodies as noted in figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting Analysis System (Amersham Biosciences).

*In Vitro Protein Kinase Assay*—Protein kinase assays were performed as previously described (26, 27). Briefly, reactions were carried out in the presence of 10 μCi of [γ-$^{32}$P] ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. Histone H2B was used as exogenous substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

*PI3K Assay*—PI3K was immunoprecipitated from the cell lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold phosphate-buffered saline, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in the immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [γ-$^{32}$P]ATP and 10 μg L-α-phosphatidylinositol 4,5-bisphosphate (Biomol) for 20 min at 25 °C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/MeOH, and phosphorylated products were separated by thin-layer chromatography as previously described (24). The conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate was detected by autoradiography and quantitated with a PhosphorImager.

*NFκB Transcriptional Activation Analysis*—HEK293 cells were seeded in 60-mm dishes and transfected with 1.5 μg of NFκB reporter plasmid (pElam-luc), 0.8 μg of pSV2-β-gal, and different forms (wild type, constitutively active, or dominant-negative) of HA-AKT2 or vector alone. The total amount of DNA transfected was increased to 6 μg with empty vector DNA. After serum starvation overnight, the cells were treated with UV (40 J/m$^2$) or TNFα (20 ng/ml) and lysed with 400 μl/dish of reporter lysis buffer (Tropix). The cell lysates were cleared by centrifugation for 2 min at 4 °C. Luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

*Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assay*—AKT2 stably transfected A2780 cells were seeded into 60-mm dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 24 h and pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. Apoptosis was determined by TUNEL using an *in situ* cell death detection kit (Roche Molecular Biochemicals). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in phosphate-

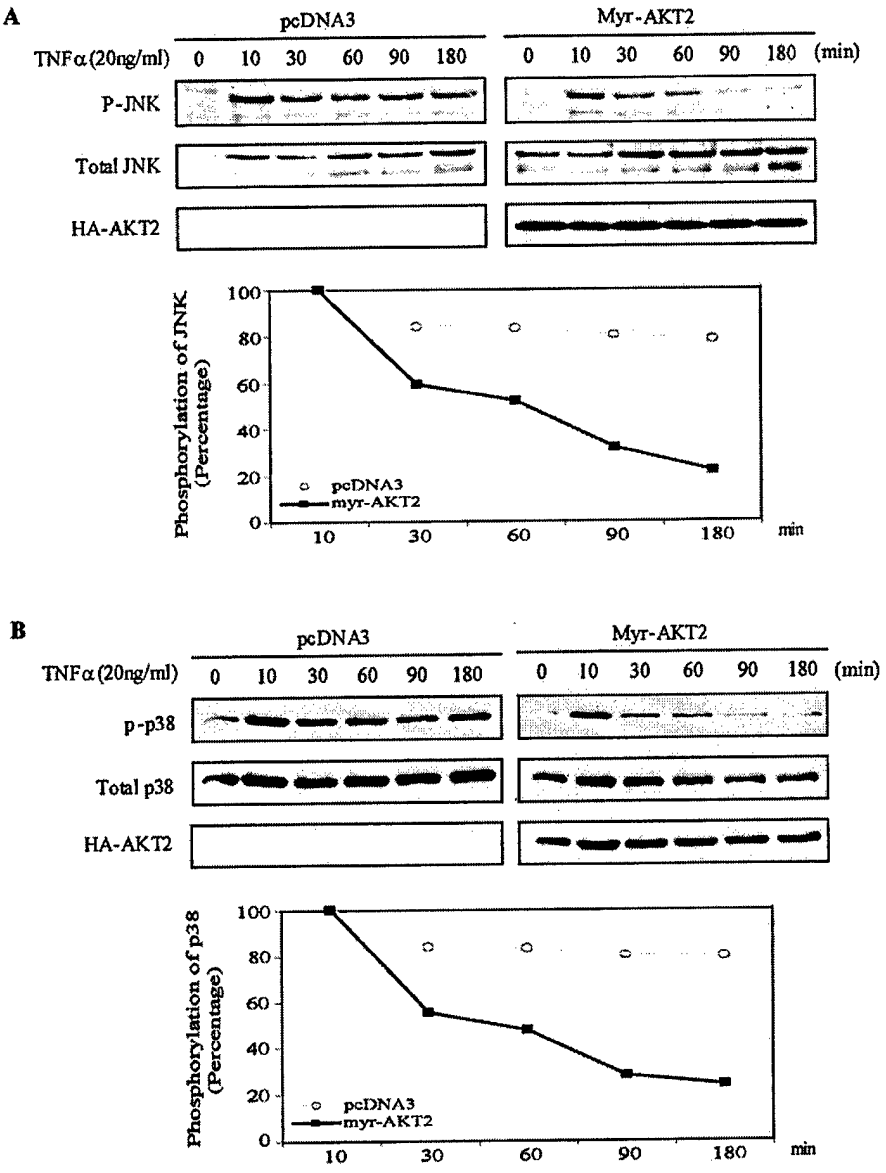



FIG. 3. **AKT2 kinase inhibits UV- and TNFα-induced JNK and p38 activation.** *A*, Western blotting analyses of HEK293 cells transfected with the indicated plasmids. Cells were lysed at indicated times after incubation with TNFα and analyzed with anti-phospho-JNK (*P-JNK*, *upper*), -total JNK (*middle*), and -HA (*lower*) antibodies. The immunoblotting analyses were repeated three times. *B*, the procedures are the same as *A*, except the membranes were probed with anti-phospho-p38 (*upper*), -total p38 (*middle*), and -HA (*lower*). Graphical presentations show the normalized density of phosphorylated JNK (*bottom* of *panel A*) and p38 (*bottom* of *panel B*), decaying from 100%.

buffered saline (pH 7.4)). Slides were rinsed with phosphate-buffered saline and incubated in permeabilization solution followed by TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37 °C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA) for 10 min at 25 °C. After an additional rinse, the slides were mounted and analyzed under a light microscope. These experiments were performed in triplicate.

## RESULTS

*AKT2 Is Activated by UV Irradiation, Heat Shock, Hyperosmolarity, and TNFα*—Previous studies showed that stress activates AKT1 and AKT3 but not AKT2 in fibroblasts (19). It has also been shown that TNFα receptor mediates UV- and heat shock-induced stress signaling (1–3). In agreement with these studies, we found that exposure of NIH 3T3 fibroblasts to UV-C, heat, or hyperosmotic conditions did not result in AKT2 activation (data not shown). It is possible, however, that stress might activate AKT2 in epithelial cells due to the fact of frequent alterations of AKT2, but not AKT1 and AKT3, in human epithelial tumors (7, 24, 27). For this reason we examined the effects of stress on AKT2 activation in two ovarian epithelial cancer cell lines, A2780 cells, which were transiently transfected with HA-AKT2, and OVCAR3 cells, which express high levels of endogenous AKT2 (7). The cells were exposed to UV-C, heat shock (45 °C), 0.4 M NaCl, or 20 ng/ml TNFα. IGF1-stimulated cells were used as controls. As assessed by *in vitro* kinase and Western blot analyses of AKT2 immunoprecipitates, all the stimuli substantially increased AKT2 activity in both A2780 and OVCAR3 cells (Figs. 1, *A* and *B*). The levels of AKT2 activity induced by these agents, however, were variable. AKT2 activity induced by TNFα and UV was comparable with that stimulated by IGF-1, whereas the effect of heat shock and hyperosmolarity (NaCl) on AKT2 activity was relatively smaller (Fig. 1). Nevertheless, these findings suggest that stresses activate AKT2 in a cell type-specific manner.

*Stress Simulates PI3K That Mediates AKT2 Activation*—To show that stress does indeed activate PI3K in epithelial cells, A2780 or HEK293 cells were exposed to UV irradiation, heat shock, and 0.4 M NaCl or TNFα, and cell lysates were immunoprecipitated with antibody to pan-p85, a regulatory subunit of PI3K. Assay of PI3K activity shows that these stress conditions as well as TNFα activated PI3K as efficiently as did IGF-1 (Fig. 2*A*). As described above, stress has been shown to activate AKT1 by both PI3K-dependent and -independent pathways

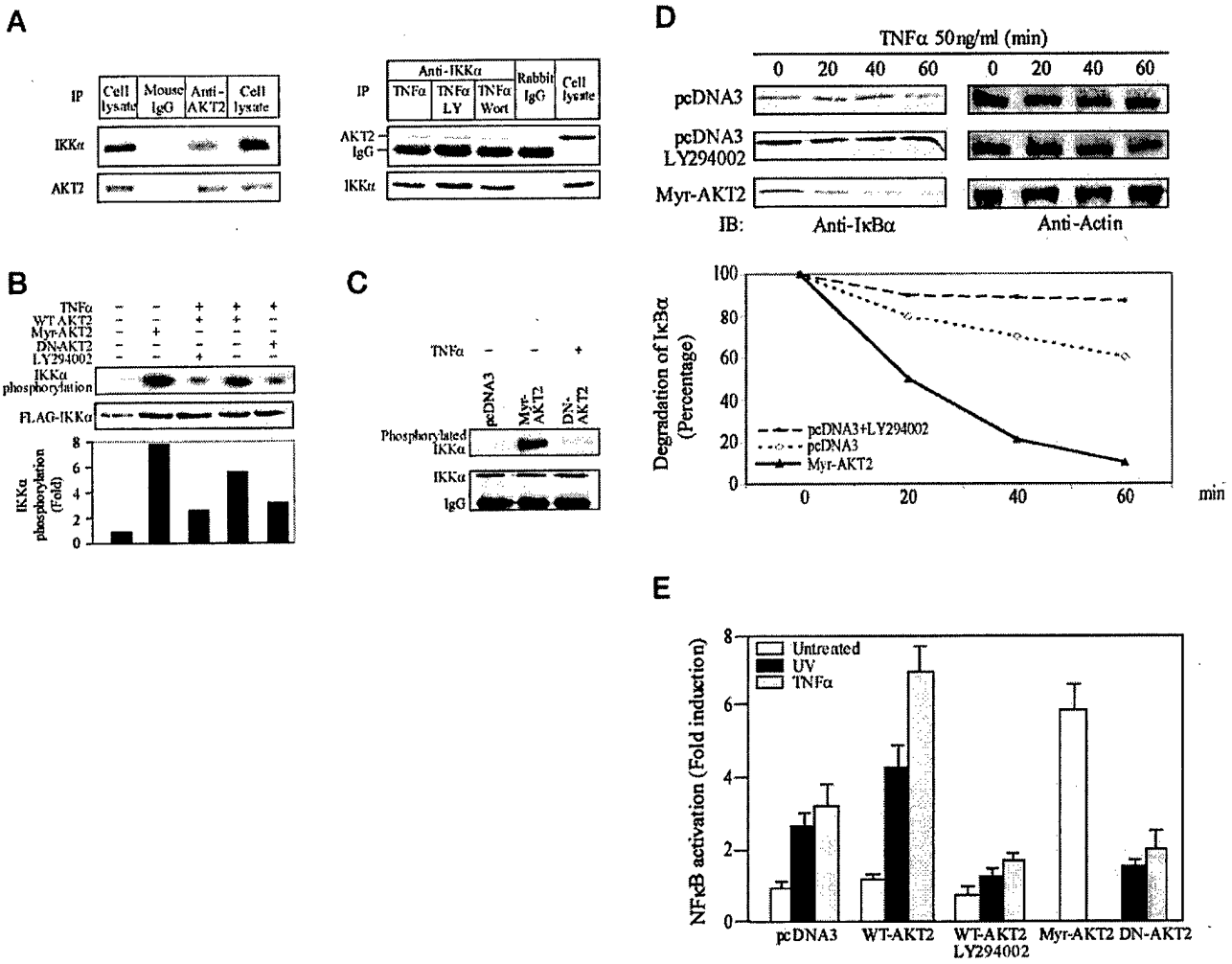



FIG. 4. **AKT2 interacts with and phosphorylates IKKα, leading to IκBα degradation and NFκB activation.** *A*, *left panel*, Western blotting analyses. HEK293 cell lysates were immunoprecipitated (*IP*) with anti-AKT2 or IgG (control) and detected with anti-IKKα (*top*) or anti-AKT2 (*bottom*) antibody. *Right panel*, HEK293 cells were treated with LY294002 (*LY*) or wortmannin for 30 min followed by TNFα for 20 min. Immunoprecipitates were prepared with anti-IKKα antibody or IgG and immunoblotted with antibody to AKT2 (*top*) or IKKα (*bottom*). *B*, *in vitro* kinase assay analyses of immunoprecipitates prepared from A2780 cells transfected with indicated plasmids using immunopurified FLAG-IKKα as substrates (*upper*). Expression of FLAG-IKKα was confirmed by immunoblotting analysis with anti-FLAG antibody (*middle*). The *bottom panel* shows the relative phosphorylation levels of IKKα by AKT2. *C*, *in vivo* labeling of IKKα from COS7 cells transfected with indicated DNA constructs treated with or without TNFα and incubated with [$\gamma$-$^{32}$P]orthophosphate for 4 h. IKKα immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, exposed to film (*top*), and then detected with anti-IKKα antibody (*bottom*). *D*, AKT2 induces IκBα degradation. HEK293 cells were transfected with indicated plasmids and treated with cycloheximide (50 μg/ml) for 1 h before treatment with 50 ng/ml TNFα for up to 60 min. Cell lysates were immunoblotted (*IB*) with antibody to IκBα (*left panels*) or β-actin (*right panels*). Degradation of IκBα was quantified with a densitometer. The *bottom panel* shows the degradation rate of IκBα by normalizing density of IκBα bands at 0 time point as 100%. *E*, reporter assays. HEK293 cells were transfected with 2×NFκB-Luc, β-galactosidase and WT-AKT2, Myr-AKT2, or DN-AKT2 pretreated with or without LY294002 and subsequently exposed to 40 J/m$^2$ UV-C or 20 ng/ml TNFα. Cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. *Error bars* represent S.D. Data were obtained from triplicate experiments.

(17, 18). To assess the role of PI3K in the stress-induced activation of AKT2, A2780 cells transfected with HA-AKT2 were exposed to 25 μM LY294002, a specific PI3K inhibitor, for 30 min before stress or TNFα treatments. LY294002 effectively inhibited stress- and TNFα-induced AKT2 activation (Fig. 2*B*). These data provide direct evidences of stress-induced activation of AKT2 through a PI3K-dependent pathway in human epithelial cells.

*Stress-induced AKT2 Activation Inhibits UV- and TNFα-induced JNK and p38 Activities*—Previous studies demonstrated that two groups of mitogen-activated protein kinases, the JNK and p38, are activated by environmental stress and TNFα (28). Therefore, we examined the effects of stress-induced AKT2 activation on the JNK and p38 to determine whether stressed-induced AKT2 activation could target these two stress kinases. A2780 cells were transfected with constitutively active AKT2 or pcDNA3 vector alone. Thirty-six hours after transfection, cells were treated with TNFα or UV and analyzed by Western blot for JNK and p38 activation using anti-phospho-JNK and anti-phospho-p38 antibodies. Both JNK and p38 were activated by TNFα and UV irradiation. The maximal activation was observed at 10 min of stimulation. Expression of constitutively active AKT2, however, exhibited inhibitory effects on the activation of JNK and p38 that was induced by TNFα and UV irradiation. Notably, the activation of JNK and p38 in constitutively active AKT2-transfected cells does not significantly differ from that of the cells transfected with pcDNA3 vector at 10 min of TNFα treatment. However, the phosphorylation levels of JNK and p38 in the cells expressing constitutively active AKT2 declined much more than that of

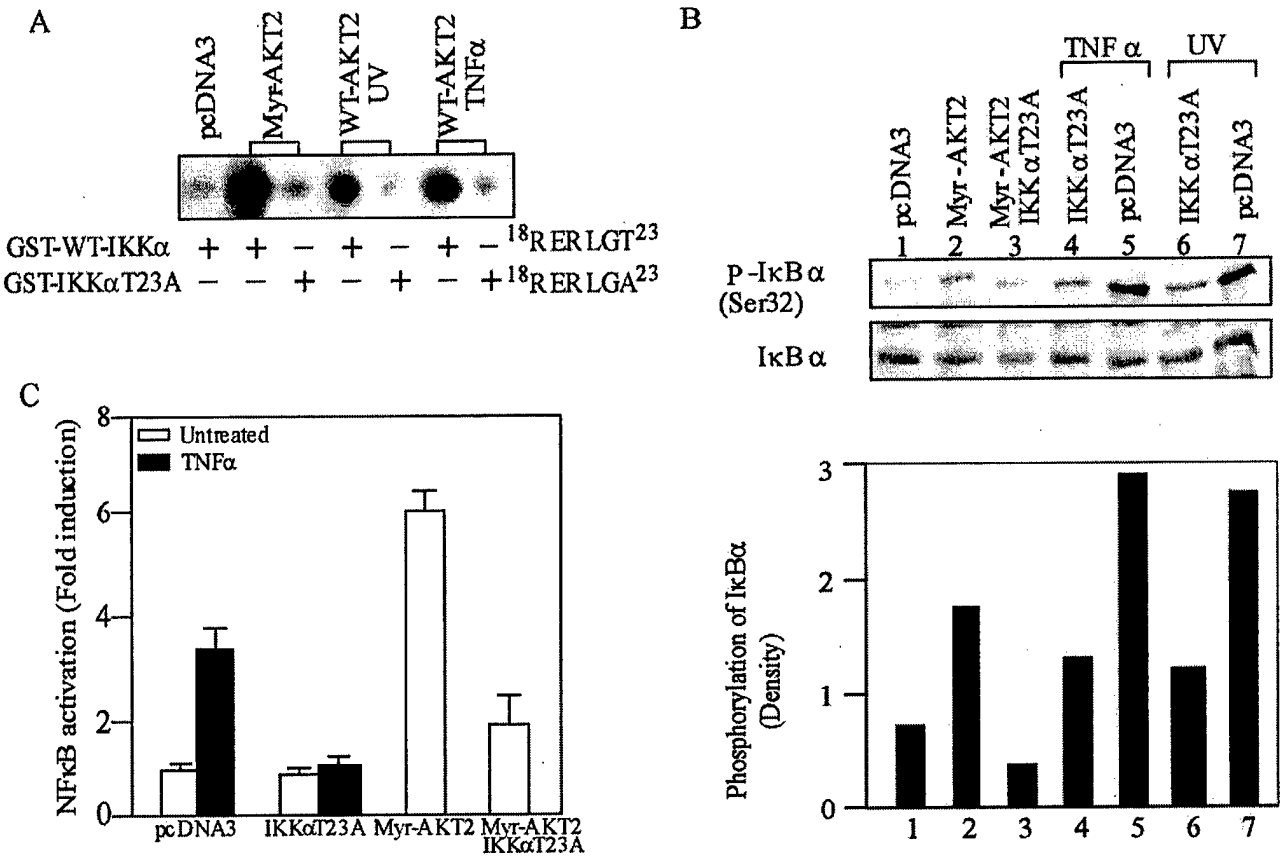



FIG. 5. **AKT2-phosphorylated IKKα at threonine 23 is required for stress-induced NFκB.** *A*, AKT2 phosphorylation of IKKα at threonine 23. *In vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transfected with the indicated plasmids and treated with or without UV or TNFα. GST-fused wild type IKKα ($^{18}$**RERLGT**$^{23}$) or mutant IKKα ($^{18}$**RERLGA**$^{23}$) was used as the substrate. *B*, AKT2 induces IκBα phosphorylation (*P-*). HEK293 cells were transfected with the indicated expression constructs. Thirty-six hours after transfection, cells were treated with 20 ng/ml TNFα for 30 min or irradiated with 40 J/m$^2$ UV followed by a 30-min incubation. Cell lysates were immunoblotted with anti-phospho-IκBα (*upper*) or anti-IκBα (*middle*) antibody. The band density of the phospho-IκBα was quantified (*bottom*). *C*, luciferase reporter assay. HEK293 cells were transfected with the indicated plasmids. After treatment with or without 20 ng/ml TNFα for 12 h, cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Results were obtained from three independent experiments.

pcDNA3-transfected cells after 30 min of stimulation (Fig. 3 and data not shown). We therefore conclude that the activation of AKT2 does not activate but rather inhibits TNFα- and UV-induced JNK and p38 activities.

*AKT2 Interacts With and Phosphorylates IKKα, but Not NIK, Leading to IκBα Degradation and NFκB Activation*—The capacity of both cellular stress and TNFα to activate the NFκB pathway is well documented (29). Previous studies also show that AKT1 induces activation of the NFκB by interaction with IKKα (13, 14). However, to date there are no reports addressing the potential role of AKT2 in the activation of the NFκB pathway. To determine whether AKT2 associates with IKKα, HEK293 cells were treated with or without TNFα, immunoprecipitated with anti-AKT2, and immunoblotted with anti-IKKα antibody or vice versa. In both instances, the association of AKT2 with IKKα was observed (Fig. 4*A*). Additional studies showed that AKT2-IKKα interaction was unaffected by treatment of cells with PI3K inhibitor, wortmannin, or LY294002 (Fig. 4*A*). These findings indicate that AKT2 constitutively associates with IKKα. In addition, we have identified putative AKT2 phosphorylation sites in the IKKα ($^{18}$**RERLGT**$^{23}$) and in NFκB-inducing kinase (NIK, $^{366}$**RSREPS**$^{371}$) (bold residue letters represent Akt consensus sequence). To determine whether IKKα and/or NIK are phosphorylated by AKT2, A2780 cells were transfected with different forms of AKT2 and treated with LY294002 and TNFα. *In vitro* AKT2 kinase assays were performed using FLAG-IKKα or HA-NIK, purified from the transfected COS7 cells, as substrate. Repeated experiments show that TNFα-induced AKT2 and constitutively active AKT2 phosphorylated IKKα (Fig. 4*B*) but not NIK (data not shown). Phosphorylation of IKKα induced by TNFα was largely attenuated by PI3K inhibitor LY294002. Quantification analyses revealed that approximate 70% of TNFα-induced IKKα phosphorylation was inhibited by pretreatment with LY294002 (Fig. 4*B*). Furthermore, we assessed AKT2 to determine if it phosphorylates IKKα *in vivo*. COS7 cells were transfected with FLAG-IKKα together with either constitutively active or dominant-negative AKT2 or vector alone and labeled with [γ-$^{32}$P]orthophosphate. IKKα immunoprecipitates prepared using anti-FLAG antibody were separated by SDS-PAGE and transferred to nitrocellulose. The phospho-IKKα was detected by autoradiography. As shown in Fig. 4*C*, IKKα was highly phosphorylated in cells expressing constitutively active AKT2 but not in the cells transfected with pcDNA3 and dominant-negative AKT2. Collectively, these data indicate that IKKα is an AKT2 physiological substrate.

Activation of NFκB requires its dissociation from its cytosolic inhibitor, IκB, a process dependent on the phosphorylation and consequent degradation of IκB by IKK. Thus, we next examined AKT2 to determine if it induces IκB degradation. Immunoblotting analyses revealed that constitutively active AKT2 significantly promoted IκBα degradation (Fig. 4*D*). To assess the involvement of AKT2 in NFκB activation, HEK293 cells were co-transfected with a NFκB-luciferase reporter and either

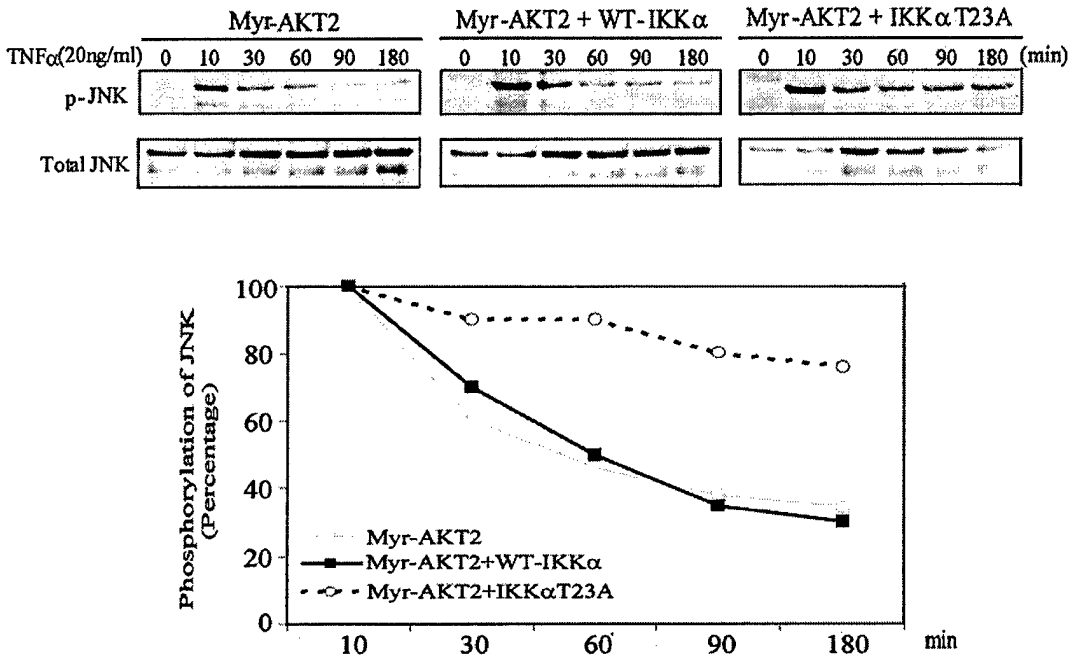


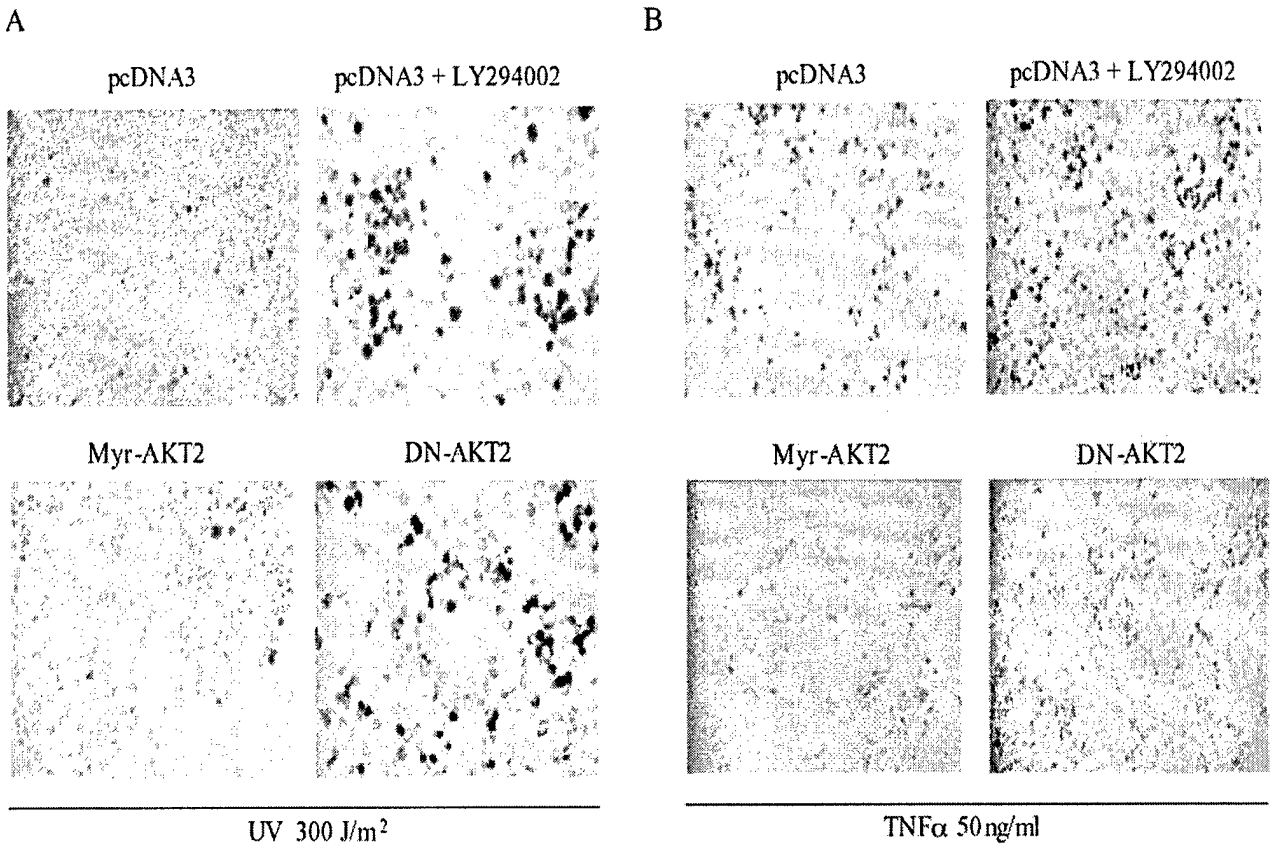

FIG. 6. **AKT2 phosphorylation of IKKα is required for inhibition of TNFα-induced JNK activity.** Immunoblotting analyses of HEK293 cells transfected with indicated expression constructs and treated with TNFα (20 ng/ml). The blots were probed with anti-phospho-JNK (*p-JNK*; *upper*) and -total JNK (*middle*) antibodies. Results represent one of three independent experiments. The *bottom panel* shows the quantification of phosphorylated JNK at the indicated time points.

vector alone, wild type, or constitutively active or dominant negative AKT2 treated with or without LY294002 before UV or TNFα stimulation. As shown in Fig. 4*E*, ectopic expression of wild-type AKT2 significantly enhanced UV- and TNFα-induced NFκB activity, which was abolished by treatment of cells with LY294002 or dominant negative AKT2. Constitutively active AKT2 alone was able to induce NFκB activity to a level comparable with UV- or TNFα-treated cells transfected with wild-type AKT2. These data show that PI3K/AKT2 mediates both stress- and TNFα-activated NFκB pathway.

To determine AKT2 phosphorylation site of IKKα, GST fusion proteins containing either wild type IKKα ($^{18}$**RERLGT**$^{23}$; termed GST-WT-IKKα) or mutant IKKα ($^{18}$**RERLGA**$^{23}$; termed GST-IKKαT23A) were prepared and used as substrates in *in vitro* AKT2 kinase assays. As seen in Fig. 5*A*, UV- and TNFα-activated AKT2 as well as constitutively active AKT2 phosphorylated GST-WT-IKKα but not GST-IKKαT23A. We next assessed the capacity of AKT2-induced IKKα to phosphorylate IκBα. Constitutively active AKT2 was expressed in HEK293 cells, and cell lysates were immunoblotted with an antibody that specifically recognizes phosphorylated IκBα at Ser$^{32}$. The results of these experiments show that constitutively active AKT2 increased IκBα phosphorylation ~2-fold and that this increase was abolished by cotransfection of pcDNA3-IKKαT23A. Expression of IKKαT23A also blocked IκBα phosphorylation induced by TNFα or UV (Fig. 5*B*). Additional luciferase reporter experiments demonstrated that expression of IKKαT23A inhibited the TNFα- or constitutively active AKT2-induced NFκB activation (Fig. 5*C*). These data indicate that phosphorylation of IKKα at Thr$^{23}$ is required for AKT2-mediated NFκB activation.

*IKKα Phosphorylation by AKT2 Is Required for Inhibition of JNK but Not p38 Activation*—Recent studies showed that NFκB exerts its cell survival function by inhibition of JNK activation in response to extracellular stress (30, 31). However, it is currently unknown whether AKT-induced NFκB activation results in inhibition of JNK. Therefore, we next attempted to determine if AKT2-activated IKKα is required for AKT2 inhibition of JNK and p38 activities induced by stress and TNFα. The activation of JNK and p38 was examined in HEK293 cells transfected with IKKα or IKKαT23A together with or without constitutively active AKT2. Western blotting analyses with phospho-JNK and -p38 antibodies revealed that wild type IKKα did not significantly enhance AKT2 inhibition of JNK (Fig. 6). However, expression of IKKαT23A abrogated the effects of constitutively active AKT2 on inhibition of JNK (Fig. 6). Similar to the results shown in Fig. 3, TNFα-induced JNK activation reached the maximal level at 10 min of stimulation, which was neither significantly inhibited by constitutively active AKT2 nor affected by expression of IKKαT23A (Fig. 6). Therefore, these data indicate that inhibition of JNK activation by AKT2/NFκB could be via a mechanism of induction of dephosphorylation of JNK by the AKT2/IKKα/NFκB cascade.

*AKT2 Activation Inhibits Stress-induced Apoptosis*—It is documented that various stresses and TNFα are capable of inducing apoptosis in different cell types through activation of JNK and p38 pathways (29). Because PI3K/AKT is essential for cell survival and activated AKT2 inhibits JNK/p38 and induces NFκB pathway, we investigated the role of PI3K/AKT2 in stress- and TNFα-induced programmed cell death. AKT2 stably transfected A2780 cells were pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. As determined by the TUNEL assay, inhibition of PI3K activity dramatically increased the percentage of cells undergoing apoptosis in response to UV or TNFα (Fig. 7). Moreover, inhibition of AKT2 activity by expression of dominant-negative AKT2 increased the percentage of apoptotic cells in the UV- and TNFα-treated populations by ~2-fold. On the other hand, cells expressing constitutively active AKT2 were resistant to UV- and TNFα-induced apoptosis. These data show that the PI3K/AKT2 pathway plays a key role in protecting cells from apoptosis induced by extracellular stress or TNFα.



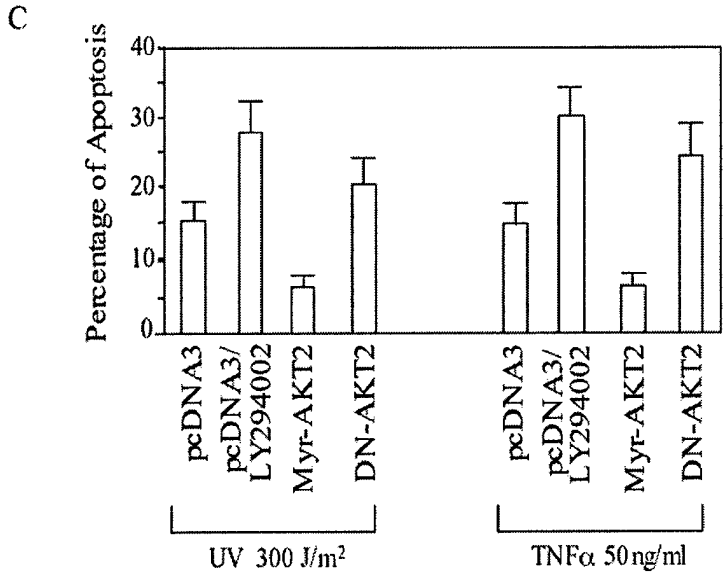




FIG. 7. **AKT2 activation inhibits stress-induced apoptosis.** A2780 cells stably transfected with constitutively active AKT2, DN-AKT2, or vector alone were pretreated with or without 25 μM LY294002 for 2 h before exposure to UV-C (300 J/m²) (*A*) or TNFα (50 ng/ml) for 24 h (*B*). Apoptosis was assessed by TUNEL assay. *C*, quantitation of data shown in *A* and *B* were derived from triplicate experiments. *Error bars* represent S.D.

## DISCUSSION

In this report, we have provided evidence that AKT2 is activated by extracellular stress and TNFα through a PI3K-dependent pathway in human epithelial cells. Most importantly, the activation of AKT2 inhibits stress- and TNFα-induced JNK and p38 activities and activates the NFκB cascade, leading to protection of cells from stress- and TNFα-induced apoptosis.

Previous studies show that stress activates cell membrane

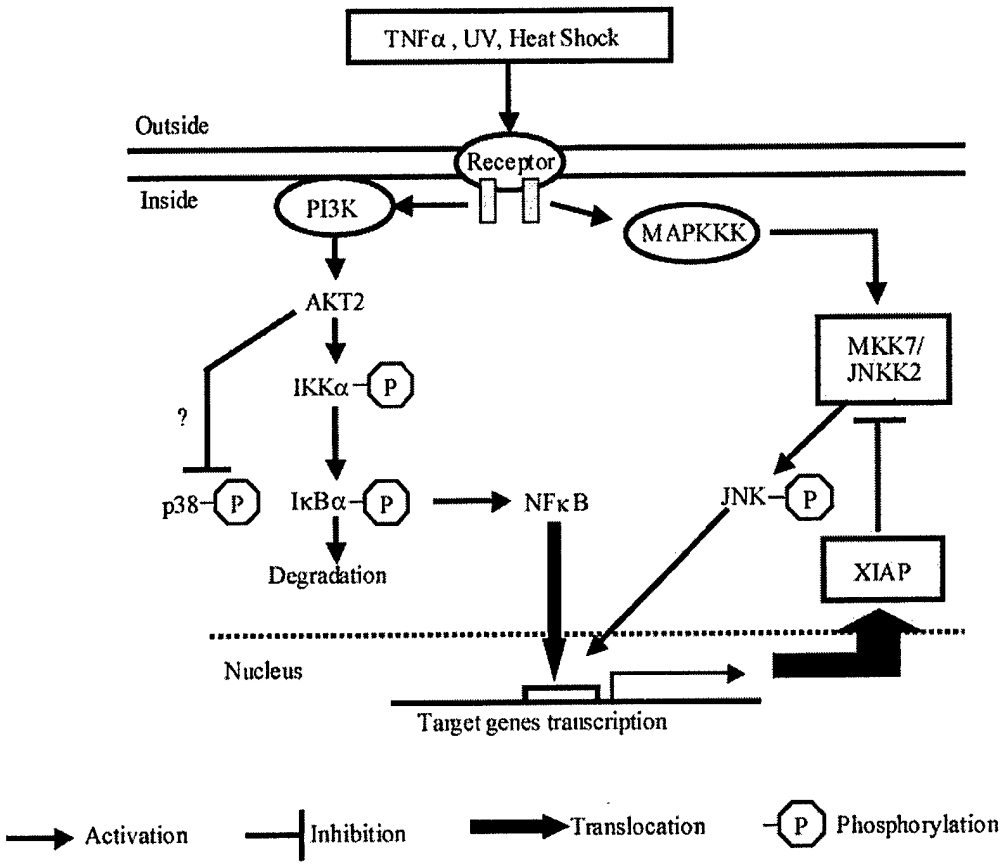



FIG. 8. **Schematic illustration of negative regulation of JNK by AKT2/NFκB.**

receptors, including those for epidermal growth factor, platelet-derived growth factor, and IGF. As a result, receptors associate with numerous proteins that activate downstream signaling molecules (1–3). One such protein is PI3K, which has been implicated in the regulation of nearly all stress signaling pathways (1). Because the AKTs are major downstream targets of PI3K, their role in the stress response has been recently investigated. In Swiss 3T3 cells, both oxidative stress and heat shock were shown to induce a marked activation of AKT1 and AKT3 but not AKT2 (19). AKT1 activation by hyperosmotic stress in COS7 and NIH 3T3 cells has also been demonstrated (17). In this study, we show that AKT2 is activated by different stress conditions including UV irradiation, hyperosmolarity, and heat shock as well as by TNFα in several human epithelial cell lines.

Three isoforms of AKT display high sequence homology and share similar upstream regulators and downstream targets as identified so far. However, there are clear differences between them in terms of biological and physiological function. In addition to the more prominent role of AKT2 in human malignancy and transformation (7, 32), the expression patterns of AKT1, AKT2, and AKT3 in normal adult tissues as well as during development are quite different (4, 8, 33). Recent studies suggest that AKT1, AKT2, and AKT3 may interact with different proteins and, thus, may play different roles in signal transduction. For instance, the Tcl1 oncoprotein preferentially binds to and activates AKT1 but not AKT2 (34). Gene knockout studies revealed that AKT1-deficient mice display defects in both fetal and postnatal growth but, unlike $AKT2^{-/-}$ mice, do not exhibit a type II diabetic phenotype; these differences suggest that the functions of AKT1 and AKT2 are non-redundant with respect to organismic growth and insulin-regulated glucose metabolism (21–23). It has been also shown that AKT2 but not AKT1 plays a specific role in muscle differentiation (35).[2] In this study, we demonstrated that AKT2 is activated by a variety of stress conditions in human epithelial cells but not in fibroblasts, suggesting that activation of different isoforms of AKT is cell type-specific in response to extracellular stress.

It is controversial whether stress-induced AKT1 activation is mediated by the PI3K pathway (17–19). Two previous reports showed that PI3K inhibitors did not block heat shock- or $H_2O_2$-induced activation of AKT1 and, thus, suggested that stress (unlike growth factors) activates AKT1 in a PI3K-indpendent manner (17, 18). However, the opposite results were observed by other groups (19, 20). Konishi *et al.* also provide evidence of AKT1 activation by $H_2O_2$ and heat shock through both PI3K-dependent and -independent pathways (18). We previously demonstrated that activation of AKT2 by growth factors required PI3K activity, whereas both PI3K-dependent and -independent pathways contributed to AKT2 activation by Ras (26). In this report, we show that PI3K inhibitors completely block AKT2 activation induced by UV-C, heat shock, and hyperosmolarity, indicating that stress activates AKT2 via the PI3K pathway.

JNK and p38 are stress mitogen-activated protein kinases that are activated by cytokines and a variety of cellular stresses (28). Like the classical mitogen-activated protein kinase kinase (MEK), direct activators for JNK and p38 have been identified. JNK is activated by phosphorylation of tyrosine and threonine by the dual specificity kinases, MKK4/SEK1 and MKK7. Similarly, p38 is activated by MKK3 and MKK6. However, biochemical studies have documented the existence of other JNK

[2] S. Kaneko, S. V. Nicosia, Z. Wu, T. Nobori, and J. Q. Cheng, submitted for publication.

and p38 activators or inhibitors in cells stimulated by a variety of cellular stresses (28). Although previous reports showed that AKT, JNK, and p38 are downstream targets of PI3K and represent parallel pathways in response to stress (17–20, 37, 38), the data presented in this study indicate that stress- and TNFα-induced activation of AKT2 inhibits the JNK and p38 activities, suggesting that AKT2 cross-talks with JNK and p38 stress pathways.

NFκB is another critical stress response pathway (29). Activation of NFκB is achieved through the signal-induced proteolytic degradation of IκB, which is associated with and inhibits the activity of NFκB in the cytoplasm. The critical event that initiates IκB degradation is the stimulus-dependent activation of the IκB kinases IKKα and IKKβ, which phosphorylate IκB at specific N-terminal serine residues ($Ser^{32}$ and $Ser^{36}$ for IκBα; $Ser^{19}$ and $Ser^{23}$ for IκBβ). Phosphorylated IκB is then selectively ubiquitinated by an E3 ubiquitin ligase and degraded by the 26 S proteasome, thereby releasing NFκB for translocation to the nucleus where it initiates the transcription of target genes (29). Moreover, two mitogen-activated protein kinase kinase kinase (MAPKKK) members, NIK and MEKK1, have been reported to enhance the activity of the IKKs and consequently trigger the phosphorylation and destruction of the IκBs and induce the activation of the NFκB pathway (29). Recent studies also showed that AKT1 induces the NFκB cascade through activation of IKK and degradation of IκB (13, 14). In this report, we show that AKT2 physically binds to and phosphorylates IKKα but not NIK even though NIK contains an AKT2 phosphorylation consensus sequence. When activated by stress or TNFα, AKT2 degrades IκB and activates NFκB-mediated transcription, indicating that stress-activated AKT2 targets the NFκB pathway.

Importantly, we have provided evidence that activation of AKT2 induced by stress and TNFα inhibits JNK activity through activation of the NFκB pathway to protect cells from apoptosis in response to these stimuli. Previous studies showed that the AKT2 pathway is important for cell survival and malignant transformation (7, 24, 32). The data presented here show that cells expressing constitutively active AKT2 are resistant to stress- and TNFα-induced apoptosis and that dominant-negative AKT2 and LY294002 sensitize cells to stress- and TNFα-induced programmed cell death. These findings indicate that stress-induced AKT2 activation promotes cell survival. Among the stress-activated kinases are JNK; recent studies demonstrated that activation of JNK and p38 plays an important role in triggering apoptosis in response to extracellular stress and TNFα (36, 39–41), whereas activation of NFκB protects cells from programmed cell death (29). Although a number of downstream targets of AKT2 have been identified, our data indicate that AKT2-inhibited JNK and p38 activities and AKT2-induced NFκB activation could play, at least in part, an important role in the AKT2 pathway that protects cells from stress- and TNFα-induced apoptosis. Recent reports demonstrate that NFκB-up-regulated Gadd45β and Xiap inhibited JNK activation and abrogated TNFα-induced programmed cell death (30, 31). Our cDNA microarray experiments showed that constitutively active AKT2 induces *Xiap*.[3] Thus, AKT2 inhibition of JNK activity could be due to up-regulation of Xiap by NFκB pathway (Fig. 8). Further studies are required to characterize the mechanism of inhibition of p38 stress pathway by AKT2 and involvement of Xiap in AKT2/NFκB inhibition of the JNK activation.

[3] M. Sun and J. Q. Cheng, unpublished data.

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# AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1

## IMPLICATION OF AKT2 IN CHEMORESISTANCE*

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**Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers. Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling downstream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects.**

Although cisplatin and its analogues, the DNA cross-linking agents, are first-line chemotherapeutic agents for the treatment of human ovarian and breast cancers, chemoresistance remains a major hurdle to successful therapy (1, 2). Several molecules have been implicated in cisplatin resistance, including decreased cellular detoxication (3, 4), increased DNA repair (5), and mutations of *p53* tumor suppressor gene (6, 7). However, the mechanisms involved in cisplatin resistance are still poorly understood. A growing body of evidence indicates that defects in the intra- and extracellular survival/apoptotic pathways are an important cause of resistance to cytotoxic agents.

Phosphatidylinositol 3-kinase (PI3K)[1]/Akt is a major cell survival pathway that has been extensively studied recently (8). PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit and converts the plasma membrane lipid phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Pleckstrin homology domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Akt (also known as PKB) represents a subfamily of serine/threonine kinases. Three member of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated in a PI3K-dependent manner by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (9–12). Downstream targets of Akt contain the consensus phosphorylation sequence R*X*R*XX*(S/T)(F/L) (13). Several targets of Akt that have been identified have roles in the regulation of apoptosis, such as the proapoptotic proteins BAD and caspase-9 and transcription factor FKHRL1. Phosphorylation by Akt blocks BAD binding to Bcl-$x_L$, inhibits caspase-9 protease activity, and blocks FKHRL1 function, reducing Fas ligand transcription (14–16).

Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies including ovarian cancer. We have demonstrated previously amplification of the AKT2 in a number of human ovarian cancer cell lines and recently detected frequently elevated protein and kinase levels of AKT2 in about a half of primary ovarian carcinoma examined (17, 18). Moreover, ectopic expression of wild type of AKT2 but not Akt1 in NIH 3T3 cells resulted in malignant transformation (19). Inhibition of PI3K/AKT2 by farnesyltransferase inhibitor-277 induced apoptosis in ovarian cancer cells that overexpress AKT2 (20). We have also shown that TNF$\alpha$ and extracellular stresses, including UV irradiation, heat shock, and hyperosmolarity, induce AKT2 kinase and that

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[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun $NH_2$-terminal kinase; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; TNF$\alpha$, tumor necrosis factor $\alpha$; GST, glutathione *S*-transferase; HEK, human embryonic kidney; MKK, mitogen-activated protein kinase kinase.

activated AKT2 inhibits JNK/p38 activity to protect cells from TNF$\alpha$ and cellular stress-induced apoptosis (21).

JNK and p38 are predominantly activated through environmental stresses, including osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors, stimulation of Fas, and inflammatory cytokines such as TNF$\alpha$ and interleukin-1. Stimulation of JNK/p38 activity has also been shown to be critical for cisplatin-induced apoptosis in some cancer cells (22, 23). Specific inhibition of JNK or p38, through small molecule inhibitors, dominant negative JNK/p38 mutants, or knock-out of JNK expression, suppresses various types of stress-induced apoptosis (24). Although it has been shown that JNK phosphorylates and inhibits antiapoptotic protein Bcl-2 (25), the mechanism of JNK/p38 induction of apoptosis is still not well understood.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase family that activates both the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (26–28). ASK1 is a general mediator of cell death in responds to a variety of stimuli, including oxidative stress (29, 30) and chemotherapeutic drugs such as cisplatin and paclitaxel (22, 23). Ectopic expression of ASK1 induced apoptosis in various cell types (26, 28). Furthermore, disruption of the *ASK1* gene in mice causes a remarkable reduction in sensitivity to stress-induced cell death, such as that promoted by TNF$\alpha$ or oxidative stress (33). These data indicate that ASK1 plays a key proapoptotic function through promoting the sustained activation of JNK/p38 mitogen-activated protein kinases.

In the present study, we show that AKT2 activity promotes resistance to cisplatin-induced apoptosis in A2780S ovarian cancer cells through the inhibition of the ASK1/JNK/p38 pathway. In A2780S cells, we show that AKT2 complexes with and phosphorylates ASK1 at Ser-83 within a consensus Akt phosphorylation site on this molecule. This results in inhibition of ASK1 activity and the blocking of JNK and p38 activation. We also show that these latter activities are required for cisplatin-induced apoptosis in A2780S cells. Furthermore, in response to cisplatin, we observe that ASK1 and JNK/p38 promote Bax conformational change. Collectively, these studies indicate that AKT2 may be an important mediator of chemoresistance through its regulatory effects on the ASK1/JNK/p38/Bax pathway.

## EXPERIMENTAL PROCEDURES

*Reagents*—Cisplatin, LY294002, and anti-Bax (6A7) were obtained from Sigma. DMEM and fetal bovine serum were purchased from Invitrogen. Anti-phospho-Akt (Ser-473), anti-cleaved PARP, anti-phospho-JNK (p54/44), anti-phospho-extracellular signal-regulated kinase 1/2(44/42), anti-phospho-p38, anti-phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2, and anti-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). GST-c-Jun and GST-ATF6 were also purchased from Cell Signaling. Anti-AKT2, anti-Bax, and anti-ASK1 were obtained from Santa Cruz Biotechnology. JNK inhibitor II and p38 inhibitor SB203580 were from Calbiochem.

*Cell Culture and Cisplatin Treatment*—The human epithelial cancer cell lines, A2780S and A2780CP, kindly provided by Benjamin K. Tsang at The Ottawa Hospital, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum. The cells were seeded in 60-mm Petri dishes at a density of $0.5 \times 10^6$ cells per dish. After 24 h, cells were treated with cisplatin (20 $\mu$M) for the appropriate time as noted in the figure legends.

*Expression Constructs*—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2 and constitutively active HA-Myr-AKT2 have been described previously (31). The $pcDNA_3$-HA-ASK1 construct was kindly provided by Hidenori Ihijo at Tokyo Medical and Dental University. HA-ASK1-S83A and ASK1-S83D, as well as dominant negative AKT2 with triple mutations (T309A, E299K, and S474A), were created using the QuikChange site-directed mutagenesis kit (Stratagene). JNK and p38 plasmids were obtained from Roger Davis at the University of Massachusetts.

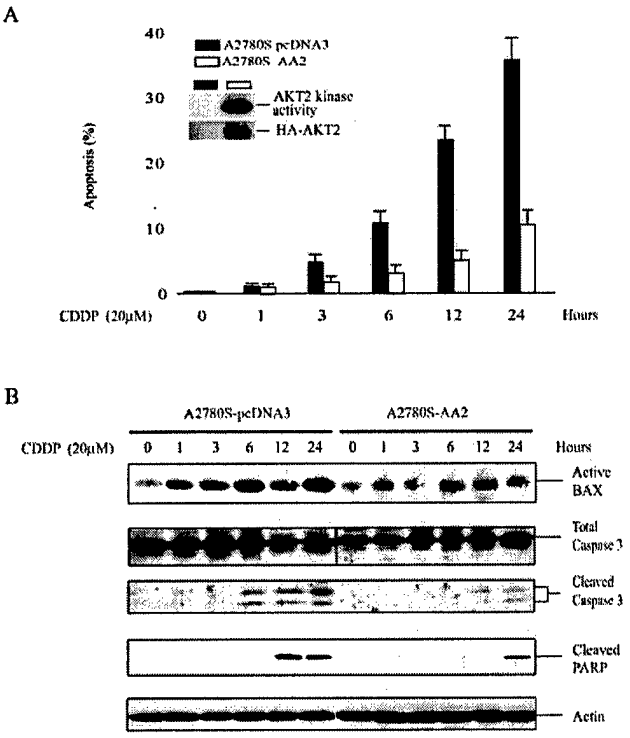


FIG. 1. **Activation of AKT2 renders cells resistant to cisplatin and inhibits cisplatin-induced Bax conformational change and caspase-3 cleavage.** *A*, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (*A2780S-AA2*) or pcDNA3 (*A2780S-pcDNA3*). Expression and kinase activity of transfected AKT2 were examined by Western blot and *in vitro* kinase assays (*inset*). The cells were treated with cisplatin (*CDDP*; 20 $\mu$M) for indicated time and analyzed by Tunel assay. Apoptotic cells were quantified in triple experiments. *B*, Western blot analysis. The cells were treated with cisplatin and lysed. A portion of lysate was immunoprecipitated with anti-active Bax (6A7) and detected with anti-total Bax antibody (*top panel*). The rest of the lysates were immunoblotted and probed with anti-caspase-3 (*second and third panels*), anti-PARP (*fourth panel*), and anti-actin (*bottom panel*) antibodies.

*Immunoprecipitation and Immunoblotting*—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 $\mu$g/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM $\beta$-glycerolphosphate. Lysates were centrifuged at 12,000 $\times$ *g* for 15 min at 4 °C prior to immunoprecipitation or Western blot. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated with appropriate antibodies in the presence of 25 $\mu$l of protein A-protein G (2:1)-agarose beads for at least 2 h at 4 °C. The beads were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10; twice with phosphate-buffered saline; and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM $\beta$-glycerolphosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the appropriate antibodies as noted in the figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting analysis system (Amersham Biosciences).

*In Vitro Kinase Assay*—Protein kinase assays were performed as described previously (21). Briefly, reactions were carried out in the presence of 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (PerkinElmer Life Sciences) and 3 $\mu$M cold ATP in 30 $\mu$l of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. 2 $\mu$g of myelin basic protein was used as the exogenous substrate. After incubation at room temperature for 30 min the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each

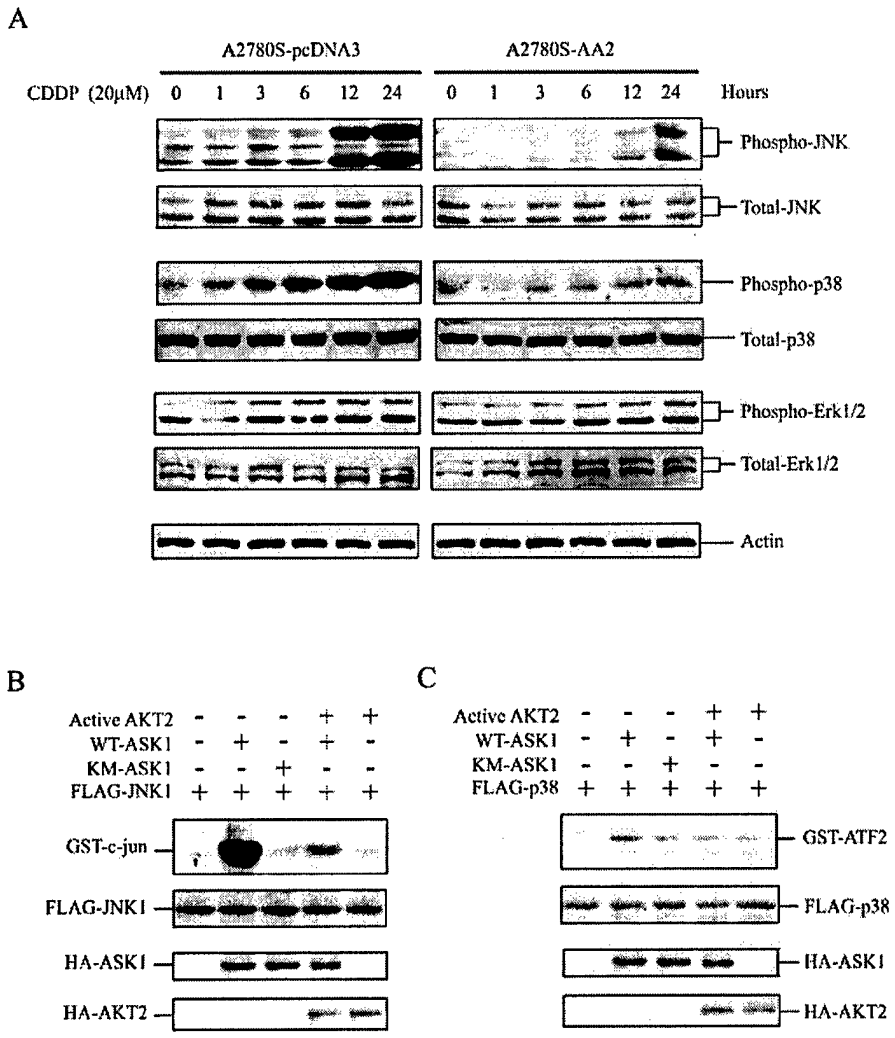


FIG. 2. **AKT2 inhibits JNK and p38 activation induced by cisplatin and ASK1.** *A*, immunoblotting analysis. Following treatment with cisplatin at indicated times, the cells were lysed and immunoblotted. The blots were detected with indicated antibodies. *B* and *C*, *in vitro* kinase assay. HEK293 cells were transfected with the indicated expression plasmids. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. The FLAG-JNK and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase using GST-c-Jun (*B*) and GST-ATF2 (*C*) as substrate, respectively (*top panel*). Expression of the transfected plasmids was shown in the *second*, *third*, and *fourth panels*.

experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

*In Vivo [$^{32}$P]$P_i$ Labeling*—HEK293 cells were co-transfected with active AKT2 and HA-tagged ASK1 or pcDNA3 and labeled with [$^{32}$P]$P_i$ (0.5 mCi/ml) in phosphate- and serum-free DMEM medium for 4 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to membranes. Phosphorylated ASK1 band was visualized by autoradiography. The expression of transfected ASK1 was detected with anti-HA antibody.

*Luciferase Reporter Assay*—Cells were seeded in 6-well plate and transfected with c-Jun or ATF6 reporter plasmid (pGl-GAL4), pSV2-β-gal, and different forms (wild type, constitutively active, or dominant negative) of HA-AKT2 together with or without different forms of ASK1 or vector alone. After 36 h of the transfection, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

*Tunel Assay*—Cells were seeded into 60-mm dishes and grown in DMEM supplemented with 10% fetal bovine serum for 24 h and treated with 20 μM cisplatin for different times. Apoptosis was determined by Tunel assay using an *in situ* cell death detection kit (Roche Applied Science). These experiments were performed in triplicate.

## RESULTS

*Activation of AKT2 Renders Cisplatin-sensitive Cells Resistant to Cisplatin and Inhibits Cisplatin-induced Bax Conformational Change*—We have shown previously (18, 34) frequent activation of AKT2 kinase in human ovarian and breast cancers. To examine whether activation of AKT2 contributes to chemoresistance in cancer cells, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (A2780S-AA2) or pcDNA3 vector alone. Expression and kinase activity of transfected constitutively active AKT2 were confirmed by Western blot and *in vitro* kinase analysis (Fig. 1A, *inset*). Following treatment with cisplatin (20 μM) for 0, 1, 3, 6, 12, and 24 h, programmed cell death in A2780S-pcDNA3 and A2780S-AA2 (active AKT2) cells were examined by Tunel assay. The number of apoptotic cells was quantified by counting three different microscopic fields. Three h after treatment, A2780S-pcDNA3 cells begun to undergo apoptosis. By 24 h of treatment, 35% of the cells were apoptotic, which is a similar response reported in the literature for parental A2780S cells (35). However, we observed a distinctly lower percentage of apoptotic cells at the time points 3, 6, 12, and 24 h in A2780S-AA2 cells (Fig. 1A), indicating that activation of AKT2 renders cisplatin-sensitive A2780S cells resistant to cisplatin.

It has been shown that Bax is required for cisplatin-induced apoptosis, *i.e.* cisplatin activates Bax by inducing its N-terminal conformation change and then targeting it to mitochondria resulting in cytochrome *c* release and activation of apoptotic pathway (36, 37). Thus, we next examined the effects of AKT2 activation on induction of Bax conformational changes by cisplatin. After treatment with cisplatin, A2780S-pcDNA3 and A2780S-AA2 cells were lysed and immunoprecipitated with anti-active Bax (6A7) antibody. The immunoprecipitates were subjected to Western blot analysis with total anti-Bax anti-

body. As shown in Fig. 1*B*, cisplatin promotes alteration of Bax conformation after 3 h of treatment in A2780S-pcDNA3 cells but not in A2780S-AA2 cells. Accordingly, cleavage of caspase 3 and its substrate, PARP, was also inhibited by expression of constitutively active AKT2 as compared with pcDNA3-transfected A2780S cells (Fig. 1*B*).

*AKT2 Inhibits Cisplatin- and ASK1-induced JNK and p38 Activation*—It has been documented that stress kinases, JNK and p38, are activated by cisplatin, and their activations are required for cisplatin-induced programmed cell death (22, 23, 38). To examine whether the effect of cisplatin on JNK and p38 is abrogated by the activation of AKT2, A2780S-pcDNA3 and A2780S-AA2 cells were treated with cisplatin at different times. As expected, JNK and p38 were activated by cisplatin in A2780S-pcDNA3 cells, and the activation of p38 took place before that of JNK. However, the activation of JNK and p38 was reduced dramatically in A2780S cells transfected with a constitutively active AKT2. No significant difference in the phosphorylation levels of extracellular signal-regulated kinase was observed between these two cell lines (Fig. 2*A*).

To explore the mechanism of AKT2 inhibition of the JNK and p38, we probed for direct interaction of these proteins by coimmunoprecipitation. We were not, however, able to demonstrate any interaction between AKT2 and JNK or p38 (data not shown). As ASK1 is known to activate JNK/p38 and be induced by cisplatin (32), and its overexpression is sufficient to induce apoptosis (26, 28), we next examined whether AKT2 restrains JNK and p38 activity through inhibition of ASK1. HEK293 cells were transfected with FLAG-JNK1 or FLAG-p38 and wild type or kinase-dead ASK1 (KM-ASK1), with or without constitutively active AKT2. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. FLAG-JNK1 and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase assays using GST-c-Jun and GST-ATF2 as substrates, respectively. Repeated experiments revealed that kinase activities of JNK1 and p38 were significantly induced by expression of wild type but not kinase-dead ASK1 and that the activation of JNK and p38 was attenuated by ectopic expression of constitutively active AKT2 (Fig. 2, *B* and *C*). These data indicate that AKT2 may negatively regulate ASK1, causing inhibition of cisplatin-induced JNK/p38 activation and apoptosis.

*AKT2 Interacts with, Phosphorylates, and Inhibits ASK1*—To examine whether ASK1 is a direct target of AKT2, co-immunoprecipitation was carried out with anti-AKT2 antibody and detected with anti-ASK1 antibody, and vice versa. As shown in Fig. 3, *A* and *B*, interaction between ASK1 and AKT2 was readily detected, and this interaction was enhanced by cisplatin treatment. Sequence analysis revealed that an AKT2 phosphorylation consensus site resides in ASK1 at residue Ser-83, which is conserved between human and mouse. To determine whether AKT2 phosphorylates ASK1, *in vitro* AKT2 kinase assays were performed using immunoprecipitated HA-ASK1 (wild type ASK1 or ASK1S83A) as substrates (Fig. 3*C*). In addition, *in vivo* [$^{32}$P] labeling and immunoblotting analyses with anti-phospho-Ser/Thr Akt substrate antibody were curried out in HEK293 cells transfected with ASK1 and constitutively active or wild type AKT2 (Fig. 3*D*). Both *in vitro* kinase and *in vivo* labeling experiments, as well as Western blot analysis, showed that wild type and constitutively active AKT2 phosphorylate ASK1 at Ser-83 with the lower phosphorylation level by wild type AKT2 (Fig. 3, *C* and *D*).

We next determined whether cisplatin-induced ASK1 activation is inhibited by AKT2 and, if it is, whether this inhibition depends upon AKT2 phosphorylation of ASK1 at Ser-83. Mutagenesis was used to create a form of ASK1 not phosphorylatable by AKT2, ASK1-S83A, prepared by converting Ser-83 of ASK1 to alanine. We also prepared ASK1-S83D, derived from mutating Ser-83 of ASK1 to aspartic acid, which mimics ASK1 phosphorylated by AKT2. A2780S cells were transfected with ASK1-S83A or ASK1-S83D, with or without constitutively active AKT2. Following cisplatin treatment, ASK1s were immunoprecipitated, and *in vitro* ASK1 kinase assays were performed using myelin basic protein as substrate. As shown in Fig. 3*E*, cisplatin significantly induced the kinase activity of both wild type ASK1 and nonphosphorylatable ASK1-S83A but not AKT2 phosphomimic ASK1-S83D. Expression of constitutively active AKT2 inhibited cisplatin-stimulated kinase activity of wild type ASK1 but not that of nonphosphorylatable ASK1-S83A. These results indicate that ASK1 kinase activity is negatively regulated by AKT2 through phosphorylation of ASK1 at Ser-83.

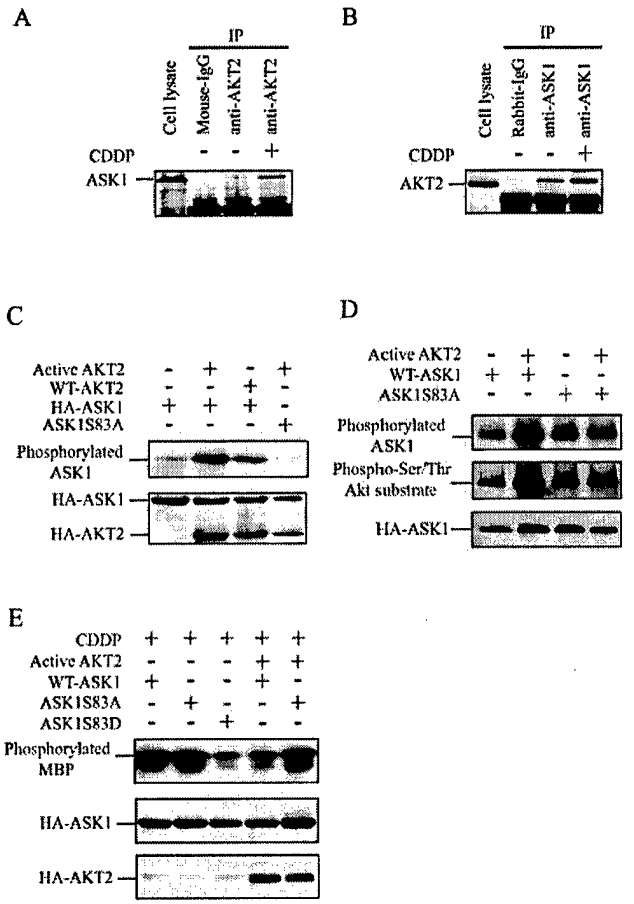


FIG. 3. **AKT2 interacts with and phosphorylates ASK1 and inhibits ASK1 kinase activity.** *A* and *B*, Western blot analyses of the immunoprecipitates prepared from A2780S cells treated with or without cisplatin. Immunoprecipitation was performed with anti-AKT2 and detected with anti-ASK1 antibody (*A*) and vice versa (*B*). *C*, *in vitro* kinase analysis of AKT2 immunoprecipitates derived from HEK293 cells that were transfected with indicated plasmids. Immunopurified HA-ASK1 or HA-ASK1-S83A was used as substrate (*top panel*). *The bottom panel* shows expression of transfected plasmids. *D*, *in vivo* [$^{32}$P]P$_i$ labeling. HEK293 cells were transfected with indicated expression constructs, labeled with [$^{32}$P]P$_i$ (0.5 mCi/ml), and immunoprecipitated with anti-HA antibody. The HA-ASK1 immunoprecipitates were separated in SDS-PAGE, blotted, and exposed to x-ray film (*top panel*). The membrane was then detected with anti-Akt substrate antibody (*middle panel*) and anti-HA antibody (*bottom panel*). *E*, *in vitro* ASK1 kinase analysis of the immunoprecipitates prepared from A2780S cells transfected with indicated plasmids and treated with cisplatin (20 $\mu$M) for 6 h. Myelin basic protein was used as substrate (*top panel*). Expression of transfected different forms of ASK1 and AKT2 was shown in the *second and third panels*.

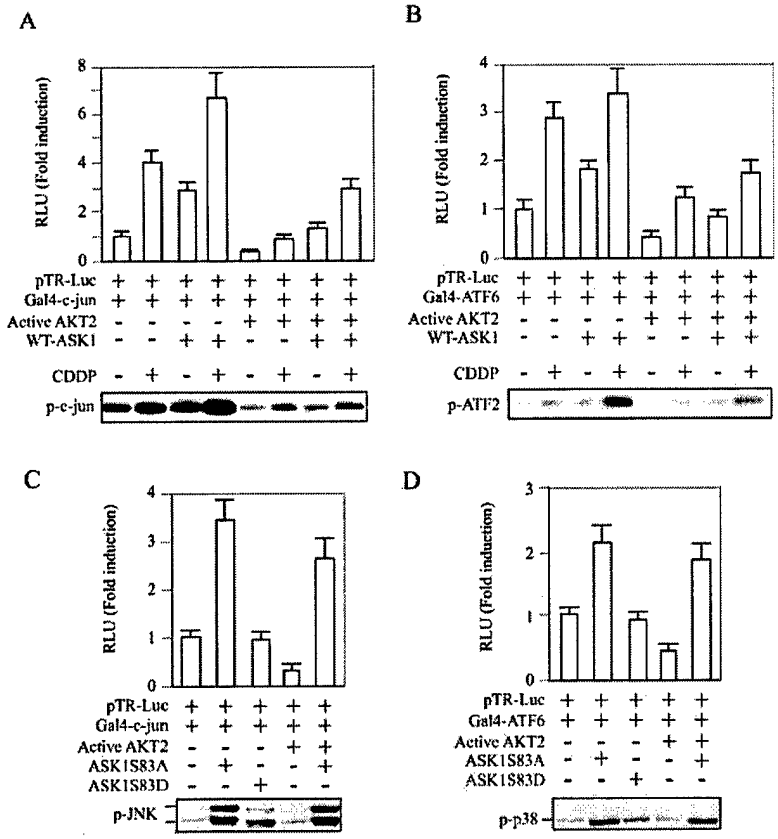


FIG. 4. **Activation of AKT2 inhibits ASK1- and/or cisplatin-induced JNK and p38 activation.** *A–D*, luciferase reporter assays. A2780S cells were transfected with indicated expression constructs and treated with or without cisplatin. Luciferase and β-galactosidase assays were performed, and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. Each experiment was repeated three times. The *bottom panels* of *A* and *B* show the results obtained from *in vitro* JNK and p38 kinase assays using GST-c-Jun and ATF2 as substrates, respectively. The effects of AKT2 and its phosphorylation of ASK1 at Ser-83 on JNK and p38 activation were shown in the *bottom panels* of *C* and *D*.

*AKT2 Inhibition of Cisplatin-stimulated JNK and p38 Is Mediated by Phosphorylation of ASK1 at Residue Ser-83*—We next determined whether phosphorylation of ASK1 on Ser-83 by AKT2 is required for AKT2 inhibition of p38 and JNK, which are downstream from ASK1. Luciferase reporter assays were performed using Gal4-c-Jun/pTR-Luc (for JNK) and Gal4-ATF6/pTR-Luc (for p38) reporter systems. A2780S cells were transfected with ASK1, ASK1-S83A, ASK1-S83D, and/or Myr-AKT2, as well as pTR-Luc, Gal4-c-Jun, or Gal4-ATF6, and treated with or without cisplatin. Three independent experiments revealed that cisplatin induces Gal4-c-Jun or Gal4-ATF-regulated reporter activities. Further, *in vitro* JNK and p38 kinase analysis revealed that the phosphorylation of c-Jun and ATF2 was also stimulated by cisplatin treatment. These effects were enhanced by ectopic expression of wild type ASK1; however, they were inhibited by expression of constitutively active AKT2 (Fig. 4, *A* and *B*). Expression of nonphosphorylatable ASK1-S83A was also sufficient to induce the reporter activities and to attenuate the inhibitory action of constitutively active AKT2. In contrast, phosphomimic ASK1-S83D failed to stimulate the reporter activities (Fig. 4, *C* and *D*). Moreover, the effects of ASK1-S83A and ASK1-S83D on cisplatin-induced JNK and p38 activation were similar to their action on Gal4-c-Jun and Gal4-ATF6 reporters (Fig. 5*A*). Therefore, we conclude that AKT2 inhibits cisplatin-induced JNK and p38 via a phosphorylation of ASK1-dependent manner.

*Cisplatin-induced Bax Conformational Change Is Regulated by AKT2 Phosphorylation of ASK1*—Previous studies have shown that JNK is required for UV- and cisplatin-induced Bax conformational change (39). Our data demonstrate that ectopic expression of constitutively active AKT2 overrides cisplatin-induced ASK1/JNK/p38 activation and prevents formation of the active Bax conformation (see Figs. 1 and 2). To more directly probe the effect of AKT2 phosphorylation of ASK1 on Bax activation, we transfected A2780S cells with nonphosphorylatable and phosphomimic ASK1 and treated the cells with or without cisplatin. As revealed by immunoprecipitation and Western blot analyses, ectopic expression of nonphosphorylatable ASK1-S83A enhance cisplatin-dependent Bax conformational change, whereas ASK1-S83D, mimicking ASK1 phosphorylated by AKT2, inhibited cisplatin-induced Bax

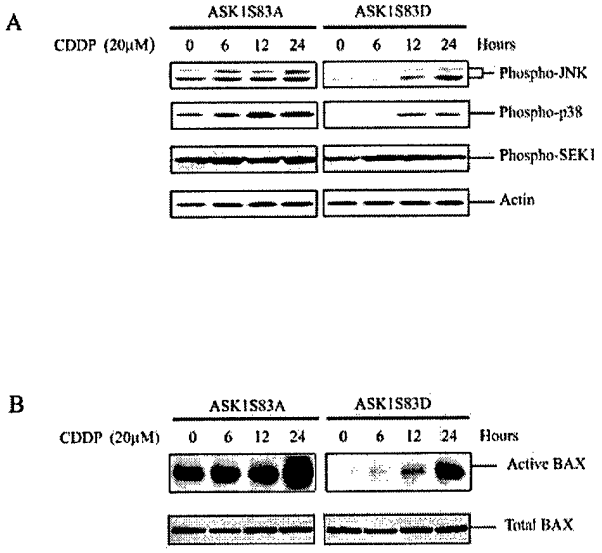


FIG. 5. **AKT2 phosphorylation of ASK1 at Ser-83 plays a critical role in cisplatin-induced JNK/p38 activation and Bax conformational change.** *A*, immunoblotting analysis of A2780S cells transfected with nonphosphorylatable and phosphomimic ASK1 prior to treatment with cisplatin. The blots were probed with the indicated antibodies. *B*, Western blot analysis. A2780S cells were transfected with indicated expression plasmids, treated with cisplatin, immunoprecipitated with anti-active Bax antibody, and detected with anti-total Bax antibody (*top panel*). Expression of Bax was shown in the *bottom panel*.

activation (Fig. 5*B versus* Fig. 1*B*). These data suggest that AKT2 inhibition of cisplatin-stimulated Bax conformational change is mediated at least to some extent by AKT2 phosphorylation of ASK1 at residue Ser-83.

Because JNK and p38 are downstream targets of ASK1, we next examined their roles in ASK1-stimulated Bax activation by using selective small molecule inhibitors of JNK and p38, JNK inhibitor II and SB 203580. As illustrated in Fig 6*A*, expression of ASK1 was sufficient to induce a Bax conformational change, and this effect was enhanced by cisplatin treatment. However, the conformational change of Bax induced by ASK1 and/or cisplatin was significantly diminished following treatment of cells with JNK inhibitor II (10 $\mu$M) and p38 inhibitor, SB 203580 (10 $\mu$M), suggesting that JNK and/or p38 mediate cisplatin-induced Bax activation. To probe the individual contributions of JNK and p38 in cisplatin-stimulated Bax activation, we further examined the effects of small molecule inhibitors of p38 and JNK and the expression of wild type and dominant negative forms of these kinases. A2780S cells were transfected with wild type or dominant negative JNK or p38, together with ASK1, and treated with or without cisplatin and/or inhibitor of JNK or p38. As shown in Fig. 6, *B* and *C*, expression of wild type JNK or p38 enhanced ASK1- and cisplatin-induced Bax activation, as expected. Furthermore, dominant negative JNK or a small molecule JNK inhibitor significantly decreased the Bax activation induced by cisplatin treatment or ectopic expression of ASK1 (Fig. 6*B*). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6*C*). These results indicate that cisplatin- and/or ASK1-induced Bax activation is mediated primarily by JNK.

*Inhibition of PI3K/AKT2 Pathway Sensitizes Cells to Cisplatin-induced Apoptosis*—Because activated AKT2 reduces the cisplatin sensitivity of A2780S cells, we next examined the ability of inhibition of the PI3K/AKT2 pathway to sensitize cells to cisplatin-induced apoptosis. Cisplatin-resistant A2780CP and A2780S cells were transfected with dominant negative AKT2 or treated with PI3K inhibitor, LY294002, together with cisplatin. Tunel assay analyses revealed that either LY294002 or ectopic expression of dominant negative AKT2 enhanced cisplatin-induced apoptosis as compared with cells treated with cisplatin alone (Fig. 7, *A* and *C*). Accordingly, cleavage of caspase-3 and PARP was increased by treatment of cells with a combination of cisplatin with LY294002 or dominant negative-AKT2 (Fig. 7, *B* and *D*). To examine the role played by AKT2 phosphorylation of ASK1 in cisplatin-induced apoptosis, we transfected A2780S cells with ASK1-S83A, which is not phosphorylated by AKT2, ASK1-S83D, which mimics AKT2 phosphorylation, and then induced apoptosis with cisplatin. Notably, ectopic expression of ASK1-S83A significantly augmented cisplatin-induced apoptosis. In contrast, expression of ASK1-S83D conferred resistance to cisplatin (Fig. 7*E*). These data further indicate that PI3K/AKT2 promotes cell survival through phosphorylation and inhibition of ASK1 signaling.

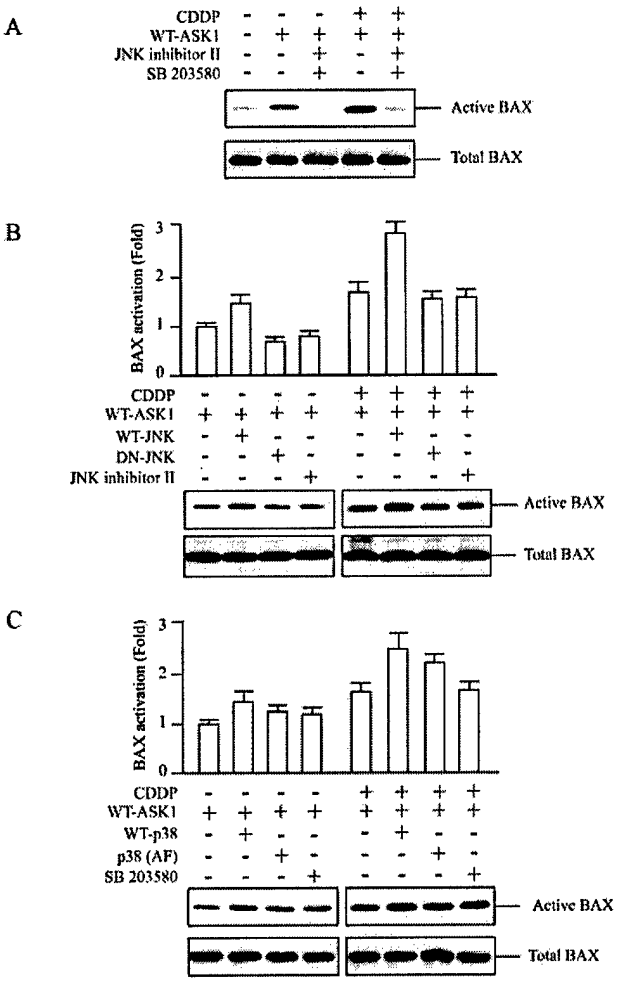


FIG. 6. **JNK and p38 mediate cisplatin- and ASK1-induced Bax conformational change.** *A*, Western blot analysis. A2780S cells were transfected with ASK1 and treated with JNK inhibitor II (10 $\mu$M) and SB 203580 (10 $\mu$M) for 1 h prior to addition of cisplatin. Following 16 h of the further treatment, Bax conformational change was examined as described above. *B* and *C*, immunoblotting analyses. A2780S cells were transfected with indicated plasmids and treated with indicated reagents. Bax conformational change was evaluated as described above. Both JNK inhibitor and dominant negative JNK exhibited more significant inhibitory effects on Bax activation than did p38 inhibitor and dominant negative p38 (AF). All the experiment was repeated three times.

## DISCUSSION

We have demonstrated previously (18, 34) that AKT2 kinase is frequently elevated in human ovarian and breast cancers and that AKT2, like Akt1, exerts its anti-apoptotic function through phosphorylation of Bad (20). However, the biological role of AKT2 activation in human cancer and the mechanism of AKT2-induced cell survival in a chemotherapeutic setting have not been well documented. In this study, we show that activation of AKT2 significantly increases the resistance of ovarian cancer cells to cisplatin. AKT2 protects cells from cisplatin-induced apoptosis by inhibiting cisplatin-induced JNK/p38 activation and Bax conformational change. AKT2 mediates these effects through its interaction and phosphorylation of ASK1.

Cisplatin-induced JNK and p38 activations are required for its anti-tumor activity (22, 23). This activation has been shown to correlate with induction of apoptosis by cisplatin (22, 23). Moreover, studies using dominant negative mutants of JNK and p38 and specific pharmacological inhibitors have shown that activation of JNK and/or p38 is necessary for stress or chemotherapeutic drug-induced apoptosis (38, 40). Also, studies on fibroblasts with targeted disruptions of all the functional *Jnk* genes established an essential role for JNK in UV- and other stress-induced apoptosis (41). ASK1, an upstream regulator of JNK/p38, has also been shown to be induced by cisplatin (32). Furthermore, oxidative stress-induced ASK1 kinase activity is inhibited by Akt1 (42). Consistent with this, we demonstrate that activation of AKT2 inhibits cisplatin-induced JNK and p38 through direct interaction with and phosphorylation of ASK1 at serine 83. We also demonstrate that phosphorylation of ASK1 by AKT2 renders cells resistant to cisplatin.

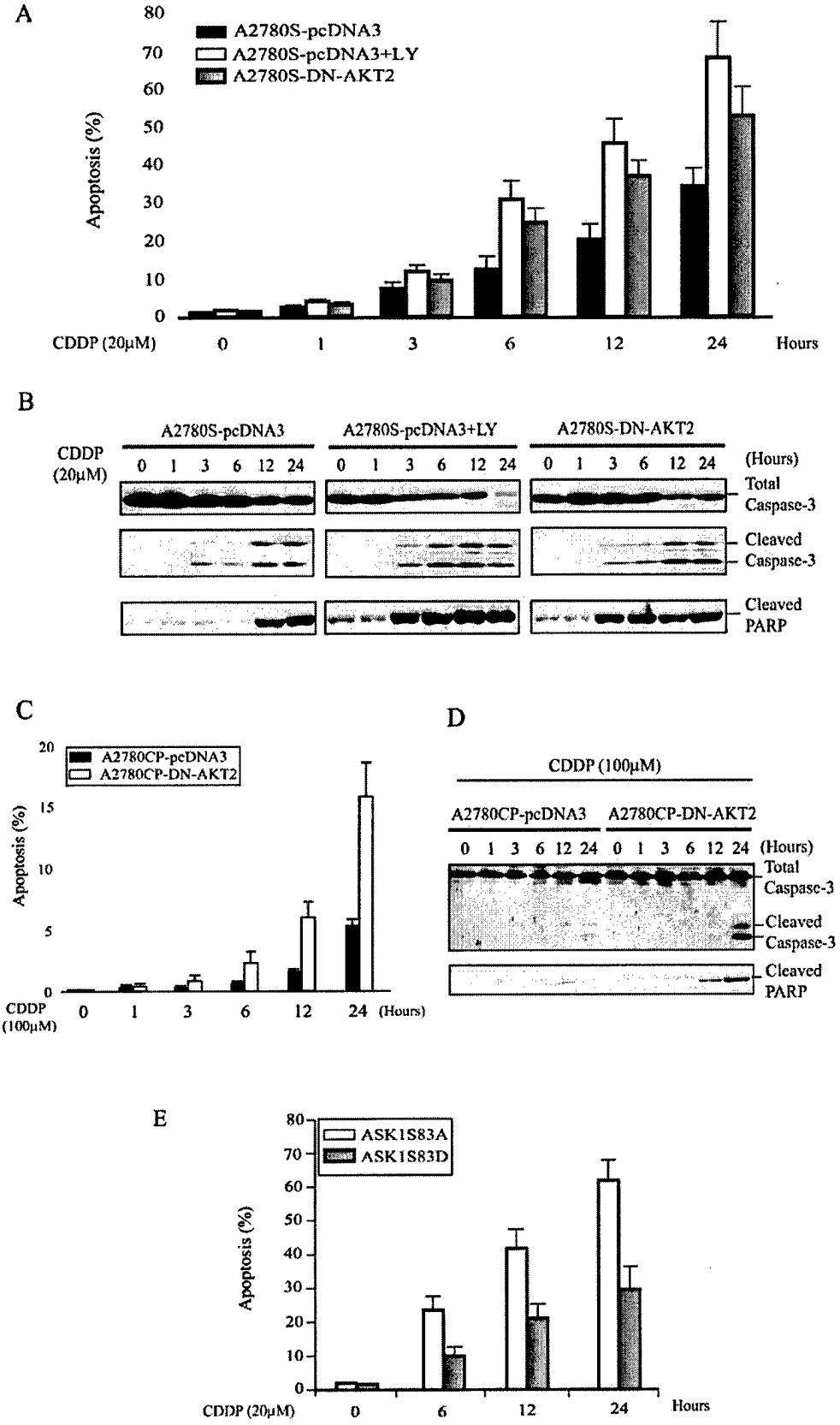


FIG. 7. **Inhibitions of PI3K/AKT2 and ASK1 phosphorylation sensitize cells to cisplatin-induced apoptosis.** *A*, Tunel assay. A2780S cells were transfected with dominant negative AKT2 or pcDNA3 vector and treated with cisplatin or cisplatin/LY294002. Apoptosis was examined and quantified after treatment for the indicated times. *B*, immunoblotting analysis of cell lysates prepared from cells treated as *A*. The blots were probed with indicated antibodies. *C* and *D*, cisplatin-resistant A2780CP cells transfected, treated, and analyzed as described in *A* and *B* except LY294002 treatment. *E*, Tunel assay. A2780S cells were transfected with indicated plasmids and treated with cisplatin. All the experiments were performed in triplicate.

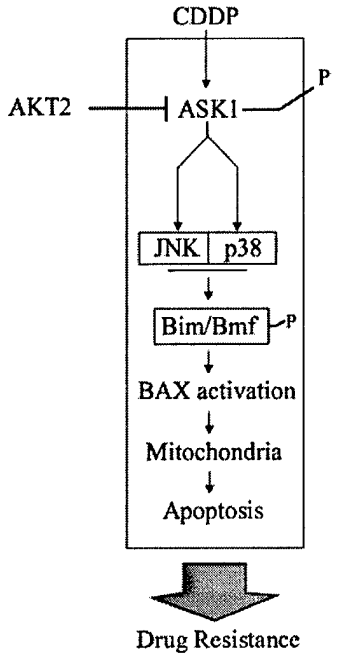


FIG. 8. **Schematic illustration of AKT2 regulation of ASK1/JNK/p38 and Bax.**

Besides the direct inhibition of ASK1, AKT2 could regulate JNK and p38 through other mechanisms. For example, NFκB-induced X chromosome-linked inhibitor of apoptosis and GADD45β down-regulate TNFα-induced JNK signaling (43, 44). We have demonstrated previously (21) that AKT2 inhibits UV- and TNFα-induced JNK and p38 by activation of the NFκB pathway (21). Therefore, we examined the possibility of AKT2 up-regulation of X chromosome-linked inhibitor of apoptosis and GADD45β. Western and Northern blot analyses, however, revealed no difference in X chromosome-linked inhibitor of apoptosis and GADD45β expression in A2780S cells transfected with constitutively active AKT2 or the control plasmid, pcDNA3 (data not shown). The possible reason is that cisplatin, unlike UV and TNFα, is incapable of inducing the NFκB pathway in A278S cells. In fact, our reporter assay revealed that cisplatin inhibits rather than activates NFκB activity in A2780S cells (data not shown).

In the present study, we observed that the ability of AKT2 to inhibit cisplatin-induced JNK/p38 was attenuated by nonphosphorylatable ASK1-S83A. Expression of phosphomimic ASK1-S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). In addition, ASK1-S83D exhibited effects similar to that of constitutively active AKT2, *i.e.* rendered cells resistant to cisplatin, whereas ASK1-S83A sensitized cells to cisplatin-induced apoptosis (Fig. 7*E*). Thus, we conclude that AKT2 inhibition of cisplatin-stimulated JNK/p38 activation leading to cisplatin resistance is mediated by AKT2 phosphorylation/inhibition of ASK1.

It has been demonstrated that cisplatin-induced Bax conformational change is also important for cisplatin-stimulated apoptosis (45). Bax is a pro-apoptotic member of the Bcl2 family. Accumulated evidence shows that death signals, including cisplatin, induce a conformational change of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and cytochrome *c* release (46, 47). Recent studies from Bax and/or Bak knock-out cells have shown that BH3-only proteins, such as tBid, Bad, Puma, and Bim, are required for inducing the activation of Bax and Bak by their direct interaction (48). Moreover, Akt has been shown to effectively inhibit Bax conformational change and contribute to chemoresistance (49). However, the mechanism by which Akt blocks Bax activation is poorly documented. We demonstrate in this report that ASK1 mediates at least in part cisplatin-induced Bax conformational change. Ectopic expression of constitutively active AKT2 attenuates cisplatin-induced Bax activation by phosphorylation and inhibition of ASK1. Downstream targets of ASK1, JNK, and p38, especially JNK, mediate AKT2 inhibition of Bax conformational change. These results are consistent with the recent findings obtained from a *Jnk*-deficient cell model (39).

Accumulated evidence shows that AKT2 plays a more significant role in human oncogenesis than AKT1 and AKT3. Frequent alterations of AKT2, but not AKT1 and AKT3, were detected in human cancers (18). Further, ectopic expression of AKT2, but not AKT1 and AKT3, leads to increased invasion and metastasis of human breast and ovarian cancer cells (50) and to malignant transformation of mouse fibroblasts (19). We observed in this study that A2780S cells expressing constitutively active AKT2 became cisplatin-resistant whereas expression of dominant negative AKT2 or treatment with PI3K inhibitor sensitized both cisplatin-sensitive (A2780S) and -resistant (A2780CP) ovarian cancer cells to cisplatin-induced apoptosis. Moreover, cisplatin-induced programmed cell death was enhanced by the expression of AKT2 nonphosphorylatable ASK1-S83A, whereas it is inhibited by phosphomimic ASK1-S83D. These data, therefore, indicate that activation of AKT2 contributes to cisplatin resistance by regulation of the ASK1/JNK/p38/Bax pathway and that the PI3K/AKT2/ASK1 cascade could be a critical therapeutic target for human cancer (Fig. 8).

A recent report (51) demonstrates that JNK and p38 phosphorylate BH3-only proapoptotic proteins Bim and Bmf, which was thought to mediate UV-induced apoptosis through a Bax-dependent mitochondrial apoptotic pathway (Fig. 8). Further investigation is required to determine the molecular mechanism by which ASK1/JNK/p38 regulates Bax activation in ovarian cancer cells, *i.e.* whether ASK1 and/or cisplatin induce Bim and Bmf phosphorylation and whether the phosphorylation is inhibited by PI3K/AKT2 pathway.

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## Publications:

**Yuan Z,** Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-23440, 2003.

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