ABSTRACT

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 AKT2, a member of the Akt family of serine/threonine kinases, renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin. Here, we report that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through regulation of the ASK1/JNK/p38 pathway by AKT2. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. AKT2 overexpression in chemoresistant cell lines results in decreased cellular detoxication, increased DNA repair, and mutations of p53 tumor suppressor gene. However, these mechanisms are still unclear. Thus, the role of AKT2 in chemoresistance is still under investigation.}

This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, portions of the total and cleaved caspase-3 immunoblots from Fig. 1B were reused in Fig. 7D. The HA-Ask1 immunoblot from Fig. 2B was reused in Fig. 2C. Lanes 4 and 5 of the HA-AKT2 immunoblot from Fig. 2B were reused in lanes 4 and 5 of the HA-AKT2 immunoblot from Fig. 2C. Lanes 4 and 5 from the total BAX immunoblot in Fig. 6A were reused in lanes 5 and 6 of the total BAX immunoblot from Fig. 6C. In Fig. 7B, lanes 1 and 2 from the cleaved caspase-3 immunoblot from A2780S-pcDNA3 were reused in lanes 1 and 2 for A2780S-pcDNA3+LY and A2780S-DN-AKT2. The authors state that they stand by the overall conclusions that were supported by the rest of the data in this work.

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. Several molecules have been implicated in cisplatin resistance, including decreased cellular detoxication, increased DNA repair, and mutations of p53 tumor suppressor gene. However, these mechanisms are still unclear. Thus, the role of AKT2 in chemoresistance is still under investigation.

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Activated AKT2 inhibits JNK/p38 activity to protect cells from TNFα 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 TNFα 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 family that activates both the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (26–28). ASK1 is a general mediator of cell death in response 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 TNFα 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 co-activates and phosphorylates ASK1 at Ser-83 within its regulatory domain and blocks JNK activity and the blocking of JNK activity also show that these latter activities are required for cisplatin-induced apoptosis in A2780S ovarian cancer cells. Moreover, in response to cisplatin, we observe that AKT2 is a key proapoptotic mediator of the ASK1/JNK/p38 pathway. Collectively, these data indicate that AKT2 may be an important mediator of chemoresistance through its regulatory effects on the ASK1/JNK/p38 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/p44), anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), anti-phospho-p38, anti-phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and anti-mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). GST-Δ-Jun and GST-ATF6 were also purchased from Cell Signaling. Anti-AKT2 anti-Rax, 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 of the University of Massachusetts. The cytomegalovirus-based expression constructs encoding wild type HA-AKT2 and constitutively active HA-Myr-AKT2 have been described previously (31). The pcDNA3-HA-ASK1 construct was kindly provided by Hidenori Iijima 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.

Immunoprecipitation and Immunoblotting—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 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-glycerophosphate. Lysates were centrifuged at 12,000 × g for 15 min at 4 °C prior to immunoprecipitation or Western blot analysis. 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 μl 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 mM 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 MgCl₂, 10 mM MnCl₂, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerophosphate 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 antibody 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 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 50 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM dithiothreitol. 2 μg of myelin basic protein was used as the exogenous substrate. After incubation at room temperature for 30 min the reaction was stopped by adding myelin basic 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 quantified with a PhosphorImager (Molecular Dynamics).

**In Vivo [32P]Pi Labeling** — HEK293 cells were co-transfected with active AKT2 and HA-tagged ASK1 or pcDNA3 and labeled with [32P]Pi (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 FLAG-JNK and FLAG-p38 immunoprecipitates were subjected to in vitro kinase assay 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.

![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.](image)

**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. 1B, 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. 1B).

**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. 2A).

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 antibodies against FLAG-JNK1 and FLAG-p38 immunoprecipitates were subjected to in vitro kinase assays using GST-c-Jun and GST-p38, respectively, as substrates, respectively. In all these experiments, we revealed that kinase activity of JNK and p38 was significantly induced by expression of wild type or kinase-dead ASK1 and that the activation was attenuated by ectopic expression of constitutively active AKT2 (Fig. 2, B and C). These data indicate that AKT2 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. 3C). In addition, in vitro [32P]Pi labeling and immunoblotting analyses with anti- phospho-Thr/Ser antibody were carried out in HEK293 cells transfected with ASK1 and constitutively active or wild type AKT2 (Fig. 3D). 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 AKT1 kinase assays were performed using myelin basic protein as substrate. As shown in Fig. 3E, 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.
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-ATF6-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. 5A). 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 enhanced cisplatin-dependent Bax conformational change, whereas ASK1-S83D, mimicking ASK1 phosphorylated by AKT2, inhibited cisplatin-induced Bax

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.

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. 5B versus Fig. 1B). 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 6A, 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 μM) and p38 inhibitor, SB 203580 (10 μM), 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. 6B). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6C). These results indicate that cisplatin- and/or JNK and p38 mediate Bax activation is mediated primarily by JNK.

Inhibition of PI3K/AKT2 Pathway Sensitizes Cells to Cisplatin-induced Apoptosis—Because activated AKT2 reduces the contributions of JNK and p38 in cisplatin-induced Bax activation, we transfected A2780S cells with ASK1 and treated with or without cisplatin and/or inhibitor of JNK or p38. As shown in Fig. 6, A 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. 6B). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6C). These results indicate that cisplatin- and/or JNK and p38 mediate Bax activation is mediated primarily by JNK.

AKT2 Inhibits ASK1/JNK/p38/Bax by Phosphorylation of ASK1

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.

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 μM) and SB 203580 (10 μM) 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.

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.
AKT2 Inhibits ASK1/JNK/p38/Bax by Phosphorylation of ASK1

Besides the direct inhibition of ASK1, AKT2 could regulate JNK and p38 through other mechanisms. For example, NHE1, a constitutively active X chromosome-linked inhibitor of apoptosis (GADD45) down-regulates TNFα-induced JNK and p38 (21, 22). We have demonstrated previously (21) that AKT2 regulates the NFκB pathway (Fig. 7). We have also shown that JNK and p38 activation is inhibited by PI3K/AKT2 pathway (Fig. 8) accumulation 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, which is normally inhibited by phosphomimetic ASK1-S83A. Therefore, we examined the possibility of AKT2 constitutively active AKT2, S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). We have demonstrated previously (21) that AKT2 inhibits 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, lead 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, which is normally inhibited by phosphomimetic ASK1-S83A. Therefore, we examined the possibility of AKT2 constitutively active AKT2, S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). We have demonstrated previously (21) that AKT2 inhibits 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).
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19. Cheng, J. Q., Altomare, D. A., Klein, M. A., Lee, W. C., Kruh, G. D., Lissy, N. A., and Testa, J. R. (1997) *Oncogene* **14**, 2793–2801
20. Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S. V., Hamilton, A. D., Sebit, S. M., and Cheng, J. Q. (2000) *Mol. Cell. Biol.* **20**, 139–148
21. Yuan, Z. Q., Feldman, R. I., Sun, M., Olashaw, N. E., Coppola, D., Sussman, G. E., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2002) *J. Biol. Chem.* **277**, 29973–29982
22. Benhar, M., Dalvit, I., Engelberg, D., and Levitzki, A. (2001) *Mol. Cell. Biol.* **21**, 6913–6926
23. Sanchez-Perez, I., Murguia, J. R., and Perona, R. (1998) *Oncogene* **16**, 533–540
24. Tibbles, L. A., and Woodgett, J. R. (1999) *Mol. Life Sci.* **2**, 1220–1254
25. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. (1999) *Mol. Cell. Biol.* **19**, 8469–8478
26. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsuomoto, K., Miyazono, K., and Gotob, Y. (1997) *Science* **275**, 90–94
27. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) *Mol. Cell* **2**, 389–395
28. Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998) *Science* **281**, 1860–1863
29. Saitoh, M., Nishitoh, H., Fuji, M., Takeda, K., Tobiume, K., Sawada, Y., Kawahata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17**, 2596–2606
30. Gotob, Y., and Cooper, J. A. (1998) *J. Biol. Chem.* **273**, 17477–17482
31. Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., and Wimalasena, J. (1998) *J. Biol. Chem.* **273**, 4928–4936
32. Chen, Z., Seimiyi, H., Naito, M., Mochida, Y., Kizuki, A., Dan, S., Inazumi, M., Ichijo, H., Miyazono, K., and Tsuruo, T. (1999) *Oncogene* **18**, 173–180
33. Ichijo, H., Nishida, E., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) *EMBO Rep.* **2**, 222–228
34. Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V., and Cheng, J. Q. (2001) *Am. J. Pathol.* **159**, 431–437
35. Asselin, E., Mils, G. B., and Tsang, B. K. (2001) *Cancer Res.* **61**, 1862–1868
36. Sugimoto, C., Fujieda, S., Seki, M., Sunaga, H., Fan, G. K., Tsuzuki, H., Bernet, C., Suito, H., and Matsuoka, S. (1999) *Int. J. Cancer.* **82**, 860–867
37. Makin, G. W., Corfem B. M., Griffiths, G. J., Thistlethwaite, A., Hickman, J. A., and Dive, C. (2001) *EMBO J.* **20**, 6306–6315
38. Sanchez-Perez, I., and Perona, R. (1999) *FEBS Lett.* **453**, 151–158
39. Lei, K., Nimmual, A., Zeng, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D., and Davis, R. J. (2002) *Mol. Cell. Biol.* **22**, 4929–4942
40. Wang, X., Martindale, J. L., Liu, Y., and Hallbrook, N. J. (1998) *Biochem. J.* **333**, 291–300
41. Tournez, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimmual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288**, 870–874
42. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) *Mol. Cell. Biol.* **21**, 893–901
43. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) *Nature* **414**, 308–313
44. Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001) *Nature* **414**, 313–317
45. Devarajan, P., Savoca, M., Castaneda, M. P., Park, M. S., Esteban-Cruciani, N., Kalinec, G., and Kalinec, F. (2002) *Hearing Res.* **174**, 45–54
46. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) *EMBO J.* **17**, 3878–3885
47. Bellosillo, B., Villanor, N., Lopez-Guillermo, A., Marco, S., Bosch, F., Campo, E., Montserrat, E., and Colomer, D. (2002) *Blood* **100**, 1810–1816
48. Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N. R. (2002) *Mol. Cell. Biol.* **22**, 3577–3589
49. Yamaguchi, H., and Wang, H. G. (2001) *Oncogene* **20**, 7779–7786
50. Arboleda, M. J., Lyons, J. F., Kabbinavar, F. F., Bray, M. R., Snow, B. E., Ayala, R., Danno, M., Karlan, B. Y., and Shamon, D. J. (2003) *Cancer Res.* **63**, 196–206
51. Lei, K., and Davis, R. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 2432–2437

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