Inhibition of Phosphorylation of BAD and Raf-1 by Akt Sensitizes Human Ovarian Cancer Cells to Paclitaxel*

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We studied the roles of the phosphatidylinositol 3-kinase (PI-3K)-Akt-BAD cascade, ERK-BAD cascade, and Akt-Raf-1 cascade in the paclitaxel-resistant SW626 human ovarian cancer cell line, which lacks functional p53. Treatment of SW626 cells with paclitaxel activates Akt and ERK with different time frames. Interference with the Akt cascade either by treatment with PI-3K inhibitor (wortmannin or LY294002) or by exogenous expression of a dominant negative Akt in SW626 cells caused decreased cell viability following treatment with paclitaxel. Interference with the ERK cascade by treatment with an MEK inhibitor, PD98059, in SW626 cells also caused decreased cell viability following treatment with paclitaxel. Treatment of cells with paclitaxel also stimulated the phosphorylation of BAD at both the Ser-112 and Ser-136 sites. The phosphorylation of BAD at Ser-136 was blocked by treatment with wortmannin or cotransfection with the dominant negative Akt. On the other hand, the phosphorylation of BAD at Ser-112 was blocked by PD98059. We further examined the role of BAD in the viability following paclitaxel treatment using BAD mutants. Exogenous expression of doubly substituted BAD2SA in SW626 cells caused decreased viability following treatment with paclitaxel. Moreover, because paclitaxel-induced apoptosis is mediated by activated Raf-1 and the region surrounding Ser-259 in Raf-1 conforms to a consensus sequence for phosphorylation by Akt, the regulation of Raf-1 by Akt was examined. We demonstrated an association between Akt and Raf-1 and showed that the phosphorylation of Raf-1 on Ser-259 induced by paclitaxel was blocked by treatment with wortmannin or LY294002. Furthermore, interference with the Akt cascade induced by paclitaxel up-regulated Raf-1 activity, and expression of constitutively active Akt inhibited Raf-1 activity, suggesting that Akt negatively regulates Raf-1. Our findings suggest that paclitaxel induces the phosphorylation of BAD Ser-112 via the ERK cascade, and the phosphorylation of both BAD Ser-136 and Raf-1 Ser-259 via the PI-3K-Akt cascade, and that inhibition of either of these cascades sensitizes ovarian cancer cells to paclitaxel.

Paclitaxel, a natural product originally isolated from the bark of Taxus brevifolia, has significant anti-tumor activity in several human tumors, particularly in advanced ovarian and breast carcinomas (1, 2). Unlike other antimicrotubule agents, paclitaxel increases tubulin polymerization, stabilizes microtubules, and prevents tubulin depolymerization, ultimately causing tubulin bundling (3–5). These effects of the drug are correlated with the arrest of cells in the G2/M phase of the cell cycle, as well as cellular toxicity (5–7). The inclusion of paclitaxel in the treatment of patients with newly diagnosed ovarian cancer has led to improved response rates and prolonged median survival compared with the results of prior regimens (8). Nevertheless, the majority of patients with advanced ovarian cancer are destined to relapse and subsequently to develop resistance to initially active drugs such as paclitaxel (9).

Insight into the mechanisms of drug resistance has been gained from a better understanding of the pathway of apoptosis. Apoptosis is the final common pathway of many if not all forms of chemotherapy-induced cell death (10, 11). Molecules known to predispose cells to apoptosis have been shown to enhance sensitivity to a variety of chemotherapeutic agents that induce damage to DNA or to the mitotic spindle (10, 12, 13). Conversely, defects in the apoptotic pathway have been observed to confer insensitivity to the cytotoxic effects of chemotherapy and may therefore represent an important mechanism for the development of chemoresistance (14–19). The apoptotic response of a cell damaged by chemotherapy partly depends upon the balance between proteins that predispose to apoptosis, such as BAX, and proteins that antagonize apoptosis, such as Bcl-XL or Bcl-2 (20, 21). Recently, a signaling pathway by which extracellular stimuli suppress apoptosis has been characterized. One of the first reports on survival signaling demonstrated an association between activation of the mitogen-activated protein (MAP)1 kinase cascade with survival in PC-12 cells (22). Recent studies (23–25) suggest that paclitaxel affects the activities of several intracellular tyrosine and serine/threonine protein kinases. For example, paclitaxel causes rapid activation of MAP kinase (26). Another signaling pathway requiring phosphatidylinositol 3-kinase (PI3K) activity was shown to be associated with anti-apoptotic signaling in neurons, fibroblasts, and hematopoietic cells (27–29). Subsequently, the serine/threonine kinase termed Akt or protein kinase B was identified as a downstream component of survival signaling through PI3K (30–34). Akt plays a central role in promoting the survival of a wide range of cell types (30–36).

1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; PTX, paclitaxel; ERK, extracellular signal-regulated (protein) kinase; PI-3K, phosphatidylinositol 3-kinase; BAD, Bcl-2 associated death protein; Ser, serine; GST, glutathione S-transferase; CMV, cytomegalovirus; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline.
Recently, BAD, a pro-apoptotic member of the Bcl-2 family, was found to be a substrate of Akt, identifying an intersection point of pro- and anti-apoptotic regulatory cascades (37, 38). Whereas BAD can be phosphorylated at either Ser-112 or Ser-136 (39), Akt phosphorylates BAD specifically at Ser-136 (37, 38). The involvement of MAP kinase/ERK kinase (MEK) upstream of BAD phosphorylation (40) and the promotion of cell survival by the Ras-MAP kinase signaling pathway by phosphorylation of BAD at Ser-112 (41-43) were recently reported. BAD is capable of forming heterodimers with the anti-apoptotic proteins Bcl-XL or Bcl-2 and antagonizes their anti-apoptotic activity (44).

Recent work (25, 45, 46) has suggested that Raf-1 is a mediator of paclitaxel-induced apoptosis. Treatment with geldanamycin to deplete intracellular Raf-1 inhibited paclitaxel-induced apoptosis (25, 45). The kinase activity of Raf-1 (47) is regulated by phosphorylation of a highly conserved serine residue (Ser-259) in the amino-terminal regulatory domain (48). The region surrounding Ser-259 in Raf-1 conforms to a consensuss sequence for phosphorylation by Akt (49, 50). It was reported recently (51) that Akt-mediated phosphorylation of Raf-1 at Ser-259 in vivo inhibits Raf-1 kinase activity.

However, the effects of the chemotherapeutic agent paclitaxel, which induces damage to the mitotic spindle, on the PI-3K-Akt-BAD cascade, the ERK-BAD cascade, and the association of Akt and Raf-1 have not yet been reported. Therefore, we sought to determine whether the PI-3K-Akt-BAD cascade, the ERK-BAD cascade, or the phosphorylation of Raf-1 by Akt plays a role in the cellular stress response to paclitaxel by using the paclitaxel-resistant SW626 human ovarian cancer cell line, which lacks functional p53. Here we provide evidence that paclitaxel induced the activation of Akt and ERK with different time frames, followed by phosphorylation of BAD. Paclitaxel also induced the phosphorylation and negative regulation of Raf-1 by Akt. Moreover, whereas inhibition of Raf-1 activity by geldanamycin markedly increased the cell viability following treatment with paclitaxel, inhibition of Akt and BAD markedly decreased the cell viability following treatment with paclitaxel.

EXPERIMENTAL PROCEDURES

Materials—Paclitaxel was a gift from Bristol-Myers Squibb Co. Wortmannin and geldanamycin were purchased from Sigma. PD98059, mouse monoclonal anti-phospho-ERK antibody, rabbit polyclonal anti-phospho-BAD antibody, and rabbit polyclonal anti-phospho-Raf antibody were obtained from New England Biolabs (Beverly, MA). The plasmids encoding the HA-tagged form of kinase-dead Akt (HA-AktK179M) and constitutively active Akt (HA-mAkt4-129 Akt) and the BAD mutant (pCDNA3-BAD Ser to Ala at 112 and 136) used in this study have been described previously (37).
Effects of Paclitaxel on BAD and Raf-1 Phosphorylation

![Diagram showing the effects of Paclitaxel on BAD and Raf-1 phosphorylation.]

**Fig. 1. Paclitaxel induces activation of both Akt and ERK.** Cells were grown in 100-mm dishes. A, cells were treated with 1 µM paclitaxel for the indicated times (lanes 2–6). Cells were pretreated with 100 nM wortmannin for 15 min (lane 7) or 100 nM PD98059 for 30 min (lane 8), followed by treatment with 1 µM paclitaxel for 3 h. For analysis of Akt activation (upper panel), lysates were subsequently immunoprecipitated with immobilized anti-Akt antibody, and the kinase reaction was carried out in the presence of cold ATP and GSK-3α fusion protein, as described under “Experimental Procedures.” After the reactions were stopped with Laemmli sample buffer, the samples were resolved by 8% SDS-PAGE, followed by Western blotting with anti-phospho-GSK-3α/β antibody. For analysis of phosphorylated Akt (middle panel) or the total amount of Akt (lower panel), 250 µg of protein from the lysate samples was resolved by 8% SDS-PAGE, followed by Western blotting with anti-Akt antibody or anti-Akt antibody, respectively. Experiments were repeated three times with essentially identical results. B, cells were treated with 1 µM paclitaxel for the indicated times (lanes 2–6). Cells were pretreated with 100 nM PD98059 for 30 min (lane 7), 100 nM wortmannin for 15 min (lane 8), or 50 µM EGF for 20 min (lane 9). For analysis of ERK activation (upper panel), lysates were subsequently immunoprecipitated with anti-ERK1 antisera, and the immunoprecipitates were incubated with [γ-32P]ATP in the presence of myelin basic protein (MBP) as described under “Experimental Procedures.” After the reactions were stopped by the addition of Laemmli sample buffer, samples were subjected to SDS-PAGE and autoradiographed. For analysis of phosphorylated ERK (middle panel) or the total amount of ERK (lower panel), 250 µg of protein from the lysate samples were resolved by 10% SDS-PAGE, followed by Western blotting with anti-phospho-ERK antibody or anti-ERK antibody, respectively. Experiments were repeated three times with essentially identical results.

**Materials and Methods.**

**Experimental Procedures.** Cells cultured in 100-mm dishes were washed twice with PBS and lysed in ice-cold lysis buffer B (50 mM Tris, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, and 1 mM dithiothreitol), and then the samples were resuspended in 30 µl of kinase assay buffer containing 10 µg of myelin basic protein and 40 µM [γ-32P]ATP (1 µCi) as described previously (52, 53). The kinase reaction was allowed to proceed at room temperature for 5 min and was stopped by the addition of Laemmli SDS sample buffer (55). Reaction products were resolved by 15% SDS-PAGE and visualized by autoradiography.

For analysis of phosphorylated ERK or the total amount of ERK, 250 µg of protein from the lysate samples was resolved by 8% SDS-PAGE, followed by Western blotting with anti-phospho-ERK antibody or anti-ERK1 rabbit polyclonal antibody, respectively.

**Phosphorylation of BAD—**Cells cultured in 100-mm dishes were transfected with 4 µg of pCDNA3-BAD using LipofectAMINE plus. At 72 h after transfection, serum-deprived cells were treated with various materials. They were then washed twice with PBS and lysed in ice-cold HNTG buffer (53). The lysate samples were immunoprecipitated with phospho-BAD (Ser-112) or phospho-BAD (Ser-136) antibody. Immune complexes were precipitated with protein A-Sepharose, and the isolated proteins were analyzed by electrophoresis on 8% SDS-PAGE. Transfer to nitrocellulose, Western blotting with phospho-BAD (Ser-112) or phospho-BAD (Ser-136) antibody, and washing were performed as described elsewhere (53).

For analysis of the total amount of BAD, 250 µg of protein from the lysate samples was resolved by 8% SDS-PAGE, followed by Western blotting with anti-BAD antibody or anti-BAD1 rabbit polyclonal antibody, respectively.

**Assay of Raf Kinase Activity—**Cells were incubated in the absence of serum for 16 h and then treated with various materials. They were then washed twice with PBS and lysed in ice-cold lysis buffer B. The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent. Five hundred µg of protein from the lysate samples was incubated with gentle rocking at 4 °C overnight with immobilized Raf-1 antibody cross-linked to protein G-agarose beads. After Raf-1 was selectively immunoprecipitated from the cell lysates, the immunoprecipitated products were washed twice in 500 µl of lysis buffer B and once in 80 µl of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). Immunoprecipitated Raf-1 was then incubated with 0.4 µg of inactive GST-MEK 1 and 1 µg of inactive GST-MAP kinase 2/Erk2 in 30 µl of kinase assay buffer (20 mM MOPS, pH 7.2, 25 mM MgCl2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 150 µM ATP) for 30 min at 30 °C. Four microliters of supernatant from the reaction were transferred to a new tube and incubated for 10 min at 30 °C with 10 µl of assay dilution buffer containing 20 µg of myelin basic protein and 40 µM [γ-32P]ATP (1 µCi) as described previously (56). The kinase reaction was stopped by the addition of Laemmli SDS sample buffer (55). Reaction products were resolved by 15% SDS-PAGE and visualized by autoradiography.

**Statistics—**Statistical analysis was performed using Student’s t test, and p < 0.05 was considered significant. Data are expressed as the mean ± S.E.

**Results.**

**Activation of Akt and ERK—**We first evaluated whether Akt was activated or phosphorylated in response to paclitaxel treatment in SW626 human ovarian cancer cells. Cultured cells were exposed to 1 µM paclitaxel for the indicated times (Fig. 1A). To examine Akt activation, cell lysates were immunoprecipitated with immobilized anti-Akt antibody, and the in vitro kinase reaction was carried out in the presence of cold ATP and [γ-32P]ATP in the presence of myelin basic protein (MBP). The kinase reaction was allowed to proceed at room temperature for 5 min and was stopped by the addition of Laemmli SDS sample buffer (55). Reaction products were resolved by 15% SDS-PAGE and visualized by autoradiography. For analysis of the total amount of BAD, 250 µg of protein from the lysate samples was resolved by 8% SDS-PAGE, followed by Western blotting with anti-BAD antibody or anti-BAD1 rabbit polyclonal antibody, respectively.
followed by treatment with the indicated concentrations of paclitaxel as described under “Experimental Procedures.”}

We therefore investigated whether paclitaxel induces the activation or the phosphorylation by paclitaxel (Fig. 1A, upper and middle panels). The lysate samples were immunoprecipitated with anti-HA antibody. To examine Akt phosphorylation, cell lysates were resolved by SDS-PAGE, followed by Western blotting with anti-phospho-Akt antibody. Induction of both activation and phosphorylation of Akt by paclitaxel in SW626 cells was detected at 30 min, reached a plateau at 3 h, and declined thereafter (Fig. 1A, lower panel). These results indicate that paclitaxel induces Akt activity and phosphorylation by paclitaxel (Fig. 1A, upper panel), indicating the absence of cross-talk between the Akt and ERK cascades in the activation induced by paclitaxel.

Recently it was reported that paclitaxel activates the MEK/ERK cascade and that MEK inhibition enhances paclitaxel-induced tumor apoptosis (57). We therefore investigated whether paclitaxel induces the activation or the phosphorylation of ERK. Cells were treated with 1 μM paclitaxel for the times indicated in Fig. 1B. To examine ERK activation, cell lysates were immunoprecipitated with anti-ERK antibody and examined for ERK activity by assayng the incorporation of $^{32}$P into MBP (Fig. 1B, upper panel). To examine ERK phosphorylation, cell lysates were resolved by SDS-PAGE, followed by Western blotting with anti-phospho-ERK antibody (Fig. 1B, middle panel). We confirmed that the total amount of ERK in each lane was the same (Fig. 1B, lower panel). Both the activation and phosphorylation of ERK were fully induced by paclitaxel by 30 min and declined thereafter, a time frame different from that of the effects of paclitaxel on Akt.

Although pretreatment with a PI-3K inhibitor, wortmannin, completely abolished the induction of Akt activity and phosphorylation by paclitaxel (Fig. 1A, upper panels, lane 7), pretreatment with either wortmannin or LY294002 had no effect on the induction of ERK activity and phosphorylation by paclitaxel (Fig. 1B, upper and middle panels, lanes 8 and 9). In addition, although an MEK inhibitor, PD98059, completely abolished the induction of ERK activity and phosphorylation by paclitaxel (Fig. 1B, upper and middle panels, lane 7), it had no effect on the induction of Akt activity or phosphorylation by paclitaxel (Fig. 1A, upper and middle panels, lane 8), indicating the absence of cross-talk between the Akt and ERK cascades in the activation induced by paclitaxel.

Kinase-deficient Akt, Wortmannin, PD98059 Sensitize SW626 Cells to Paclitaxel—To determine whether Akt activation is necessary for cell survival signaling after paclitaxel-induced cell damage, the effect of paclitaxel treatment on the viability of SW626 cells either pretreated with wortmannin or LY294002 (Fig. 2A) or expressing a kinase-
deficient Akt (AktK179M), which is an Akt derivative rendered kinase-inactive by a point mutation within the catalytic domain (30, 37) (Fig. 2C), was compared with its effect on the viability of the parental SW626 cells or an empty vector (CMV-6)-expressing control line, respectively. We first confirmed the overexpression of ectopically expressed Akt protein products (Fig. 2B, lower panel) and the negative effects of the expression of HA-AktK179M on Akt activity (Fig. 2B, upper panel). The viability of SW626 cells was not detectably affected by increasing concentrations of paclitaxel of >200 nM. Further titrations revealed IC_{50} values of 1900 and 1980 nM for parental (Table I) and empty vector-expressing (Table I) SW626 cells, respectively (Fig. 2B, lower panel). SW626 cells pretreated with wortmannin or LY294002 exhibited IC_{50} values as low as 190 and 198 nM, respectively, indicating over 10.0- and 13.8-fold greater sensitivity to paclitaxel than the untreated cells, respectively (Fig. 3), indicating over 15.2- or 14.6-fold greater sensitivity to paclitaxel than the untreated cells, respectively (Fig. 3A, lane 4). Pretreatment with either wortmannin or LY294002 appeared to be due to interference with the activation of Akt.

Moreover, to determine whether ERK activation is necessary for cell survival signaling after paclitaxel-induced cell damage, the effect of paclitaxel treatment on the viability of SW626 cells pretreated with PD98059 was compared with the effect of the parental cells (Fig. 2A). The cells pretreated with PD98059 exhibited IC_{50} values as low as 190 nM, indicating over 10.0-fold greater sensitivity to paclitaxel than the untreated cells (Fig. 2A and Table I). Thus, the sensitization to paclitaxel caused by pretreatment with PD98059 appeared to be due to interference with the activation of ERK, as reported previously (57).

LY294002 and PD98059 Sensitize Both Paclitaxel-sensitive and -resistant Cells to Paclitaxel—We further examined whether blockade of the PI-3K-Akt and ERK cascades in ovarian cancer cells that are sensitive to paclitaxel has the same effect. A2780 and Caov-3 cells exhibited IC_{50} values of 180 and 210 nM, respectively, indicating over 1.8- and 2.6-fold greater resistance to paclitaxel than the parental cells, respectively (Fig. 3D and Table III). Treatment of A2780 (Fig. 3, left panel) and Caov-3 (Fig. 3, right panel) cells with paclitaxel induced Akt activation (Fig. 3A, lane 2) and ERK activation (Fig. 3B, lane 2). Although pretreatment with LY294002 completely abolished the induction of Akt activity by paclitaxel (Fig. 3A, lane 4), pretreatment with PD98059 had no effect on the induction of ERK activity by paclitaxel (Fig. 3B, lane 3). In addition, although PD98059 completely abolished the induction of ERK activity by paclitaxel (Fig. 3B, lane 4), it had no effect on the induction of Akt activity by paclitaxel (Fig. 3A, lane 3), indicating the absence of cross-talk between the Akt and ERK cascades in the activation induced by paclitaxel in A2780 and Caov-3 cells, as in the case of SW626 cells (Fig. 1). A2780 and Caov-3 cells pretreated with LY294002 exhibited IC_{50} values as low as 68 and 52 nM, respectively, indicating over 1.47- and 1.54-fold greater sensitivity to paclitaxel than the untreated cells, respectively (Fig. 3C and Table III). A2780 and Caov-3 cells pretreated with PD98059 exhibited IC_{50} values as low as 67 and 34 nM, respectively, indicating over 1.49- and 2.35-fold greater sensitivity to paclitaxel than the untreated cells, respectively (Fig. 3C and Table III). Thus, blockade of each cascade in cells which are sensitive to paclitaxel also further sensitized the cells to paclitaxel.

Moreover, we examined whether blockade of the PI-3K-Akt and ERK cascades in A2780/PTX and Caov-3/PTX cells, both of which have acquired in vitro resistance to paclitaxel, has the same effect. A2780/PTX and Caov-3/PTX cells exhibited IC_{50} values of 180 and 210 nM, respectively, indicating over 1.8- and 2.6-fold greater resistance to paclitaxel than the parental cells, respectively (Fig. 3D and Table III). A2780/PTX and Caov-3/PTX cells pretreated with PD98059 exhibited IC_{50} values as low as 110 and 100 nM, respectively, indicating over 1.49- and 2.35-fold greater sensitivity to paclitaxel than the untreated cells, respectively (Fig. 3C and Table III). Thus, blockade of either cascade in cells that have acquired in vitro resistance to paclitaxel also sensitized the cells to paclitaxel.

These findings have broad implications for the potential clinical use of inhibitors of the PI-3K-Akt and ERK cascades for improving the response rate to paclitaxel in the treatment of
resistant tumors, an anticancer strategy similar to that proposed for MEK inhibitors (57).

Phosphorylation of BAD—Recently, BAD was identified as an intersection point of pro- and anti-apoptotic regulatory cascades (37, 38). BAD function is modulated by phosphorylation at two sites, Ser-112 and Ser-136 (39). The presence of two phosphorylation sites on BAD suggests that the simultaneous activation of different survival cascades may result in the con-
comitant phosphorylation of BAD Ser-112 and Ser-136 (37). In recent studies (37–39), the region surrounding Ser-136 in BAD was shown to conform to a consensus sequence for phosphorylation by Akt, and BAD was identified as a potential target of Akt, linking the PI-3K pathway directly to the apoptotic machinery. In addition, the promotion of cell survival by the Ras-MAPK signaling pathway by phosphorylation of BAD at Ser-112 (41–43, 58) was recently reported. Therefore, we next examined the effect of paclitaxel on the phosphorylation of BAD at Ser-112 and Ser-136. Cells were transfected with pCDNA3-BAD and exposed to 1 μM paclitaxel for 3 h. Cell lysates were immunoprecipitated with either anti-phospho-Ser-112 (Fig. 4A, upper panel) or anti-phospho-Ser-136 (Fig. 4B, upper panel) BAD antibody, followed by Western blotting with the same antibodies. Paclitaxel induced the phosphorylation of BAD at Ser-112 and Ser-136. Moreover, we confirmed that the total amount of BAD in each lysate was the same by Western blotting with anti-BAD antibody (Fig. 4, lower panel). In accordance with reports showing that Akt phosphorylates BAD specifically at Ser-136 (37, 38), paclitaxel-induced phosphorylation of BAD at Ser-136 was completely inhibited by wortmannin (Fig. 4B, lane 3) but was not completely inhibited by PD98059 (Fig. 4B, lane 4). In addition, paclitaxel-induced phosphorylation of BAD at Ser-136 was completely inhibited by the expression of a kinase-deficient Akt (AktK179M) (Fig. 4B, lane 5). On the other hand, paclitaxel-induced phosphorylation of BAD at Ser-112 was not inhibited by wortmannin (Fig. 4A, lane 3) but was completely inhibited by PD98059 (Fig. 4A, lane 4). Thus, paclitaxel induced the phosphorylation of BAD at Ser-112 via the ERK cascade and that at Ser-136 via the Akt cascade, as does cisplatin (58).

Interference with Phosphorylation of BAD at Ser-112 and Ser-136 Sensitizes SW626 Cells to Paclitaxel—To determine whether BAD phosphorylation is necessary for cell survival signaling after paclitaxel-induced cell damage, the effect of paclitaxel treatment on the viability of SW626 cells expressing mutant BAD constructs, in which both Ser-112 and Ser-136 were converted to alanine (BAD2SA) so that BAD could no longer be phosphorylated at these sites (37, 58), was compared with the effect on the viability of an empty vector (pCDNA3)-expressing control line. We first confirmed the over-expression of the BAD protein products (Fig. 5A, lower panel) and the negative effects of the expression of BAD2SA on BAD phosphorylation at both Ser-112 (Fig. 5A, upper panel) and Ser-136 (Fig. 5A, middle panel). The BAD2SA-expressing SW626 cells exhibited an IC50 as low as 90 nM, indicating over 20.0-fold greater sensitivity to paclitaxel than the empty vector-expressing SW626 cells (Fig. 5B and Table II). Expression of wild-type BAD did not affect the sensitivity to paclitaxel compared with the sensitivity of cells expressing the empty vector (pCDNA3)-expressing control lines (data not shown).

Thus, the sensitization to paclitaxel observed in BAD2SA-expressing cells appeared to be due to interference with the activation of BAD.

Negative Regulation of Raf-1 by Akt—Several studies have shown that paclitaxel-mediated apoptosis is mediated by activation of Raf-1 (25, 45, 46). The Hsp90 chaperone is required for maintenance of the Raf-Ras complex and for protecting Raf from degradation. An inhibitor of Hsp90, geldanamycin, disrupted the complex containing Raf, Ras, and Hsp90 and caused a marked decrease in the half-life of the Raf protein due to an increase of the rate of its degradation (59). To determine whether Raf-1 activation is necessary for paclitaxel-induced cell damage, the effect of paclitaxel treatment on the viability of SW626 cells pretreated with geldanamycin was compared with

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**Table III**

Effects of LY294002 and PD98059 on Paclitaxel-induced cytotoxicity in A2780 and Caov-3 cells

| IC50 Sensitizationa | IC50 Sensitizationa |
|---------------------|---------------------|
| **Control**         | LY294002            | PD98059            |
| A2780               | 68                  | 1.47               |
| 100 nM              | 67                  | 1.49               |
| Caov-3              | 130                 | 1.38               |
| 180 nM              | 110                 | 1.64               |
| Caov-3/PTX          | 52                  | 1.54               |
| 80 nM               | 34                  | 2.35               |
| Caov-3/PTX          | 210                 | 1.91               |
| 210 nM              | 100                 | 2.10               |

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*a Control cells were analyzed in parallel with equal concentrations of paclitaxel in the range 0–200 nM in quadruplicate.

*b Sensitization is defined as the ratio of the IC50 value for the parental cells to the IC50 value for the cells treated with the kinase inhibitor.

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**Fig. 4.** Paclitaxel induces phosphorylation of both BAD Ser-112 and BAD Ser-136. Cells grown in 100-mm dishes were transfected with pCDNA3-BAD. A, after 72 h, cells were treated with 1 μM paclitaxel for 3 h (lanes 2–4). Cells were pretreated with 100 nM wortmannin for 15 min (lane 3) or 100 nM PD98059 for 30 min (lane 4) and then treated with 1 μM paclitaxel for 3 h. Lysates were subsequently immunoprecipitated with anti-phospho-Ser-112 antibody, and the immunoprecipitated samples were subjected to SDS-PAGE followed by Western blotting with anti-phospho-Ser-112 antibody. B, after 72 h, cells were treated with 1 μM paclitaxel for 3 h (lanes 2–5). Cells were either pretreated with 100 nM wortmannin for 15 min (lane 3) or 100 nM PD98059 for 30 min (lane 4) or transfected with AktK179M, followed by treatment with 1 μM paclitaxel for 3 h. Lysates were subsequently immunoprecipitated with anti-phospho-Ser-136 antibody, and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-phospho-Ser-136 antibody. For analysis of the total amount of BAD (lower panel of A and B), 250 μg of protein from the lysate samples were resolved by 8% SDS-PAGE, followed by Western blotting with anti-BAD antibody. Experiments were repeated three times with essentially identical results. C, control.
phospho-Raf-1 antibody. The treatment with paclitaxel for 3 h induced the phosphorylation of Raf-1 at Ser-259 (Fig. 6B, lane 2). Pretreatment with either of the PI-3K inhibitors, LY294002 (Fig. 6B, lane 3) or wortmannin (Fig. 6B, lane 4), inhibited paclitaxel-induced phosphorylation of Raf-1 at Ser-259, whereas pretreatment with PD98059 had no effect (Fig. 6B, lane 5). Moreover, we confirmed that the total amount of Raf-1 in each lane was the same (Fig. 6B, lower panel). In addition, the expression of an activated Akt mutant that is constitutively targeted to the plasma membrane (HA-mAkt)–129 Akt (37, 60) markedly induced the phosphorylation of Raf-1 at Ser-259 (Fig. 6D, ii). Moreover, we examined the effect of Akt on the activation of Raf-1. Cell lysates were immunoprecipitated with anti-Raf-1 antibody, and the immunoprecipitates were subjected to Western blotting with anti-Akt and endogenous Raf-1 were co-immunoprecipitated from SW626 cells that had been stimulated with paclitaxel. Next, we examined the effect of paclitaxel-induced Akt activation on the phosphorylation of Raf-1 at Ser-259 (Fig. 6B). Cell lysates were immunoprecipitated with anti-Raf-1 antibody, and the immunoprecipitates were subjected to Western blotting with anti-

DISCUSSION

The signaling pathway involving Raf, MEK (ERK kinase), and ERK functions downstream of the small guanine nucleotide-binding protein Ras and mediates several apparently contradictory cellular responses, such as proliferation, apoptosis, growth arrest, differentiation, and senescence, depending on the duration and strength of the external stimulus and on the cell type. For example, although prolonged activation of ERK by a GnRH agonist in ovarian cancer cells was reported to be involved in growth arrest (53), transient activation of ERK by cisplatin in ovarian cancer cells was reported to be involved in the resistance to cisplatin (i.e. proliferation) (52). It was reported recently (61) that prolonged activation of the ERK cascade induced by paclitaxel was not linked to activation of the cell death machinery. In the present report, we demonstrated that ERK is activated transiently by paclitaxel (Fig. 1B) and that interfering with the MEK/ERK cascade sensitizes SW626 cells to paclitaxel (Fig. 2). It was also reported more recently (57) that paclitaxel enhances the activation of the MEK/ERK pathway, which is expected to promote cell proliferation and survival. Another pathway that acts downstream of Ras involves PI-3 kinase and Akt and also regulates the cellular responses listed above, acting either synergistically with (62) or in opposition to (63) the Raf pathway. Overexpression of Akt was reported to confer resistance to paclitaxel (64). However, it was recently reported that paclitaxel induced apoptosis independently of Akt in human ovarian carcinoma cells that expressed wild-type p53 (65). In the present report, we showed that paclitaxel induced Akt activation transiently without cross-talk with the ERK cascade (Fig. 1A) and that interfering with the Akt cascade either by the addition of specific inhibitors, wortmannin or LY294002 or by expression of dominant negative Akt sensitizes SW626 cells (which lacks functional p53) to paclitaxel (Fig. 2) as is also true in the case of cisplatin (58). Coordination of the two signaling pathways in a given cellular response may depend on the cell type or the stage of differentiation (66–68). We recently reported (58) that the ERK and Akt signaling cascades converge at BAD to suppress the apoptotic effect of BAD in cisplatin-treated ovarian cancer
FIG. 6. Negative regulation of Raf-1 by Akt. A, association between Akt and Raf. Serum-deprived cells were treated with 1 μM paclitaxel for 3 h, after which endogenous Raf and Akt were immunoprecipitated (IP) with their cognate antibodies. The resulting immunoprecipitates were then subjected to immunoblot (I.B.) analysis with the same antibodies. Molecular mass markers are noted at the right. B, Akt phosphorylates Raf-1 at Ser-259. Cells were pretreated with 50 μM LY294002 for 20 min (lane 3), 100 nM wortmannin for 15 min (lane 4), or 100 nM PD98059 for 30 min (lane 5), and then treated with 1 μM paclitaxel for 3 h (lanes 2–5). Lysates were subsequently immunoprecipitated with anti-Raf-1 antibody, and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-phospho-Raf-1 (Ser-259) antibody (upper panel) or Raf-1 antibody (lower panel). C, cells were grown in 100-mm dishes. Cells were pretreated with (lane 2) or without (lane 1) 50 μM LY294002 for 20 min and then treated with 1 μM paclitaxel for 3 h. For the Raf-1 kinase assay, equal amounts of Raf-1 (as judged by Western blotting with Raf-1 antibody (iii)) were immunoprecipitated with carboxyl-terminal Raf-1 antibody and assayed for activation of MAP kinase kinase, which in turn was assayed for its ability to activate MAP kinase, measured by phosphorylation of myelin basic protein (i) as described under “Experimental Procedures.” To assess the phosphorylation of Raf-1 at Ser-259 (ii), lysates were subsequently immunoprecipitated with anti-Raf-1 antibody, and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-phospho-Raf-1 (Ser-259) antibody. To assess Akt activation (iv), lysates were subsequently immunoprecipitated with immobilized anti-Akt antibody, and the kinase reaction was carried out in the presence of cold ATP and GSK-3 α fusion protein, as described under “Experimental Procedures.” D, cells grown in 100-mm dishes were transfected with CMV-6 or constitutively active Akt (HA-mAkt129). After 72 h, cells were treated with 1 μM paclitaxel for 3 h. The lysate samples were immunoprecipitated with anti-HA antibody. For analysis of the effects of ectopically expressed Akt on Raf-1 activity (i), immune complexes were precipitated with protein A-Sepharose, and the bound proteins were eluted with 1% SDS, and the Raf-1 activity eluted thereby was measured as described under “Experimental Procedures.” For analysis of the effects of ectopically expressed Akt on Raf-1 phosphorylation at Ser-259 (ii) or Raf-1 expression (iii), immune complexes were precipitated with protein A-Sepharose, and the immunoprecipitated samples were subjected to SDS-PAGE followed by Western blotting with anti-phospho Raf-1 (Ser-259) antibody (ii) or Raf-1 antibody (iii). For analysis of the effects of ectopically expressed Akt on Raf-1 phosphorylation at Ser-259 (ii) or Raf-1 expression (iii), immune complexes were precipitated with protein A-Sepharose, and the bound proteins were eluted with 1% SDS, and the Akt activity eluted thereby was measured as described under “Experimental Procedures.”
cells. In the present study, we also demonstrated that paclitaxel induces the phosphorylation of BAD both at Ser-112 via the ERK cascade (Fig. 4A) and at Ser-136 via the Akt cascade (Fig. 4B). Moreover, interference with either of these cascades sensitizes SW626 cells to paclitaxel (Fig. 5B). It was recently shown (13) that paclitaxel-induced apoptosis of the SW626 ovarian cancer cell line is enhanced by stable BAX overexpression in a p53-independent manner. In addition, expression of HA-BAD in ovarian cancer cell lines was found to enhance significantly the cytotoxic effects of paclitaxel (69). Thus, the ERK and Akt signaling cascades converging at BAD are involved in the mechanisms of maintaining the cell viability following both cisplatin and paclitaxel treatment in ovarian cancer cells.

Although the expression of Raf-1 has no relationship with cisplatin (70), the level of paclitaxel-induced apoptosis seems to have some dependence on Raf-1 kinase activity. It was reported that phosphorylation by Akt inactivates the function of Akt substrates such as BAD (37), FKHR1 (71), and Raf-1 (51). The regions surrounding Ser-136 in BAD and Ser-259 in Raf-1 conform to a consensus sequence for phosphorylation by Akt (49, 50). The present study also showed that paclitaxel induced the phosphorylation of BAD at Ser-136 (Fig. 4B) and Raf-1 at Ser-259 (Fig. 6B) in an Akt-dependent mechanism. Interference with the Akt-BAD cascade sensitizes SW626 cells to paclitaxel (Figs. 2 and 5), suggesting that the phosphorylation of BAD at Ser-136 by paclitaxel inhibits the pro-apoptotic function of BAD. Akt reduced the level of activation of Raf-1, whose destabilization by geldanamycin increased the resistance of cells to paclitaxel (Table 1) (Fig. 6, C and D), via direct interaction between Akt and Raf-1 (Fig. 6A) and the phosphorylation of Raf-1 at Ser-259 (Fig. 6B). Thus, Akt appears to play a central role in the mechanism of a protective response to paclitaxel.

Are there any differences among the ERK-BAD, Akt-BAD, and Akt-Raf-1 cascades with respect to the sensitivity to paclitaxel? We did not detect any differences in basal kinase activation or paclitaxel-induced kinase activation between SW626 cells, which are resistant to paclitaxel, and A2789 and Caov-3 cells, both of which are sensitive to paclitaxel (Figs. 1 and 3). In addition, blockade of the PI-3K-Akt and ERK cascades increased the sensitivity to paclitaxel, independent of the paclitaxel sensitivity of cells (Figs. 2 and 3). Extensive research has identified several potential mechanisms of paclitaxel-induced cell death; most prominent is the effect on Bcl-2 family members and p53. Several reports (45, 72–75) have indicated that paclitaxel causes the phosphorylation and inactivation of Bcl-2 and its family members, whereas other studies (76–78) have found that paclitaxel sensitivity varies with p53 status. SW626 cells show resistance to paclitaxel because they lack functional p53. The BAD protein is a newly described pro-apoptotic member of the Bcl-2 family which is capable of binding to both Bcl-XL and Bcl-2 and also of displacing BAX (79). Paclitaxel-stimulated phosphorylations of c-Raf-1 and Bcl-2 are tightly coupled (45). Because interference with these cascades changed the sensitivity to paclitaxel (Fig. 5, Table I, and Table II), these cascades seem to be p53-independent mechanisms. In other words, these cascades might be involved in repair mechanisms of cells treated with paclitaxel, as in the case of cisplatin (52, 58).

Raf-1 located at the plasma membrane phosphorylates ERK, which in turn phosphorylates Bcl-2 (45), thereby disrupting the association between Bcl-2 and the pro-apoptotic BAX protein (73), which then induces apoptosis. Prior exposure of human tumor cells to the drug geldanamycin has been reported to diminish concomitantly Raf-1 kinase activity and paclitaxel-induced apoptosis (45), as we showed in Table I. Thus, although Raf-1 is known to function upstream of ERK, the link between Raf-1 and ERK activation and paclitaxel-induced cell death might not be always straightforward. It is noteworthy that c-Raf-1 contains multiple potential serine and tyrosine phosphorylation sites (48, 80). Phosphorylation of different residues has been shown to regulate differentially the enzymatic and biological activity of the protein (48, 80). It was reported that at least some of the residues on c-Raf-1, which undergo phosphorylation in response to paclitaxel treatment, differ from those phosphorylated in response to growth factor stimulation, suggesting that the kinase(s) that are responsible for c-Raf-1 phosphorylation in response to paclitaxel treatment may be unique to this agent (46). In contrast, recent studies have shown that the Bcl-2 protein localizes Raf-1 at the mitochondrial membrane, where Raf-1 phosphorylates and inactivates the pro-apoptotic BAD protein (81). In addition, it was reported that Raf-1 kinase activity is a major determinant of paclitaxel resistance in human cervical tumor cells (82) and in ovarian cancer cells that harbor a mutated p53 protein (83). Thus, Raf-1 can act as both an agonist and antagonist of paclitaxel-induced apoptosis. It is possible that, depending on the subcellular localization of the phosphorylated Raf-1, the paclitaxel-induced elevation of Raf-1 kinase activity could lead to either high or low levels of apoptosis.

We were not able to clarify completely all of the mechanisms of resistance to paclitaxel in this work. One of these mechanisms might overlap the mechanism of resistance to cisplatin. There might also be specific mechanisms of resistance to paclitaxel. Recently, it was reported that Akt binds to Forkhead (71) and cAMP-response element-binding protein (41), which function in cascades known to be involved in transcription factor-dependent anti-apoptotic mechanisms. Further investigations will reveal whether these molecules are also involved in regulating cell viability following paclitaxel treatment. We are currently investigating these possibilities.

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