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Akt Phosphorylation and Stabilization of X-linked Inhibitor of Apoptosis Protein (XIAP)*

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**Akt negatively regulates apoptotic pathways at a pre-mitochondrial level through phosphorylation and modulation of proteins such as Bad, Forkhead proteins, and GSK-3β. Akt has also been shown to protect cell death at a post-mitochondrial level, although its downstream targets have not been well documented. Here, we demonstrate that Akt, including AKT1 and AKT2, interacts with and phosphorylates X-linked inhibitor of apoptosis protein (XIAP) at residue serine-87 *in vitro* and *in vivo*. Phosphorylation of XIAP by Akt protects XIAP from ubiquitina
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Akt, also named protein kinase B (PKB)[1] or RAC kinase, is a family of phosphatidylinositol 3-OH-kinase-regulated serine/threonine kinase (1–3). Three isoforms of Akt have been identified: Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors in a phosphatidylinositol 3-OH-kinase-dependent manner (4–6). 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 (7).

Accumulated evidence shows that Akt and its downstream targets constitute a major cell survival pathway. Akt promotes cell survival and suppresses apoptotic death in a
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which causes their nuclear exclusion and inactivation (7). Akt also phosphorylates and activates the cyclic AMP-response element-binding protein, which increases the transcription of anti-apoptotic genes, such as Bcl-2 (8, 9). In addition, recent studies have shown that Akt phosphorylates and inactivates apoptosis signal-regulating kinase-1, a mitogen-activated protein kinase kinase kinase that mediates stress- and cytokine-induced cell death (10, 11). Akt also phosphorylates the murine double minute-2 protein promoting its translocation to the nucleus and the destabilization of p53 (12). By promoting the degradation of p53, Akt impairs the cellular stress response, increasing the survival of tumor cells. A role for an anti-apoptotic function of Akt at a post-mitochondrial step has also been proposed (13), and Akt has been reported to directly phosphorylate and inactivate the cell death protease caspase-9 (14). In addition, Akt has also been shown to inhibit the activation of Bax during apoptosis through an uncharacterized mechanism (11, 15).

The inhibitor of apoptosis proteins (IAPs) are a family of intracellular anti-apoptotic proteins, first identified in baculovirus, which play a key role in cell survival by modulating death-signaling pathways at a post-mitochondrial level. They currently include X-linked IAP (XIAP), human IAP-1 (Hiap-1),

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[1] The abbreviations used are: PKB, protein kinase B; XIAP, X-linked inhibitor of apoptosis protein; IAP, inhibitor of apoptosis protein; HA, hemagglutinin; HEK, human embryonic kidney; GST, glutathione *S*-transferase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; siRNA, short interference RNA; E3, ubiquitin protease ligase; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone.

human IAP-2 (Hiap-2), neuronal apoptosis inhibitory protein (Naip), Survivin, and Livin. These proteins are characterized by the presence of a caspase-recruitment domain and an N-terminal baculovirus-inhibitor-of-apoptosis-repeat motif, which is necessary for biological activity. With the exception of Naip and survivin, IAPs also contain a C-terminal RING-zinc finger domain believed to be involved in protein-protein and protein-nucleic acid interactions (16). Recent studies have shown that the RING finger domain has ubiquitin protease ligase (E3) activity and is responsible for the autoubiquitination and degradation of IAPs after an apoptosis stimulus (17). Among human IAPs, XIAP is the most potent inhibitor of caspases and apoptosis. It has been shown that XIAP is a direct inhibitor of caspase-3 and caspase-9 and modulates the Bax/cytochrome *c* pathway by inhibiting caspase-9 (18).

In the present report, we demonstrate that XIAP is a physiological substrate of Akt. Akt interacts with and phosphorylates XIAP at serine 87. Phosphorylation of XIAP by Akt inhibits both its autoubiquitination and cisplatin-induced ubiquitination. These effects reduce XIAP degradation and the increased levels of XIAP are associated with decreased cisplatin-stimulated caspase 3 activity and programmed cell death.

MATERIALS AND METHODS

Cell Lines and Transfection—The human ovarian cancer epithelial cell line A2780S 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 transfected with appropriate DNA indicated in the figure legends, using LipofectAMINE Plus (Invitrogen).

Plasmid Constructs—Akt plasmids have previously been descri
(19, 20). FLAG-tagged XIAP was generated by reverse trans
PCR and subcloned to p3XFLAG-CMV-10 vector (Sigma)
and XIAP-S87D mutant constructs were created usi
site-directed mutagenesis kit (Stratagene). Myc
Flg-Bcl2 constructs was kindly provided by D
Wang. The glutathione *S*-transferase (G
were created by PCR and subclone

*Terminal Deoxynucleotidyltr
beling (TUNEL) and Caspase-*
detected with TUNEL using an
Applied Science). For caspase-3 ac
cells were harvested, lysed, and ass

*GST Fusion Protein, Immunoprecip
Vitro Kinase Assay*—GST-XIAP fusion
and immunoblotting were performed as
For *in vitro* kinase assay, Akt immunopre
a kinase buffer (19). GST-XIAP fusion proteins were used as the exogenous substrate. Each experiment was repeated three times.

In Vivo [^{32}P]Orthophosphate Cell Labeling and Pulse-Chase Experiments—COS7 cells were transfected with FLAG-XIAP together with or without constitutively active Akt and labeled with [^{32}P]orthophosphate (0.5 mCi/ml) in minimum Eagle's medium without phosphate for 2 h. FLAG-XIAP was immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated XIAP was detected by autoradiography. Pulse-chase was performed as described previously (20).

RESULTS

Akt Stabilizes XIAP—XIAP is a mammalian prototype of the IAP family and suppresses the programmed cell death by direct inhibition of caspase-9 and caspase-3 activity (16). Down-regulation of XIAP is an important mechanism for caspase activation in response to different apoptotic stimuli, including chemotherapeutic agents (21). We and others (22, 23) have previously shown that cisplatin-induced programmed cell death is accompanied by a decrease in XIAP protein content. We also found that activation of the Akt pathway induces cisplatin resistance by inhibition of apoptosis (24). Together, these findings prompted us to examine whether Akt inhibits cisplatin-induced XIAP degradation. A2780S ovarian cancer cells, which are sensitive to cisplatin, were stably transfected with constitutively active AKT2 (Myr-AKT2) or pcDNA3 vector alone (Fig. 1*A*). In pcDNA3-transfected cells, cisplatin (10 and 20 μM)) treatment induces a significant decline of XIAP protein expression. In contrast, XIAP levels were stable in Myr-AKT2-transfected A2780S cells (Fig. 1, *B* and *C*). TUNEL assay analysis showed that Myr-AKT2 also protected A2780S cells from cisplatin-induced apoptois (Fig. 1*C*). In addition, cisplatin-induced down-regulation of XIAP is cisplatin-dose-dependent (Fig. 1, *B* and *C*). To determine whether Akt effects XIAP at the transcriptional level, we performed Northern blot analysis using total RNA prepared from pcDNA3-A2780S or Myr-AKT2-transfected A2780S cells following treatment with cisplatin. As shown in Fig. 1*D*, levels of XIAP mRNA were similar in both cell lines and not effected by cisplatin, ruling out transcriptional regulation of XIAP by cisplatin and Akt. A second possibility is that XIAP expression and its down-regulation by cisplatin treatment of cells is predominantly regulated post-translationally via a protein degradation mechanism. To test this hypothesis, we performed a pulse-chase assay. pcDNA3-A2780S and Myr-AKT2-A2780S cells were metabolically labeled with [^{35}S]methionine for 1 h. Following change of medium, immunoprecipitation was carried out with anti-XIAP antibody at 0, 2, and 4 The amount of labeled XIAP was visualized by SDS d autoradiography of the gels. As shown in Fig. most completely degraded within 4 h in pcD ereas the degradation rate was signi pressing constitutively active t promotes increased levels ecting the protein from

AP family are transcriptionally way (16) and ectopic expression of Akt ade (25, 26), we further examined whether AP translation and/or transcription. HEK293 ransfected with increasing amount of constitutively Akt. Western and Northern blot analyses showed that topic expression of constitutively active Akt did not induce XIAP at protein and mRNA levels (Fig. 2, *A* and *B*), suggesting that Akt rescuing cisplatin-down-regulated XIAP is not resulted from up-regulation of XIAP at translational/transcriptional level.

Recent reports have demonstrated that mitochondria released Omi/HtrA2 cleavages IAP family proteins, including c-IAP1 and XIAP, during the apoptosis (27, 28). As cisplatin induces programmed cell death via mitochondrial pathway (11, 22), we next examined the possible involvement of Omi in cisplatin-induced XIAP degradation. A2780S cells were stably transfected with Bcl2, which is known to stabilize mitochondria in response to cisplatin treatment (29). The transfected cells were treated with cisplatin for 12 h. Immunoblotting and TUNEL assay analyses showed that unlike constitutively active Akt, Bcl2 only protected XIAP from cisplatin-induced down-regulation at about 20% even though it significantly rescued cisplatin-caused apoptosis (Fig. 2*C*). A second possibility of cisplatin-induced XIAP degradation is via either caspase or proteasome pathway. To this end, A2780S cells were treated with cisplatin together with or without caspase inhibitor Z-VAD-fmk or proteasome inhibitor MG132 and/or lactacystin. As shown in Fig. 2*D*, the XIAP degradation was significantly inhibited by proteasome inhibitors but not by Z-VAD-fmk. These data indicate that the XIAP degradation induced by cisplatin is primarily through proteasome pathway.

Akt Inhibits Autoubiquitination and Cisplatin-induced Ubiquitination of XIAP—XIAP has been shown to possess E3 ubiquitin ligase activity, which is responsible for its autoubiquitination and ubiquitination in response to apoptotic stimuli (16, 21, 29). To examine whether Akt inhibits autoubiquitination of

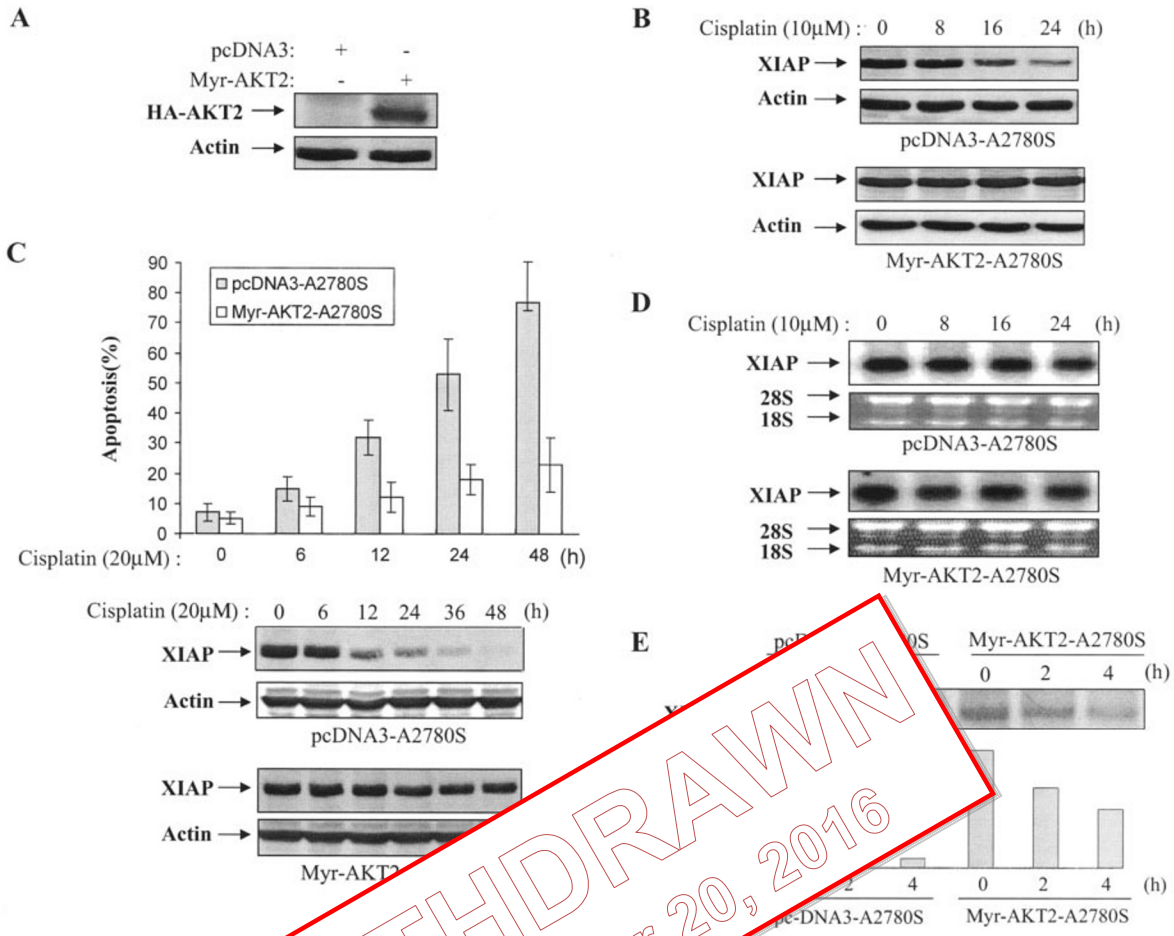

FIG. 1. **Constitutively active Akt ... gulation.** *A*, Western blotting analysis of human ovarian cancer A2780S cells stably tra... AKT2 (Myr-AKT2-A2780S) or pcDNA3 (pcDNA3-A2780S) with anti-HA (*top panel*) and anti-a... ing analysis. pcDNA3-A2780S (*top panels*) and Myr-AKT2-A2780S (*bottom panel*) cells were treated ... times and lysed and then immunoblotted with the indicated antibodies. *C*, pcDNA3-A2780S and Myr-AKT... platin (20 μM) for indicated time. Apoptosis (*top*) was examined with TUNEL assay. Expression of XIAP and eq... ected with the indicated antibodies (*bottom panels*). *D*, Northern blot analysis. pcDNA3-A2780S and Myr-AKT2-A2... ith cisplatin for indicated time. Total RNAs were isolated and subjected to Northern blot analysis with [^{32}P]dCTP-labeled ... *first* and *third panels*). Equal loadings are indicated by 28 S and 18 S ribosomal RNA in the *second* and *fourth panels*. *E*, pulse-c... pcDNA3-A2780S and Myr-AKT2-A2780S cells were labeled with [^{35}S]methionine, chased at the indicated times and immunoprecipit... anti-XIAP antibody. The immunoprecipitates were separated by SDS-PAGE, exposed to x-ray film (*top panel*), and quantified (*bottom panel*). Each experiment was repeated three times.

XIAP, HEK293 cells were transfected with Myc-tagged ubiquitin together with or without constitutively active Akt (Myr-Akt). Consistent with previous reports, ectopic expression of ubiquitin induces autoubiquitination of XIAP and its degradation in a manner sensitive to proteasome inhibitor MG132 (22, 29). Ectopic expression of constitutively active Akt significantly inhibited ubiquitin-induced autoubiquitination of XIAP (Fig. 3*A*) and prevented proteasome-mediated degradation of XIAP (Fig. 3*B*).

We next determined the effects of Akt on cisplatin-induced XIAP ubiquitination. HEK293 and A2780S cells were transfected with FLAG-XIAP and Myc-ubiquitin together with constitutively active or wild type Akt and then treated with cisplatin. Ubiquitination of XIAP was assayed by immunoprecipitation and immunoblotting after 12 h of the treatment. As shown in Fig. 3, *C* and *D*, cisplatin induces ubiquitination of XIAP that was significantly inhibited by expression wild type or/and constitutively active Akt. Taken together, we conclude that Akt stabilizes XIAP by inhibition of its autoubiquitination/ubiquitination.

Akt Phosphorylates and Interacts with XIAP—The fact that Akt regulates XIAP expression at a post-translation level suggests that XIAP may be a substrate of Akt. Indeed, protein sequence analysis revealed a consensus Akt phosphorylation sequence (R*X*R*XX*S/T) at residue serine 87 of XIAP, which is contained within the baculovirus-inhibitor-of-apoptosis-repeat 1 (BIR1) domain (Fig. 4*A*). To examine whether Akt phosphorylates XIAP, COS7 cells were co-transfected with FLAG-XIAP and constitutively active Akt1 and AKT2. After 36 h of transfection, cells were labeled with [^{32}P]orthophosphate for 3 h. Western blot analysis of FLAG-XIAP immunoprecipitates showed that both constitutively active Akt1 and AKT2 increase phosphorylation levels of XIAP as compared with cells transfected with XIAP and pcDNA3 vector (Fig. 4*B*). To examine whether Akt phosphorylates endogenous XIAP, HEK293 cells were either transfected with constitutively active AKT2 or serum-starved and then treated with LY294002 or vehicle (Me_2SO) prior to insulin stimulation. Endogenous XIAP was immunoprecipitated with anti-XIAP antibody, and the immunoprecipitates were subjected to immunoblotting analysis with anti-Akt substrate antibody. As shown in Fig. 4*B*, insulin and Akt2 induced phosphorylation of endogenous of XIAP. The phosphorylation induced by insulin was partially inhibited by phosphatidylinositol 3-OH-kinase inhibitor LY294002, suggesting that insulin also induce other kinase(s), in addition to Akt, to phosphorylate XIAP. To determine whether Akt phosphorylates XIAP at residue of sering-87, we generated GST

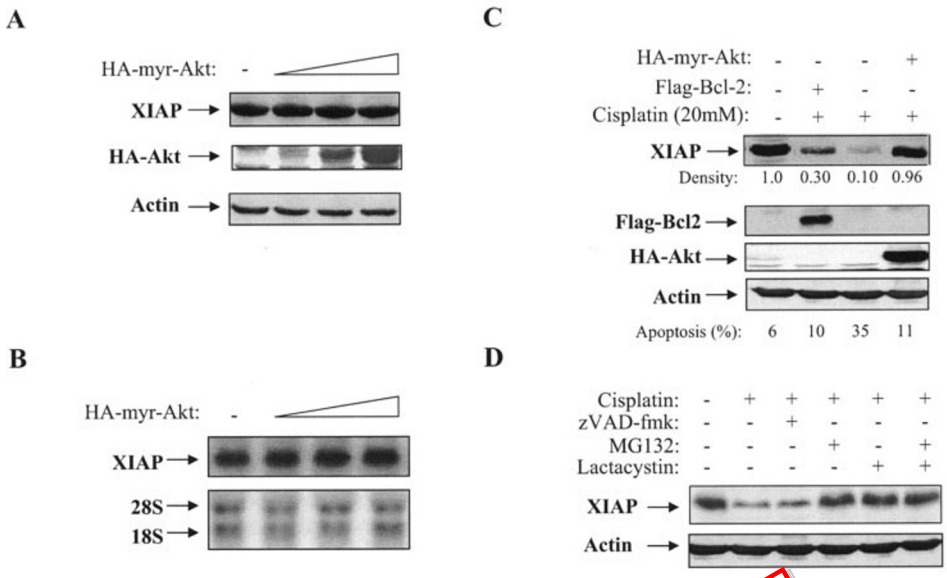

FIG. 2. **Akt does not regulate XIAP at transcriptional and translational levels; cisplati[n] -regulates XIAP primarily through proteasome pathway.** Western (*A*) and Northern (*B*) blot analysis of expression of XIAP in [transfected with increasing amount of constitutively active Akt. The *top panels* show expression of XIAP protein (*A*) and mRNA [transfected HA-Myr-Akt was shown in *middle panel* of *A*. The *bottom panels* indicate equal loadings of protein (*A*) and RNA [TUNEL assays. Stable FLAG-Bcl2- and HA-Myr-Akt-transfected A2780S cells were treated with cisplatin for 12 h a[nd TUNEL analysis. The *top panel* shows expression of XIAP. Quantification of XIAP protein levels is indicate[d] transfected Bcl2 and Akt was detected with anti-FLAG (*second panel*) and -HA (*third panel*) antibodies [*panel*). The *bottom panel* shows the percentage of apoptosis. *D*, Western blot analysis of XIAP expres[latin (20 μM), Z-VAD-fmk (20 μM), MG132 (25 μM), and/or lactacystin (25 μM) for 12 h (*top pan*[antibody. All experiments were repeated three times.

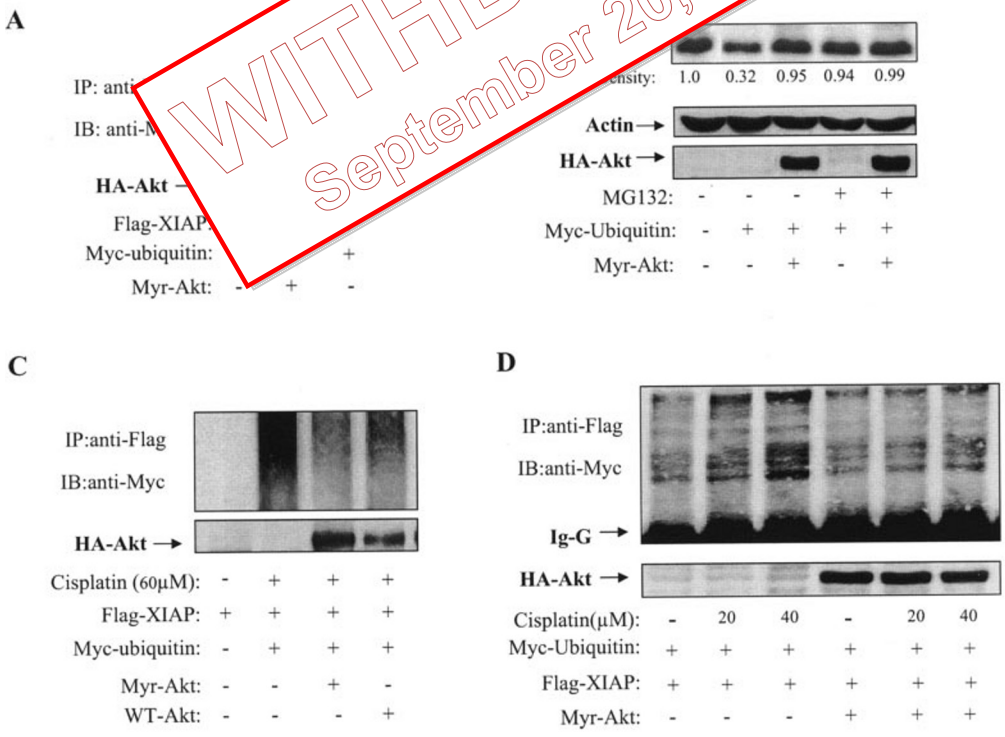

FIG. 3. **Ubiquitination and degradation of XIAP are reduced by expression of constitutively activated Akt.** *A*, Akt inhibits auto-ubiquitination of XIAP. HEK293 cells were transfected with indicated plasmids. After 48 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were immunoblotted with anti-Myc antibody (*top panel*). The *bottom panel* shows expression of transfected constitutively active Akt. *B*, constitutively active Akt and proteasome inhibitor suppress XIAP degradation. HEK293 cells were transfected with Myc-ubiquitin and constitutively active Akt. After 36 h of transfection, cells were treated with or without MG132 (100 μM) proteasome inhibitor for 12 h and subjected to immunoblotting analysis with anti-XIAP antibody (*top panel*). Expression of transfected constitutively active Akt and equal loading are shown in the *second* and *third panels*. *C* and *D*, Akt reduces cisplatin-induced XIAP ubiquitination. HEK293 (*C*) and A2780S (*D*) cells were transfected with indicated plasmids. After 36 h of transfection, cells were treated with the indicated amount of cisplatin for 12 h and lysed. Immunoprecipitation was performed with anti-FLAG antibody and immunoblotted with anti-Myc antibody.

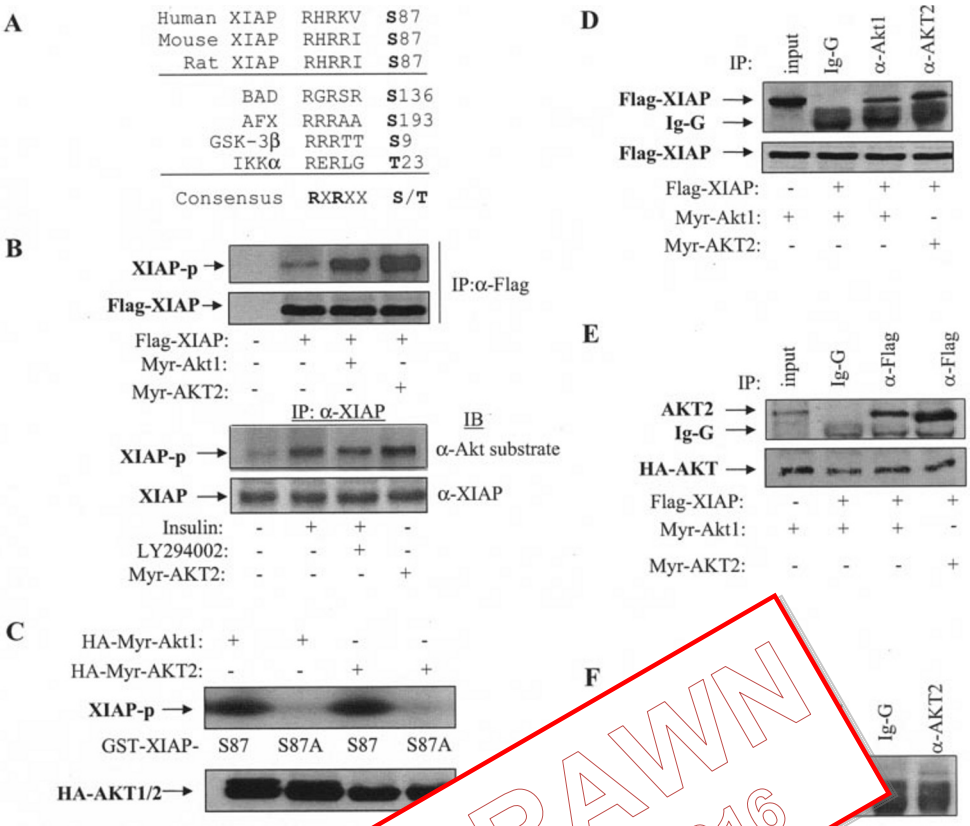

FIG. 4. **Akt phosphorylates and interacts with XI** putative Akt phosphorylation sites in human, mouse, and rat XIAP with the sequences of p ces. The phosphorylated residues are labeled by number, and a consensus sequence is denoted *vivo*. COS7 cells were transfected with indicated expression constructs, labeled with [^{32}Pl nti-FLAG antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to ected with anti-FLAG antibody (*second panel*). The *third* and *fourth panels* are Western blot AP in HEK293 cells. The cells were transfected and treated with the indicated plasmid an P antibody. The immunoprecipitates were subjected to Western blot analysis with anti-Akt sub es (*fourth panel*). *C*, Akt1 and AKT2 phosphorylate XIAP at residue serine 87 *in vitro*. *In vitro* kina tive Akt1 and AKT2 immunoprecipitates prepared from HEK293 cells transfected with HA-Myr-Akt1 a d type XIAP and XIAP-S87A proteins were used as substrates (*top panel*). Expression of transfected Akt1 and m panel. *D* and *E*, Akt interacts with XIAP. HEK293 cells were transfected with indicated plasmids. Following 48 h re lysed, immunoprecipitated with anti-Akt1 or -AKT2 antibody, and detected with anti-FLAG antibody (*D*) or vice versa of transfected plasmids is shown in the *bottom panels*. *F*, endogenous AKT2 and XIAP associates with each other. HEK293 ce ed, immunoprecipitated with anti-AKT2, and detected with anti-XIAP antibody.

fusion proteins of wild type XIAP and XIAP-S87A, which was created by converting serine 87 residue into alanine. *In vitro* Akt kinase assay was carried out using the GST-XIAP and -XIAP-S87A fusion proteins as substrates. Repeated experiments revealed that Akt1 and AKT2 can efficiently phosphorylate wild type XIAP but not XIAP-S87A (Fig. 4*C*). These data indicate that XIAP is a potential physiological substrate of Akt kinase.

A number of Akt substrates have been shown to interact with Akt, which include IKKα, tuberin, and apoptosis signal-regulating kinase-1 (7, 10, 11). We therefore examined whether XIAP physically associates with Akt. HEK293 cells were co-transfected with HA-tagged Akt1, AKT2, and FLAG-XIAP. Immunoprecipitation was carried out with anti-HA and immunoblot of the immunoprecipitates was detected with anti-FLAG antibody or vice versa. As shown in Fig. 4, *D* and *E*, both Akt1 and AKT2 interact with XIAP. The binding affinity between AKT2 and XIAP is higher than that of Akt1 and XIAP. Furthermore, an endogenous protein-protein interaction between XIAP and Akt was also detected in HEK293 cells (Fig. 4*F* and data not shown).

Akt Inhibition of XIAP Ubiquitination/Degradation Depends on Phosphorylation—As demonstrated above, ectopic expression of Akt efficiently phosphorylates serine 87 of XIAP and inhibits XIAP ubiquitination and degradation. We next performed experiments to determine whether phosphorylation of XIAP at serine 87 accounts for the protective effect of Akt on XIAP ubiquitination/degradation. To test this hypothesis, Akt nonphosphorylatable (XIAP-S87A) and phosphomimic (XIAP-S87D) forms of XIAP were created and their capacity of autoubiquitination and cisplatin-induced ubiquitination was examined in HEK293 and A2780S cells, respectively. As shown in Fig. 5*A*, nonphosphorylatable XIAP-S87A and wild type XIAP underwent autoubiquitination when the cells were co-transfected with ubiquitin. Furthermore, autoubiquitination levels of phosphomimic XIAP-S87D decreased as compared with nonphosphorylatable and wild type XIAP. Unlike wild type XIAP (also seen in Fig. 3*B*), however, constitutively active Akt did not exhibit effects on either XIAP-S87A or XIAP-S87D autoubiquitination (Fig. 5*A*). Taken together, these results provide strong evidence that phosphorylation of XIAP at serine 87 occurs in cells, and this modification results in protection of XIAP from autoubiquitination.

To examine the impact of Akt phosphorylation of XIAP on cisplatin-induced ubiquitination and degradation of XIAP, A2780S cells were transfected with XIAP-S87A and XIAP-S87D. Following treatment with cisplatin for 12 h, we found that nonphosphorylatable XIAP-S87A was ubiquitinated in a

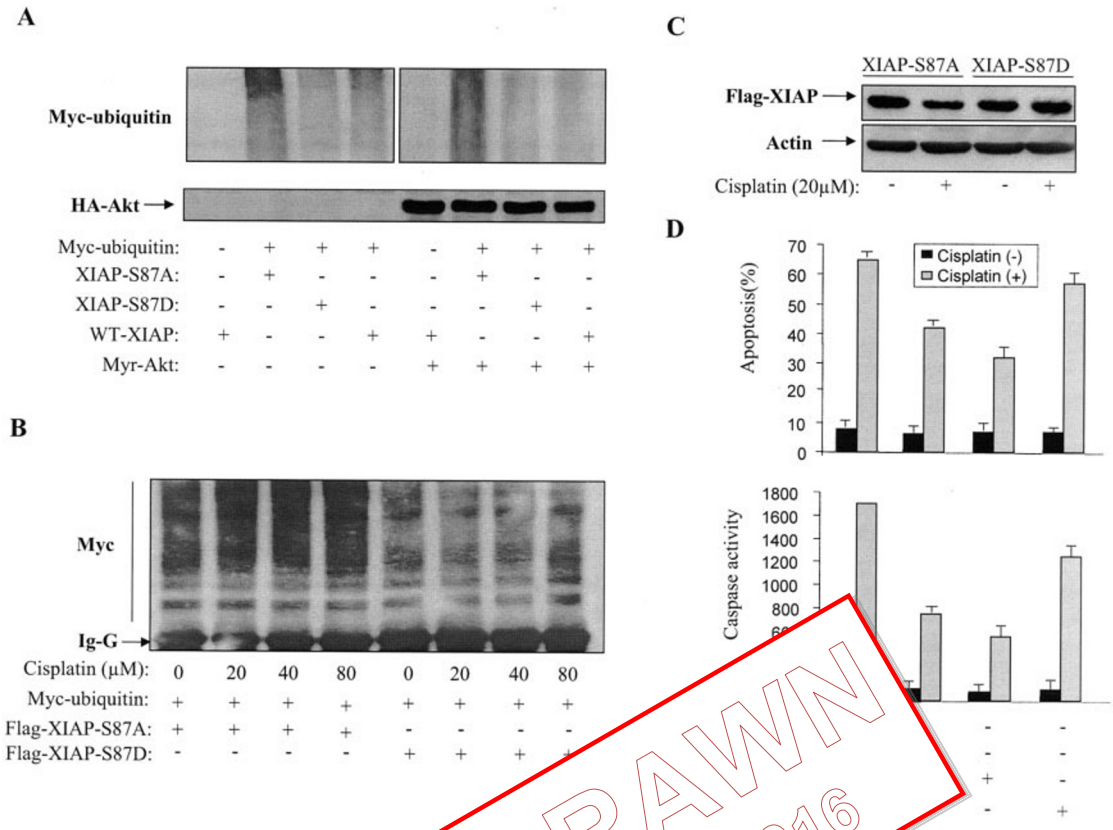

FIG. 5. **Akt phosphorylation of XIAP is required fo degradation.** *A*, the phosphorylation of XIAP inhibits its autoubiquitination. HEK293 cel ter 48 h of transfection, cells were lysed, immunoprecipitated with anti-FLAG antibody, panel). The *bottom panel* shows expression of transfected Myr-Akt. *B*, cisplatin-induced XI , but not nonphosphorylatable, form. A2780S cells were transfected with indicated plasmid 12 h, lysed, and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates v immunoblotting analysis of XIAP expression. A2780S cells were transfected with indicated munoblotted with anti-FLAG antibody (*top panel*). Equal loading is shown in the *second panel*. ctivity analysis (*bottom panel*). A2780S cells were transfected with indicated plasmids. After 36 h o without cisplatin (20 μM) for 24 h. Apoptotic cells and capase-3 activity were examined by TUNEL assay olecular Probes). The values represent three independent experiments.

cisplatin-dependent manner, w mic XIAP-S87D was resistant to cisplatin-i quitination (Fig. 5*B*). Accordingly, we also observed cantly reduced protein levels of nonphosphorylatable XIAP-S87A but not phosphomimic XIAP-S87D in response to cisplatin treatment (Fig. 5*C*). From these studies, we conclude that Akt inhibition of cisplatin-induced ubiquitination and degradation of XIAP are also done in a phosphorylation-dependent manner.

Effect of Akt Phosphorylation of XIAP on Its Antiapoptotic Function—As XIAP exhibits its anti-apoptotic effects by inhibiting caspase-3 and caspase-9, and our data show Akt inhibition of ubiquitination/degradation of XIAP by phosphorylation of XIAP, we next examined whether the phosphorylation of XIAP affects its antiapoptotic function. Wild type XIAP, nonphosphorylatable XIAP-S87A, and phosphomimic XIAP-S87D were introduced into A2780S cells. Following cisplatin treatment for 24 h, caspase-3 activity and apoptosis were analyzed. As shown in Fig. 5*D*, ectopic expression of all three forms of XIAP inhibits cisplatin-induced apoptosis and caspase-3 activity as compared with the cells transfected with pcDNA3 vector alone. However, nonphosphorylatable XIAP-S87A exhibited significantly lower antiapoptotic activity, whereas phosphomimic XIAP-S87D showed relatively higher anti-apoptotic function when compared with wild type XIAP.

To further determine the significance of Akt phosphorylation/stabilization of XIAP in Akt survival signaling, Myr-AKT2- and pcDNA3-A2780S cells were transfected or infected with siRNA or adenovirus of antisense of XIAP and then treated with cisplatin or vehicle (Me_2SO) for 12 h. Immunoblotting analysis showed that expression of XIAP was significantly inhibited by siRNA and antisense of XIAP (Fig. 6, *A* and *C*). The suppression of XIAP expression induces apoptosis in both AKT2- and pcDNA3-A2780S cells as compared with the cells treated with control siRNA or adenovirus of LacZ, which is consistent with recent reports in other cancer cells (30, 31). Furthermore, siRNA and adenovirus of antisense of XIAP largely abrogated constitutively active AKT2 protection of the cells from cisplatin-induced apoptosis (Fig. 6, *B* and *D*). These data indicate that XIAP is an important survival molecule and mediates Akt-induced cell survival and cisplatin resistance in A2780S cells.

DISCUSSION

XIAP is regulated at transcriptional and post-transcriptional levels. Activation of NFκB pathway induces RNA level of XIAP (16, 32). Since it possesses E3 ubiquitin ligase activity, XIAP can be autoubiquitinated and ubiquitinated in response to DNA damage, including treatment with chemotherapeutic agents (16, 21, 29). Recent studies showed that XIAP is cleaved by the mature serine protease Omi/HtrA2, a proapoptotic protein released from the mitochondria into the cytosol during apoptosis (27, 28). In this study, we demonstrate that the phosphorylation of XIAP by Akt regulates its ubiquitination and degradation. Akt, including Akt1 and AKT2, interacts with and phosphorylates XIAP, leading to inhibition of XIAP autoubiquitination and cisplatin-induced ubiquitination. We fur-

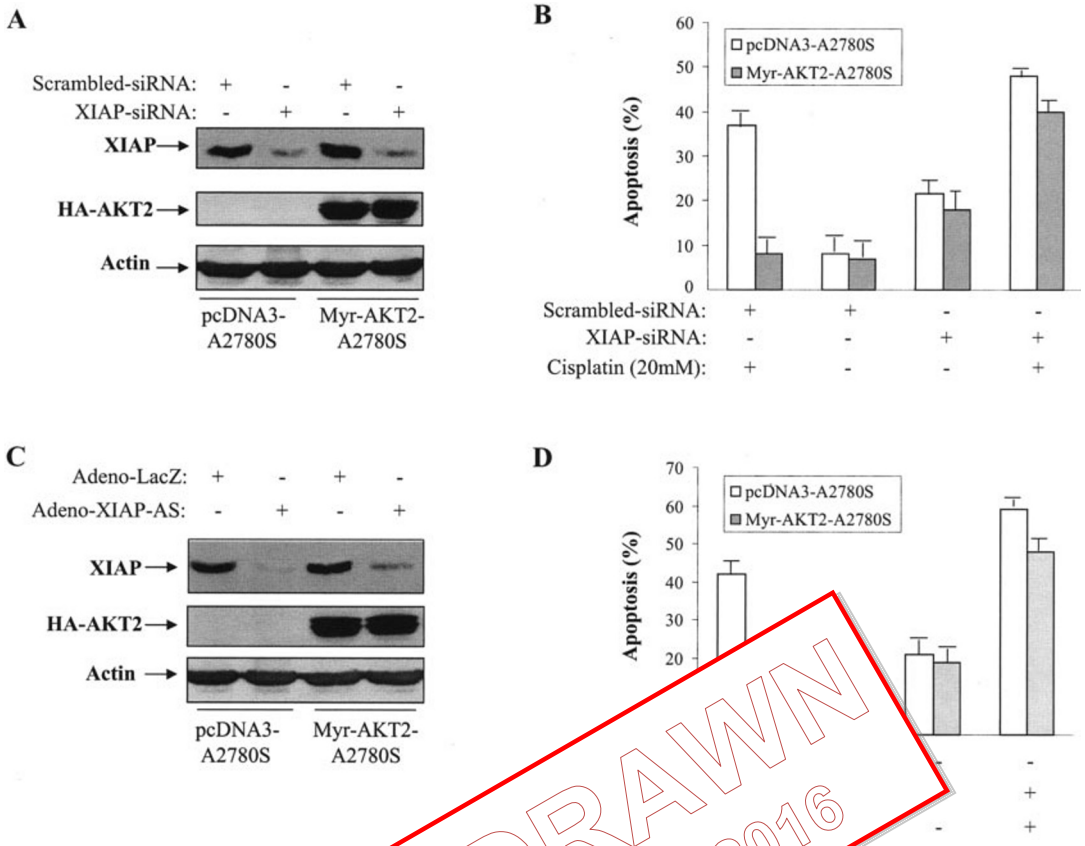

FIG. 6. **Inhibition of XIAP expression triggers … cells from cisplatin-induced apoptosis.** Stable pcDNA3- and HA-Myr-AKT2-transfected … novirus of antisense (*C*) of XIAP and subjected to Western blot analysis with anti-XIAP (*top* … The apoptosis was examined with TUNEL assay (*B* and *D*). Each experiment was repeated …

ther demonstrate that Akt … and that the inhibition of u… depends on phosphorylation … shown that Akt regulation of … ptional level (Figs. 1 and 2). However, … the possibility of Akt regulation of XIAP a… ial level as Akt has been shown to activate mT… ^{S6K} pathway (7). Nevertheless, these data indicate that the phosphorylation by Akt may be one of key mechanisms regulating XIAP levels and function in cells.

Accumulated evidence shows that Akt pathway exerts an anti-apoptotic action by regulation of molecules at both pre- and post-mitochondrial levels. The proapoptotic protein Bad has been shown to be a major target of Akt at the pre-mitochondrial level (13). However, the target(s) of Akt at the post-mitochondrial level have not been well documented, although a study demonstrated Akt phosphorylation and inhibition of caspase-9 (14). We show in this report that Akt interacts with and phosphorylates XIAP resulting in inhibition of caspase-3 activity and apoptosis in response to cisplatin treatment. Unlike caspase-9, in which Akt phosphorylation consensus site only exists in human (14, 33), the phosphorylation site of XIAP (^{82}**R**H**R**K**V**S^{87}) is well conserved among human, mouse, and rat (Fig. 4*A*). Thus, we conclude that XIAP could be a major target of Akt antiapoptotic function at post-mitochondrial level.

We have previously demonstrated that overexpression and/or activation of Akt contributes to cisplatin resistance in human ovarian cancer (24). XIAP content has been shown to be a determinant of cisplatin resistance in human ovarian cancer (21, 22). In this study, we have observed that cisplatin decreases XIAP protein content in A2780S cells without a signif… change in XIAP mRNA level (Figs. 1 and 2) and suggest that *XIAP* gene transcription is not involved in XIAP down-regulation by cisplatin. Furthermore, ectopic expression of zinc RING finger domain deleted mutant XIAP, in which ubiquitin ligase activity was disrupted, rendered cells more resistant to cisplatin as compared with expression of wild type XIAP (34). These findings indicate that XIAP degradation is an important mechanism to regulate the steady-state XIAP and determine the sensitivity to cisplatin in human ovarian cancer cells. We demonstrate in this report that ectopic expression of constitutively active Akt protects XIAP from ubiquitination and degradation induced by cisplatin. Furthermore, phosphomimic XIAP-S87D is more resistant to ubiquitination/degradation induced by cisplatin than wild type XIAP. Conversely, the non-phosphorylatable XIAP-S87A shows increased ubiquitination relative to wild type XIAP (Fig. 5). In addition, suppression of XIAP by siRNA or antisense of XIAP abrogated Akt-induced cisplatin resistance and cell survival (Fig. 6). These data indicate that Akt stabilization of XIAP could be a major mechanism accounting for Akt-induced cisplatin resistance in human ovarian cancer cells.

In summary, the data presented here demonstrate for the first time that XIAP is regulated by phosphorylation. Akt phosphorylates XIAP at residue serine 87 *in vitro* and *in vivo* and interacts with XIAP at physiological protein concentration. The phosphorylation of XIAP at serine 87 by Akt results in inhibition of its autoubiquitination and ubiquitination and thus confers resistance to cisplatin-induced XIAP degradation, caspase-3 activation, and apoptosis. These results suggest that XIAP, in addition to Bad, is a major physiological substrate of Akt in the regulation of apoptosis, especially at the post-mitochondrial level.

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Akt Phosphorylation and Stabilization of X-linked Inhibitor of Apoptosis Protein (XIAP)

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