
Mechanisms of Signal Transduction:

# Evidence for Two Modes of Development of Acquired Tumor Necrosis Factor-related Apoptosis-inducing Ligand Resistance: INVOLVEMENT OF Bcl-xL

Jae J. Song, Jee Young An, Yong Tae Kwon and Yong J. Lee

*J. Biol. Chem. 2007, 282:319-328.*
*doi: 10.1074/jbc.M608065200 originally published online November 15, 2006*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 1, pp. 319–328, January 5, 2007


# Evidence for Two Modes of Development of Acquired Tumor Necrosis Factor-related Apoptosis-inducing Ligand Resistance

## *INVOLVEMENT OF Bcl-xL*[*]

Received for publication, August 22, 2006, and in revised form, November 13, 2006 Published, JBC Papers in Press, November 15, 2006, DOI 10.1074/jbc.M608065200

**Jae J. Song**[‡], **Jee Young An**[§], **Yong Tae Kwon**[§], **and Yong J. Lee**[‡1]

*From the* [‡]*Department of Surgery and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213 and* [§]*Center for Pharmacogenetics and Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261*

**Previous studies have shown that repeated application of TRAIL induces acquired resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Using human prostate adenocarcinoma DU-145 and human pancreatic carcinoma MiaPaCa-2 cells as a model, we now demonstrate for the first time that two states of acquired TRAIL resistance can be developed after TRAIL treatment. Data from survival assay and Western blot analysis show that acquired TRAIL resistance was developed within 1 day and gradually decayed within 6 days after TRAIL treatment in both cell lines. After TRAIL treatment, the level of Bcl-xL increased and reached a maximum within 2 days and gradually decreased in both cell lines. Bcl-xL-mediated development of acquired TRAIL resistance was suppressed by knockdown of Bcl-xL expression. Protein interaction assay revealed that during the development of TRAIL resistance, Bcl-xL dissociated from Bad and then associated with Bax. Overexpression of mutant-type Bad (S136A), which prevents this dissociation, partially suppressed the development of acquired TRAIL resistance. Thus, our results suggest that (a) dissociation of Bad from Bcl-xL and (b) an increase in the intracellular level of Bcl-xL are responsible for development of acquired TRAIL resistance.**

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)[2] is a membrane-bound cytokine molecule that plays a critical role as an inducer of apoptosis in a variety of cancer cells *in vitro* and has been shown to limit tumor growth efficiently *in vivo*, with minimal damage to normal tissues (1–3). It is thought that TRAIL induces apoptosis by binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), members of the tumor necrosis factor receptor superfamily, which results in conformational changes that expose a binding surface for Fas-associated death domain, an adaptor protein (4, 5). The adaptor molecule recruits the initiators caspase-8 and -10 to promote formation of the death-inducing signaling complex (DISC). The activation of caspases has been documented by several observations, providing evidence that caspase-8, an initiator caspase, cleaves Bid and triggers mitochondrial damage and subsequently induces the release of cytochrome *c* from the mitochondria (6). Cytochrome *c* in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9 to form the apoptosome protein complex. The formation of the apoptosome results in the activation of caspase-9. Caspase-9 cleaves and activates procaspase-3 (7). This results in the activation of effector caspases such as caspase-3. Caspase-3 plays an important role in both death receptor- and mitochondria-mediated apoptosis (8). Previous studies have shown that caspase-3 is required for the DNA fragmentation and membrane blebbing associated with apoptosis (9). MCF-7, a breast cancer cell line, is caspase-3-deficient (9) and relatively insensitive to many chemotherapeutic agents (10). Reconstitution of caspase-3 sensitizes MCF-7 cells to TRAIL (11).

Although the unique feature of selectivity for cancer cells has drawn considerable attention to TRAIL as a potential therapeutic agent against human cancers, the physiological role of TRAIL is certainly more complex than merely activating the caspase-dependent apoptosis of cancer cells (12–17). Previous studies have shown that repeated application of TRAIL induces acquired resistance to TRAIL (18). Several possible molecular mechanisms have been suggested for cellular resistance to TRAIL-induced apoptotic death (19). One possibility is dysfunctions of DR4 and DR5. Mutations in these receptors can lead to a loss of their functions and result in suppression of apoptosis (20–25). Another possibility is defects in the DISC (26–28). A third possibility is a defect of effector caspases such as caspase-3. Finally, a fourth possibility is that changes in proteins which affect caspase activation may produce TRAIL resistance. This includes the mutational inactivation of pro-apoptotic molecules (Bax, Bak, Bad, Bim, or Bid) or the overexpression of death inhibitors (FLIP, FAP-1, Bcl-2, Bcl-xL, or inhibitor of apoptosis (IAP)). These death inhibitors operate by

* This work was supported by NCI, National Institutes of Health Grants CA95191, CA96989, and CA121395, Department of Defense Prostate Cancer Research Program Fund PC020530 Grant, and by the Susan G. Komen Breast Cancer Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Surgery, University of Pittsburgh, Hillman Cancer Center, 5117 Centre Ave., Rm. 1.46C, Pittsburgh, PA 15213. Tel.: 412-623-3268; Fax: 412-623-7709; E-mail: leeyj@upmc.edu.

[2] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DISC, death-inducing signaling complex; DR, death receptor; IAP, inhibitor of apoptosis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; BH, Bcl-2 homology.

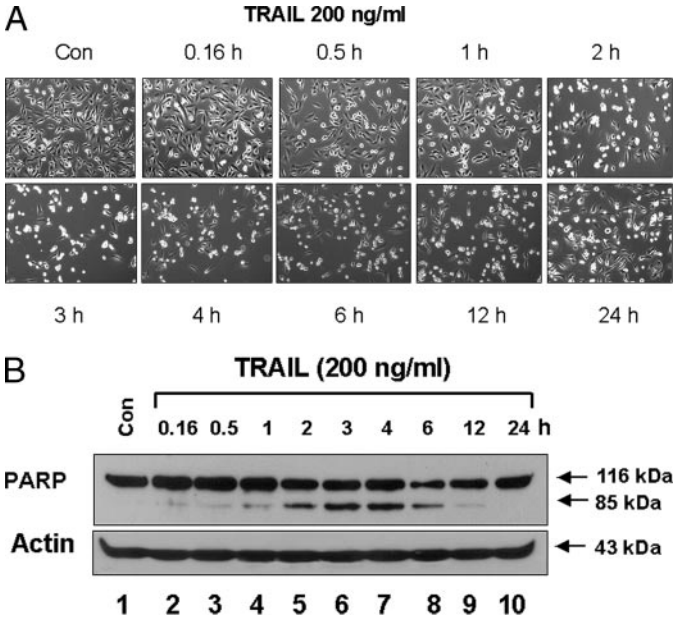


FIGURE 1. **TRAIL-induced cytotoxicity in human prostate adenocarcinoma DU-145 cells.** Cells were treated with TRAIL (200 ng/ml) for various times (0–24 h). *A*, morphological features of each cell were analyzed with a phase-contrast inverted microscope. *Con*, control. *B*, cell lysates were subjected to immunoblotting for PARP. Immunoblots of PARP show the 116-kDa PARP and the 85-kDa apoptosis-related cleavage fragment. Actin was used to confirm the amount of proteins loaded in each lane.

several different mechanisms. The anti-apoptotic molecules of the Bcl-2 family (Bcl-2, Bcl-xL) heterodimerize with pro-apoptotic members of the Bcl-2 family (Bax, Bak) and interfere with release of cytochrome *c* by pore-forming proteins (Bid, Bik) (29). Members of the IAP family (c-IAP1, c-IAP2, XIAP) directly bind and inhibit activation of caspases including caspase-3, -7, and -9 (30). Two endogenous forms of FLIP detected on the protein level, $FLIP_L$ and $FLIP_S$ (31, 32), prevent caspase-8 activation at different stages of procaspase-8 processing at the DISC (33). Thus, the overexpression of these anti-apoptotic Bcl-2 family, IAP family, and FLIP family proteins or loss of pro-apoptotic Bcl-2 family proteins can result in TRAIL resistance.

In this study we investigated possible mechanisms for acquired resistance to TRAIL. We observed that repeated application of TRAIL induced acquired TRAIL resistance that is transient and is developed through two different modes, activation of Bcl-xL and increase in the intracellular level of Bcl-xL.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Survival Assay*—Human prostate adenocarcinoma DU-145 cells and human pancreatic cancer MiaPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT) and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% $CO_2$ and air at 37 °C. For trypan blue exclusion assay (34), trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined

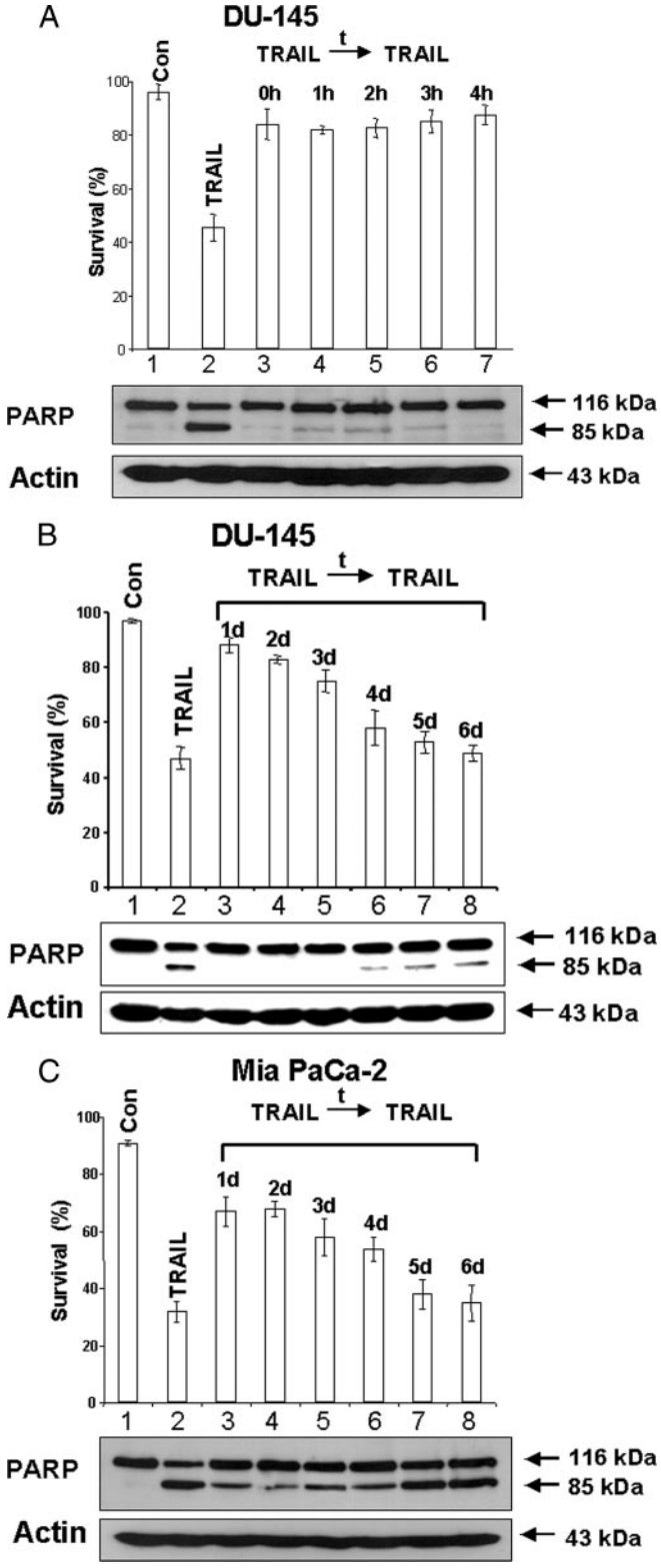


FIGURE 2. **Effect of repeated TRAIL treatment on survival and proteolytic cleavage of PARP in DU-145 cells (*A* and *B*) or human pancreatic carcinoma Mia PaCa-2 cells (*C*).** Cells were first treated with TRAIL (200 ng/ml) for 4 h, and then detached cells were removed by washing with PBS. After removal of the detached cells, fresh media were added onto the remaining attached cells and incubated for the time indicated (0–4 h or 1–6 d) and then treated a second time with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay (*upper panel*), and cell lysates were subjected to immunoblotting for PARP or actin (*lower panels*). *Con*, untreated control cells. *Error bars* represent the S.E. from three separate experiments. *t*, time.

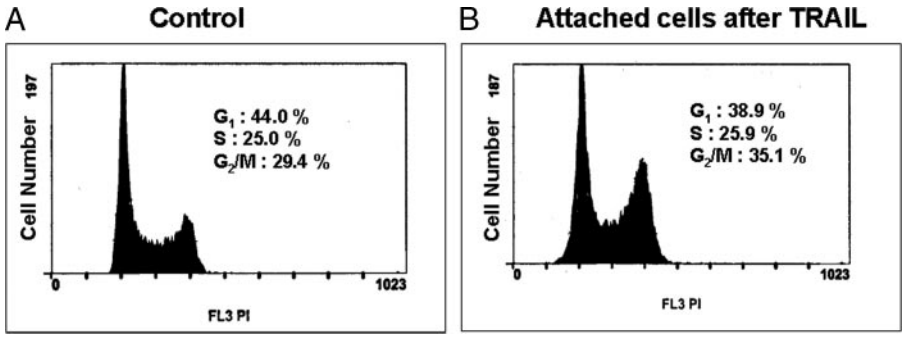


FIGURE 3. **Role of the cell cycle in TRAIL sensitivity.** DU-145 cells were treated with TRAIL (200 ng/ml) for 4 h, and then detached cells were removed by washing with PBS. Untreated control cells (*A*) and attached cells (*B*) were stained with propidium iodide (*PI*) and analyzed by a FACScan flow cytometer.

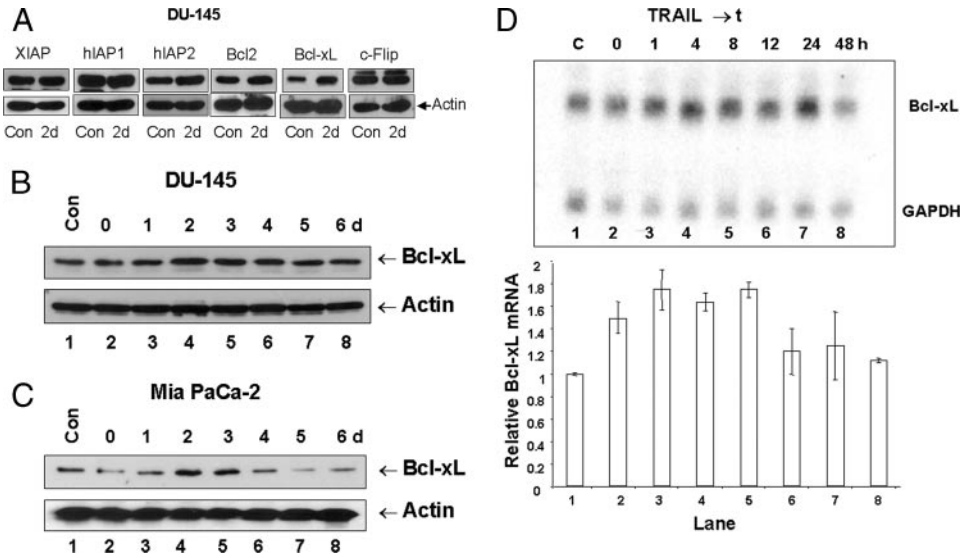


FIGURE 4. **Intracellular levels of various anti-apoptotic molecules at 2 days after TRAIL treatment in DU-145 cells (*A*), changes in Bcl-xL levels for various times after TRAIL treatment in DU-145 (*B*) or Mia PaCa-2 cells (*C*), and up-regulation of Bcl-xL expression in DU-145 cells (*D*).** Cells were treated with TRAIL (200 ng/ml) for 4 h and washed out, and then remaining attached cells were incubated for 2 days (*2d*, *A*) or various times (*B–D*). After incubation, cells were harvested, and Western blot analysis was performed for detecting various anti-apoptotic molecules including Bcl-xL (*A–C*). Actin was used to confirm that similar amounts of proteins were loaded in each lane. Northern blot analysis was performed for detecting Bcl-xL. Total electrolytically fractionated RNA was probed with the *BCL-XL* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA fragment, autoradiographed (*upper panel*) and analyzed with a densitometer (*bottom panel*) (*D*). *Con*, control; *hIAP*, human IAP; *t*, time.

under a light microscope. At least 300 cells were counted for each survival determination.

*Reagents and Antibodies*—Anti-cIAP-1 and anti-cIAP-2 were purchased from R&D Systems (Minneapolis, MN). Anti-Flip antibody was purchased from EMD Biosciences (San Diego, CA). Anti-PARP was purchased from Biomol (Plymouth Meeting, PA). Anti-caspase-8, anti-phospho-Ser-473-Akt, anti-Akt, anti-Bad, and anti-phospho-Ser-136-Bad were purchased from Cell Signaling (Beverly, MA). Anti-Bcl-xL, anti-Bcl2, anti-Bax, anti-caspase-9, and anti-caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin was purchased from ICN (Costa Mesa, CA), and other chemicals were purchased from Sigma-Aldrich.

*Production of Recombinant TRAIL*—A human TRAIL cDNA fragment (amino acids 114–281) obtained by reverse transcription-PCR was cloned into pET-23d (Novagen, Madison, WI) plasmid, and His-tagged TRAIL protein was purified using the Qiaexpress protein purification system (Qiagen, Valencia, CA).

*RNA Interference by siRNA of Bcl-xL*—To stably express siRNA for the long-term knockdown, p*Silencer* 2.1-U6 hygro vector (Ambion, Inc., Austin, TX) was used for clonal cell lines. The inserts for hairpin siRNA into p*Silencer* were prepared by annealing two oligonucleotides. For human Bcl-xL siRNA, the top strand sequence was 5′-GATCCAGGATACAGCTGGAGTCAGTTCAAGAGACTGACTCCAGCTGTATCCTTTTTTTGGAAA-3′, and the bottom strand sequence was 5′-AGCTTTTCCAAAAAAAGGATACAGCTGGAGTCAGTCTCTTGAACTGACTCCAGCTGTATCCT-3′. The annealed insert was cloned into p*Silencer* 2.1-U6 hygro digested with BamHI and HindIII. The correct structure of p*Silencer* 2.1-U6 hygro-Bcl-xL was confirmed by nucleotide sequencing. The resultant plasmid, p*Silencer*-Bcl-xL, was transfected into DU-145 cells, and hygromycin B (250 $\mu$g/ml)-resistant cell clones were isolated. The interference of Bcl-xL protein expression was confirmed by immunoblot using anti-Bcl-xL antibody.

*Transfection*—To generate Bcl-xL overexpressing DU-145 cells, cells were transfected with 2 $\mu$g of pcDNA3-neo or pcDNA3-Flag-Bcl-xL, which was kindly provided by Dr. G. Nunez (University of Michigan, Ann Arbor, MI), using Lipofectamine Plus (Invitrogen). The expression level was determined by immunoblot analysis.

*Protein Extracts and PAGE*—Cells were lysed with 1× Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate, 0.3 mM bromphenol blue) and boiled for 10 min. Protein content was measured with BCA protein assay reagent (Pierce). The samples were diluted with 1× lysis buffer containing 1.28 M $\beta$-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli (35) using a Hoefer gel apparatus.

*Immunoblot Analysis*—Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS-Tween 20 (0.1%, v/v) at 4 °C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein

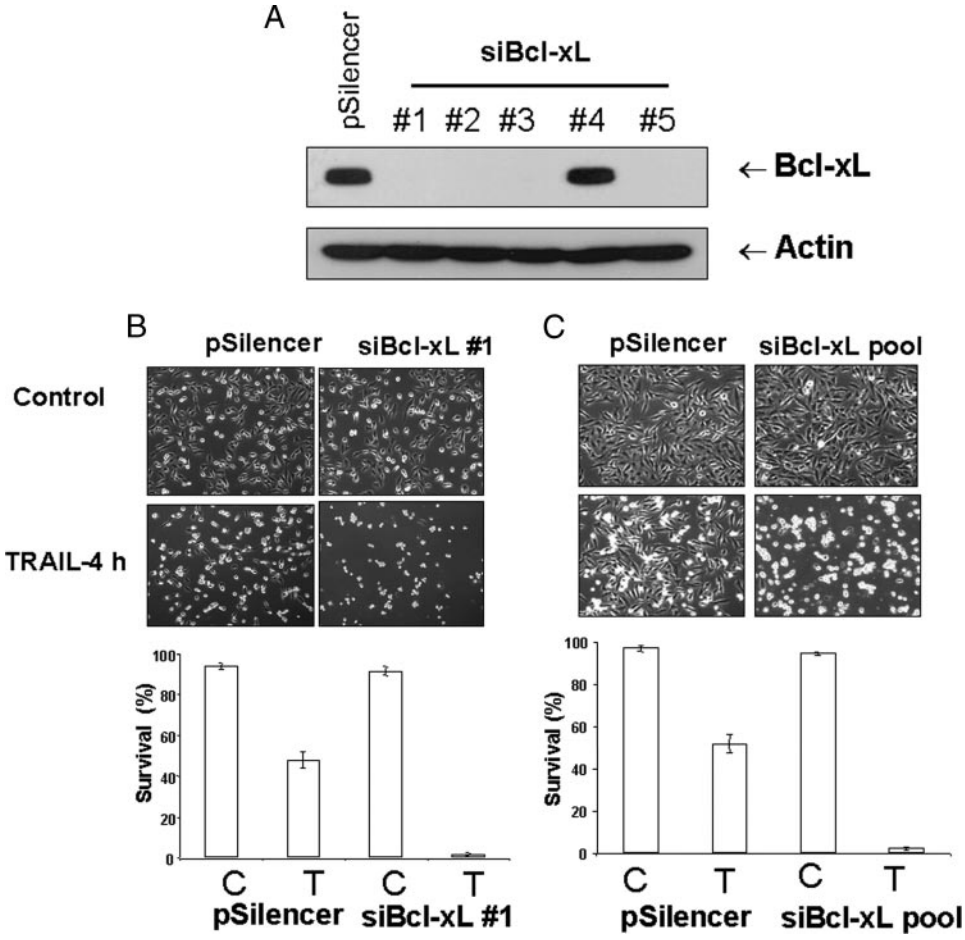


FIGURE 5. **TRAIL-induced cytotoxicity, proteolytic cleavage of PARP, and activation of caspases in control plasmid (*pSilencer*) or pSilencer-siBcl-xL stably transfected DU-145 cells.** *A*, immunoblots were made of Bcl-xL expression in control vector transfected (*pSilencer*) or pSilencer-siBcl-xL stably transfected single cell clones from DU-145 cells. Lysates containing equal amounts of protein (20 $\mu$g) were separated by SDS-PAGE and immunoblotted with anti-Bcl-xL antibody. *B* and *C*, control plasmid (*pSilencer*) or pSilencer-siBcl-xL stably transfected cells (clone #1 or pooled) were treated with TRAIL (200 ng/ml) for 4 h, and morphological features of each cell were analyzed with a phase-contrast inverted microscope (*upper panels*), or cell survival was determined by trypan blue exclusion assay (*lower panels*). *Error bars* represent the S.E. from three separate experiments. *C*, control; *T*, trail.

was visualized by the chemiluminescence protocol (ECL, Amersham Biosciences). Quantitation of x-ray film was carried out by scanning densitometer (Personal Densitometer, GE Healthcare) using area integration.

*In Vivo Binding of Bcl-xL and Bad or Bax*—To examine the interaction between Bcl-xL and Bad/Bax, DU-145 cells in 100-mm culture plates were transfected with 2 $\mu$g of pcDNA3Bad or pcDNA3BadS136A (kindly provided by Dr. Zieg, Harvard Medical School, Boston, MA) by using Lipofectamine Plus (Invitrogen) for 2 days. Then the cells were treated with TRAIL (200 ng/ml) for 4 h, and attached cells were cultured for 1 or 2 days additionally. For immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 80 $\mu$M aprotinin, and 2 mM leupeptin, and the lysates transfected with Bad were incubated with 1 $\mu$g of anti-Bad, or the lysates without transfection were incubated with anti-Bax antibody for 2 h. After the addition of protein G-agarose, the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by SDS-PAGE, and immunoblotted

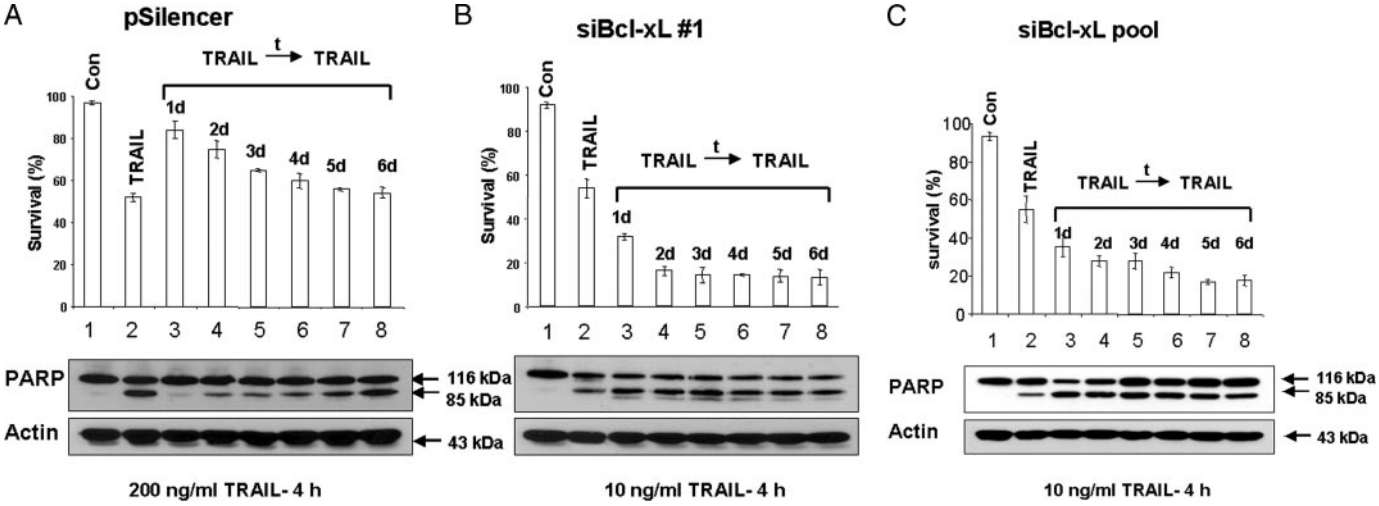


FIGURE 6. **Role of Bcl-xL in the development of acquired TRAIL resistance in DU-145 cells.** *A–C*, control plasmid (*pSilencer*) or pSilencer-siBcl-xL stably transfected (siBcl-xL #1 or pooled) cells were first treated with 200 ng/ml TRAIL or 10 ng/ml TRAIL for 4 h, respectively, and then washed out. The attached cells were incubated for various days (1–6 days (*d*)) and then treated a second time with TRAIL (200 or 10 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay, and cell lysates were subjected to immunoblotting for PARP and actin. Actin was used to confirm that similar amounts of proteins were loaded in each lane. *Error bars* represent the S.E. from three separate experiments. *Con*, control; *t*, time.

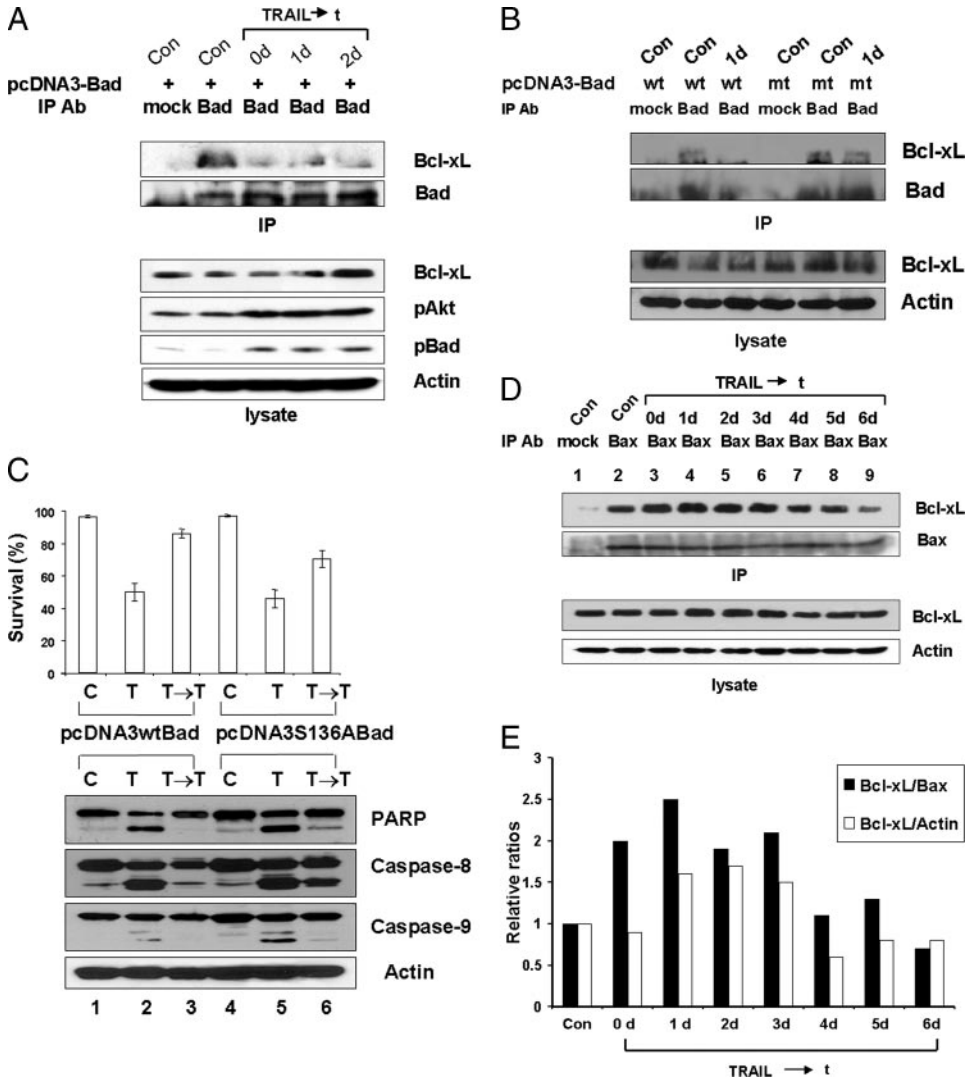


FIGURE 7. **Dissociation of Bcl-xL from Bad and association of Bcl-xL with Bax after TRAIL treatment in DU-145 cells.** *A* and *B*, cells were transfected with pcDNA3 vector containing wild-type (*wt*) Bad or mutant-type (mt;S136A) Bad. After 48 h of incubation, cells were treated with TRAIL (200 ng/ml) for 4 h, and then the remaining attached cells were incubated for various times (0–2 days (*d*)). Cell lysates were immunoprecipitated (*IP*) with anti-Bad antibody (*Ab*) and immunoblotted with anti-Bcl-xL or anti-Bad antibody (*upper panels*). The presence of Bcl-xL, phospho-Akt, phospho-Bad, or actin in the lysates was verified by immunoblotting (*lower panels*). Actin was used to confirm that similar amounts of proteins were loaded in each lane. *C*, cells were transfected with pcDNA3 vector containing wild-type (*wt*) Bad or mutant-type (mt;S136A) Bad. After 48 h incubation cells were treated with TRAIL (200 ng/ml) for 4 h (*T*), and then the remaining attached cells were incubated for 1 day before being challenged to TRAIL (200 ng/ml) for 4 h (*T*→*T*). Cell lysates were subjected to immunoblotting for PARP, caspase-8, and caspase-9. Actin was used to confirm that similar amounts of proteins were loaded in each *lane*. *D* and *E*, cells were treated with or without TRAIL (200 ng/ml) for 4 h, and then the remaining attached cells were incubated for various times (0–6 days) before harvest. Cell lysates were immunoprecipitated with anti-Bax antibody or mock antibody (IgG) and immunoblotted with anti-Bcl-xL or anti-Bax antibody (*upper panels*). The presence of Bcl-xL in the lysates was verified by immunoblotting (*lower panel*). The immunoblot was analyzed with a densitometer. *Con*, control; *C*, control; *T*, trail; *t*, time.

with anti-Bcl-xL antibodies. The proteins were detected with the enhanced chemiluminescence reaction.

*Northern Blot Analysis*—For Northern blot hybridization, total RNAs (10 $\mu$g) isolated from cells were fractionated by electrophoresis in formaldehyde, 1.2% agarose gels, blotted onto Nytran Plus (Schleicher & Schuell), and hybridized with the $^{32}$P-labeled *BCL-XL* or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe.

*Propidium Iodide Staining and Flow Cytometry*—Propidium iodide staining and flow cytometry were used for cell cycle analysis. Untreated control cells or attached cells after TRAIL treatment were washed with PBS, then harvested by trypsinization. Cells were suspended in PBS containing 2% fetal bovine serum. After washing again, each cell was resuspended at $1 \times 10^6$ cells/ml. 3 ml of cold absolute ethanol was added dropwise into 1 ml of cells while gently vortexing, then cells were kept for 1 h at 4 °C. Fixed cells were spun down to remove ethanol and washed twice with PBS, and 1 ml of propidium iodide staining solution (50 $\mu$g/ml) containing 0.5 mg RNase A was added. Then cells were incubated in the dark for 3 h at 4 °C and analyzed by a FACScan flow cytometer (Beckman Coulter, Inc., Hialeah, FL).

## RESULTS

*Development and Decay of Acquired TRAIL Resistance*—We investigated whether TRAIL resistance develops after TRAIL treatment. In the first step in this study we examined the time course of TRAIL-induced cytotoxicity. TRAIL treatment led to apoptosis, as shown by cell surface blebbing and the formation of apoptotic bodies (Fig. 1*A*). These observations were consistent with poly(ADP-ribose) polymerase cleavage (Fig. 1*B*), which is the hallmark feature of apoptosis. Fig. 1*A* also shows that ~50% of the cells died and were lysed within 4 h of treatment with 200 ng/ml TRAIL. Interestingly, repopulation occurred among the remaining cells. We further examined whether these repopulated cells had the same TRAIL sensitivity. DU-145 (Fig. 2, *A* and *B*) or MiaPaCa-2 (Fig. 2*C*) cells were exposed to 200 ng/ml TRAIL for 4 h, detached cells (>90% dead cells) were washed out, and then attached cells were incubated for various times before being challenged to 200 ng/ml TRAIL for 4 h. Data from survival assay and Western blot analysis show that acquired TRAIL resistance developed immediately, was sustained for 3 days, and then gradually decayed within 6 days in both cell lines (Fig. 2). These data suggest that acquired TRAIL resistance is transient rather than intrinsic.

*Role of the Cell Cycle in the Development of Acquired TRAIL Resistance*—Previous studies have shown that cells in the $G_0/G_1$ phase are more susceptible to TRAIL than those in the late $G_1$, S, or $G_2$/M phases (36). It is possible that cell cycle-dependent differential sensitivity to TRAIL may result in selection of a

TRAIL resistant population. This possibility was examined with cell cycle analysis by comparing untreated control cells with attached cells after TRAIL treatment. Fig. 3 shows that TRAIL treatment reduced the $G_1$ population by 5.1% and increased the $G_2$/M population by 5.7%. These results suggest that even though TRAIL treatment increases the TRAIL-resistant population, the amount is not significant enough to be responsible for the development of acquired TRAIL resistance.

*Involvement of Bcl-xL in Acquired TRAIL Resistance*—We hypothesized that expression of anti-apoptotic molecules is responsible for transient TRAIL resistance after TRAIL treatment. To test this hypothesis, the levels of several well known anti-apoptotic molecules were examined 2 days after TRAIL treatment. As shown in Fig. 4*A*, Bcl-xL, which increased by a factor of 3, was distinctly overexpressed compared with the other anti-apoptotic molecules. Data from kinetic studies illustrate that after TRAIL treatment, the level of Bcl-xL increased and reached a maximum within 2 days and gradually decreased in both DU-145 and MiaPaCa-2 cells (Fig. 4, *B* and *C*). To confirm the changes in Bcl-xL expression after TRAIL treatment, Bcl-xL gene expression was examined various times after treatment with 200 ng/ml TRAIL for 4 h. Fig. 4*D* shows an increase in Bcl-xL mRNA immediately after TRAIL treatment of DU-145 cells. We further examined whether Bcl-xL plays an important role in the development of acquired TRAIL resistance by using siRNA of Bcl-xL. DU-145 cells were stably transfected with either pSilencer control plasmid or pSilencer-siBcl-xL vector. We selected several stable transfectants and chose one transfectant for further studies. Fig. 5*A* shows that the expression of Bcl-xL was effectively reduced in the siBcl-xL #1, #2, #3, and #5. Fig. 5, *B* and *C*, show that a single clone (siBcl-xL #1) and a pool of transfected (siBcl-xL #1, #2, #3, and #5) cells were sensitive to TRAIL-induced cytotoxicity. TRAIL-induced proteolytic cleavage of PARP as well as activation of caspases were increased in a single clone (siBcl-xL #1) and a pool of transfected cells (data not shown). To examine the role of Bcl-xL in the development of acquired TRAIL resistance, pSilencer control plasmid-transfected cells, single clone (siBcl-xL #1) cells, or a pool of transfected cells were treated for 4 h with 200 or 10 ng/ml (isosurvival dose) of TRAIL, respectively. After a first TRAIL treatment, detached cells were removed, and attached cells were incubated various times before a second TRAIL treatment. Fig. 6, *A–C*, shows that development and decay of acquired TRAIL resistance was observed in pSilencer control plasmid-transfected cells but not in siBcl-xL #1 and the pool of transfected cells. Moreover, the second TRAIL treatment enhanced cytotoxicity in siBcl-xL #1 cells. Similar results were observed with the pool of transfectants (Fig. 6*C*). In contrast, overexpression of Bcl-xL protected cells from TRAIL-induced cytotoxicity and PARP cleavage as well as activation of caspases (data not shown).

*Evidence for Two Modes of Development of Acquired TRAIL Resistance*—Our results suggest that an increase and decrease in Bcl-xL level after TRAIL treatment is responsible for the development and decay of acquired TRAIL resistance. However, the kinetics of development of acquired TRAIL resistance does not exactly correspond to the kinetics of elevation of intracellular Bcl-xL level (Fig. 2 and 4, *B* and *C*). Immediately after TRAIL treatment, acquired TRAIL resistance can develop without a significant increase in Bcl-xL protein level (Fig. 2*A*). How can we reconcile this discrepancy? We hypothesized that two separate pathways are involved in the development of acquired TRAIL resistance after TRAIL treatment. One is dependent on the elevation of Bcl-xL level, which was described above. The other is activation of Bcl-xL, achieved by phosphorylating Bad so that Bad becomes inactive and can no longer inhibit Bcl-xL. Previous studies have shown that biologically active Bad, a pro-apoptotic molecule, is a dephosphorylated form that interacts with the Bcl-2 or Bcl-xL to inhibit its anti-apoptotic function (37, 38). In contrast, the inactive form of Bad is highly phosphorylated and binds to 14-3-3 scaffold proteins and cannot interact with Bcl-xL (37, 38). We postulated that association of Bad with Bcl-xL inhibits the biological function of Bcl-xL, and conversely, dissociation of Bcl-xL from Bad restores Bcl-xL function. To examine whether interaction between Bcl-xL and Bad is altered by treatment with TRAIL, DU-145 cells were treated with 200 ng/ml TRAIL for 4 h and incubated for 0–2 days. Fig. 7*A* shows that Bad dissociated from Bcl-xL immediately after treatment with TRAIL, and the dissociation was sustained. Fig. 7*A* also shows that Akt and Bad were phosphorylated after treatment with TRAIL. We hypothesized that Bad is phosphorylated by activated (phosphorylated) Akt, and TRAIL-induced phosphorylation of Bad, through Akt, is responsible for dissociation of Bad from Bcl-xL. To test the hypothesis, we employed wild-type Bad against mutant-type Bad, which cannot be phosphorylated by Akt (39, 40). Fig. 7*B* shows that TRAIL treatment dissociated Bcl-xL from wild-type Bad but not mutant-type Bad (S136A). Overexpression of mutant-type Bad (S136A) partially suppressed the develop-

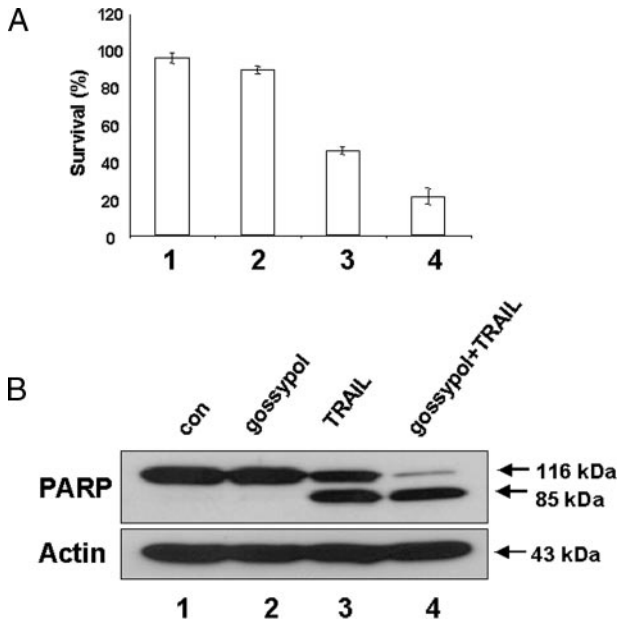


FIGURE 8. **Effect of gossypol on TRAIL-induced cytotoxicity in DU-145 cells.** Cells were pretreated with 10 $\mu$M gossypol for 12 h and treated with 200 ng/ml TRAIL for 4 h. Cell survival was determined by trypan blue exclusion assay (*A*), and cell lysates were subjected to immunoblotting for PARP or actin (*B*). Actin was used to confirm that similar amounts of proteins were loaded in each lane. *Error bars* represent the S.E. from three separate experiments. *con*, control.

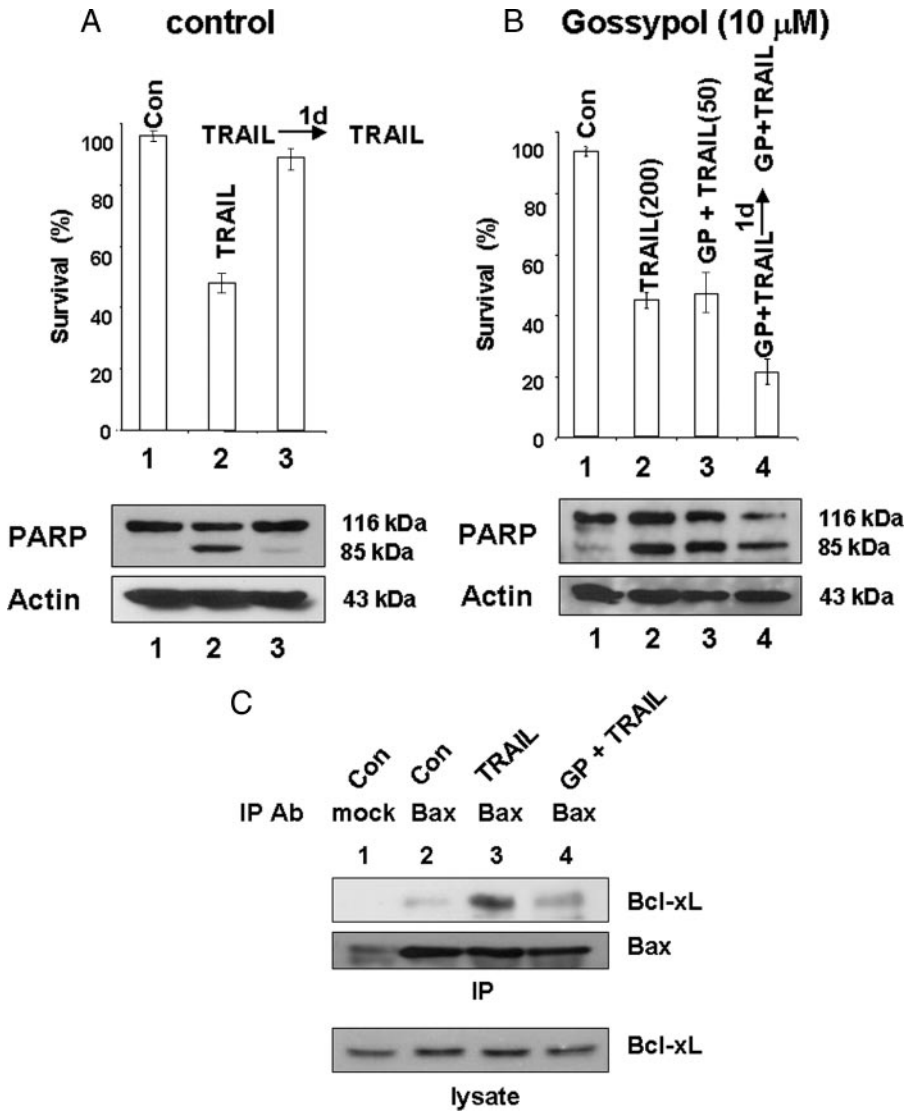


FIGURE 9. **Effect of gossypol on the development of acquired TRAIL resistance in DU-145 cells.** *A*, cells were first treated with TRAIL (200 ng/ml) for 4 h, and then detached cells were removed by washing out with PBS. After removed detached cells, fresh media were added onto the remaining attached cells and incubated for 1 day (*d*) and then treated a second time with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay (*upper panel*), and cell lysates were subjected to immunoblotting for PARP or actin (*lower panels*). *Con*, untreated control cells. *B*, cells were pretreated with 10 μM gossypol for 12 h and treated with 50 ng/ml TRAIL for 4 h (*lane 3*). These cells were washed out and incubated for 1 day in the presence of 10 μM gossypol (*GP*). Cells were then treated with 50 ng/ml TRAIL for 4 h in the presence of 10 μM gossypol (*lane 4*). Cells were treated with only TRAIL (200 ng/ml) for 4 h (*lane 2*). Cell survival and biochemical assay were performed as described in Fig. 8*A*. *C*, cells were pretreated with 10 μM gossypol for 12 h and treated with 50 ng/ml TRAIL for 4 h (*lane 4*). Cells were treated with 200 ng/ml TRAIL for 4 h (*lane 3*). Cell lysates were immunoprecipitated (*IP*) with anti-Bax antibody or mock antibody (IgG) and immunoblotted with anti-Bcl-xL or anti-Bax antibody (*Ab*, *upper panels*). The presence of Bcl-xL in the lysates was verified by immunoblotting (*lower panel*). *Con*, untreated control cells. *Error bars* represent the S.E. from three separate experiments.

*Effect of the Bcl-2 Homology 3 (BH3) Mimetic, Gossypol, on Development of Acquired TRAIL Resistance*—Previous studies have shown that gossypol, the levorotatory isomer of a natural product isolated from cottonseeds and roots, binds to the BH3 binding groove of Bcl-xL and Bcl-2 and subsequently inhibits the heterodimerization of Bcl-xL or Bcl-2 with proapoptotic proteins such as Bax, Bad, and Bcl-xS (43, 44). We hypothesized that gossypol inhibits development of acquired TRAIL resistance by inhibiting interaction between Bcl-xL and Bax. To test this hypothesis, we first examined the effect of gossypol on TRAIL cytotoxicity. Fig. 8 shows that pretreatment with gossypol enhanced TRAIL-induced apoptotic death. To investigate the effect of gossypol in the development of acquired TRAIL resistance, gossypol-pretreated cells were treated with 50 ng/ml TRAIL, which is the isosurvival dose to 200 ng/ml TRAIL alone-treated cells. After a first TRAIL treatment, detached cells were washed out, and attached cells were incubated for 1 day in the presence of gossypol before a second TRAIL treatment. Fig. 9, *A* and *B*, show that development of acquired TRAIL resistance was suppressed in the presence of gossypol. Moreover, TRAIL-induced association of Bax with Bcl-xL was inhibited by treatment with gossypol (Fig. 9*C*).

*Role of Akt in the Development of Acquired TRAIL Resistance*—Our data in Fig. 7 show that activated Akt-mediated Bad phosphorylation is responsible for dissociation of Bad from Bcl-xL. We further examined the role of Akt in the development of acquired TRAIL resistance. Fig. 10*A* shows a distinctive difference between attached cells and detached cells. PARP cleavage and caspase activation were prominent in detached cells. Interestingly, TRAIL treatment decreased the level of phosphorylated Akt in detached cells. In contrast, phosphorylation of Akt was increased in attached cells. We hypothesized that phosphorylation (activation) of Akt plays an important role in the development of acquired TRAIL resistance. To test our hypothesis we examined whether inhibition of Akt inhibits the development of acquired TRAIL resistance. For this study, LY294002 and wortmannin, inhibitors of phosphatidylinositol

ment of acquired TRAIL resistance (Fig. 7*C*). After Bcl-xL has dissociated from Bad, it seems to be associated with Bax (Fig. 7*D*). Data from densitometer analysis clearly show that an increase in association of Bax with Bcl-xL occurred before an increase in the intracellular level of Bcl-xL after TRAIL treatment (Fig. 7*E*). Previous literatures illustrated that Bcl-xL heterodimerizes with Bax, and its heterodimerization abrogates the biological apoptotic function of Bax (41, 42). Thus, these results suggest that the final effect of TRAIL-induced acquired resistance is to mediate the inhibition of the pro-apoptotic molecule, Bax, by association with Bcl-xL.

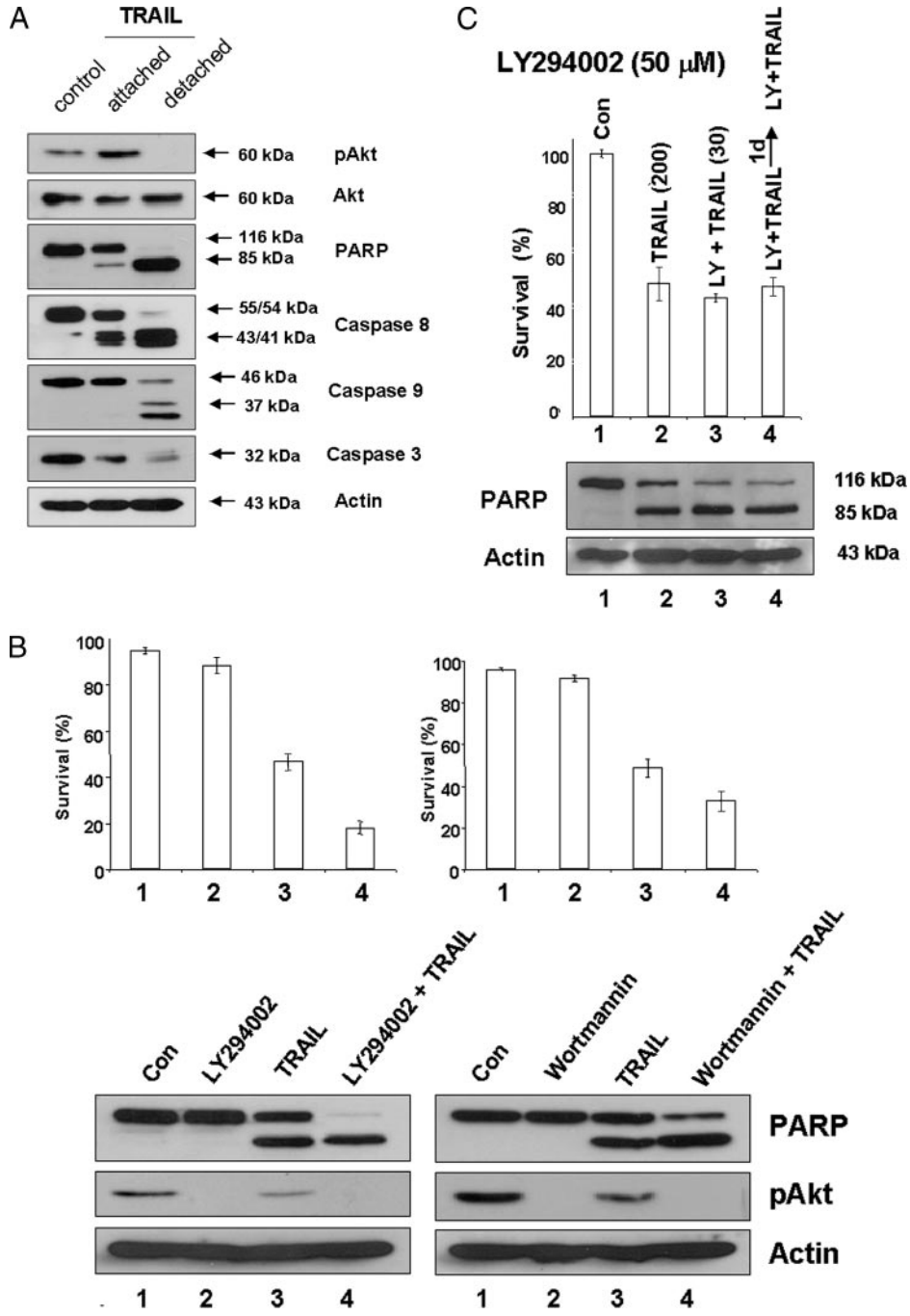


FIGURE 10. **Role of Akt in the development of acquired TRAIL resistance in DU-145 cells.** *A*, cells were treated with TRAIL (200 ng/ml) for 4 h and divided into attached cells and detached cells. Cell lysates were subjected to immunoblotting for phospho-Akt (*pAkt*), Akt, PARP, caspase-8, caspase-9, and caspase 3. Actin was used to confirm that similar amounts of proteins were loaded in each lane. *d*, day. *B*, cells were pretreated with LY294002 (50 μM) or wortmannin (1 μM) for 1 h followed by TRAIL treatment (200 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay (*upper panel*), and cell lysates were subjected to immunoblotting for PARP and pAkt (*lower panels*). Actin was used to confirm that similar amounts of proteins were loaded in each lane. *C*, cells were first treated with TRAIL (200 ng/ml) for 4 h (*lane 2*) or pretreated with LY294002 (50 μM) for 1 h followed by TRAIL treatment (30 ng/ml) for 4 h (*lane 3*). And then detached cell were removed by washing out with PBS. Attached cells were incubated for 1 day in the presence of LY294002 (50 μM). Cells were then treated a second time with TRAIL (30 ng/ml) in the presence of LY294002 (50 μM) for 4 h. Cell survival was determined by trypan blue exclusion assay (*upper panel*), and cell lysates were subjected to immunoblotting for PARP or actin (*lower panels*). *Error bars* represent the S.E. from three separate experiments. *Con*, control.

3-kinase, were employed. Fig. 10*B* shows that the level of phosphorylated Akt (attached + detached cells) was somewhat decreased in TRAIL-treated cells. LY294002 and wortmannin effectively suppressed the phosphorylation of Akt and enhanced TRAIL-induced apoptotic death. To further examine the effect of LY294002 on the development of acquired TRAIL resistance, LY294002-pretreated cells were treated with 30 ng/ml TRAIL, which is the isosurvival dose to 200 ng/ml TRAIL alone-treated cells. After a first TRAIL treatment, detached cells were washed out, and attached cells were incubated for 1 day in the presence of LY294002 before a second TRAIL treatment. Fig. 10*C* shows that development of acquired TRAIL resistance was inhibited by treatment with LY294002.

## DISCUSSION

In this study we demonstrated that TRAIL treatment could induce two states of acquired TRAIL resistance. One is dependent on the elevation of the intracellular level of Bcl-xL. The other is the restoration of biological function of Bcl-xL. Our data show that the latter case plays an important role in the development of acquired TRAIL resistance in the early stage after TRAIL treatment. The former case is probably prominent in the later stage. We also observed that the decay of acquired TRAIL resistance corresponds to the decrease in the level of Bcl-xL. These results suggest that acquired TRAIL resistance is transient, and it is not due to genetic alterations.

Previous studies have shown that Bcl-xL exerts its antiapoptotic function by inhibiting cytochrome *c* release, and overexpression of Bcl-xL can confer resistance to most apoptotic stimuli (45, 46). Bcl-xL contains four conserved domains, called BH domains: BH1, BH2, BH3, and BH4. The three-dimensional structure of Bcl-xL reveals that Bcl-xL consists of seven amphipathic α-helices joined by flexible loops (47). The BH domains coincide with α-helix loops and the domains in combination form the borders of a hydrophobic pocket located on the surface of the Bcl-xL protein. The BH1 domain, which coincides with a loop located upstream of the fifth α-helix in Bcl-xL, plays an important role in dimerization with Bax

and results in abrogation of the Bax pro-apoptotic function (41, 42). Protein-protein interaction between the BH3 domain of Bax and the BH3 binding pocket of Bcl-xL leads to reducing Bax/Bak formation and prevents mitochondrial membrane permeabilization and cytochrome *c* release. Our data (Fig. 9) and the literature have demonstrated that small molecular inhibitors such as gossypol, antimycin A, and BH3I-2′ (3-iodo-5-chloro-*N*-[2-chloro-5-((4-chlorophenyl)sulfonyl)phenyl]-2-hydroxybenzamide), which bind the BH3 binding pocket of Bcl-xL, block protein-protein interaction and inhibit the anti-apoptotic function of Bcl-xL (48–50).

In this study we demonstrated that acquired TRAIL-resistance is probably due to up-regulation of Bcl-xL. Our observations were similar to previous reports (18). Previous studies have shown that the transcription factor nuclear factor-κB (NF-κB) is critical for the expression of Bcl-xL (51, 52). There are two functional NF-κB DNA binding sites in the upstream promoter region of Bcl-xL (53, 54). The activation of NF-κB activity is mediated through promoting the phosphorylation and degradation of the inhibitory subunit IκB (55). Although the expression of Bcl-2 family and IAP family proteins is known to be regulated by NF-κB, our data show that TRAIL treatment preferentially promotes Bcl-xL gene expression (Fig. 4*A*). Thus, a fundamental question that remains unanswered is how TRAIL treatment selectively promotes the expression of the Bcl-xL gene among all the Bcl-2 family and IAP family genes. It is well known that the NF-κB family of proteins, including NF-κB1, NF-κB2, RelA, RelB, and c-Rel, can form homo- and heterodimers *in vitro*, except for RelB. In mammals, the most widely distributed NF-κB is a heterodimer composed of p50 and p65 (also called RelA) subunits (56). NF-κB activity is regulated by the IκB family of proteins that interacts with and sequesters the transcription factor in the cytoplasm. IκB proteins become phosphorylated by the multisubunit IκB kinase complex, which subsequently targets IκB for ubiquitination and degradation by the 26 S proteasome (57). At this time only speculation can be made concerning the role of NF-κB in the up-regulation of Bcl-xL gene expression by treatment with TRAIL. One possibility is that differential activation of NF-κB may be responsible for selective expression of the Bcl-xL gene. Previous studies suggest that Bcl-xL is regulated by RelA/p52 or RelA/p50 rather than p50/p50 or c-Rel/p50 (53, 58, 59). We believe that many critical questions still remain to be answered to understand the mechanisms of the regulation of Bcl-xL gene expression by treatment with TRAIL. However, this model will provide a framework for future studies.

It is well known that NF-κB activity can be regulated through the phosphatidylinositol 3-kinase-Akt-IκB kinase-IκB signal transduction pathway. Phosphatidylinositol 3-kinase consists of a regulatory subunit (P85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, which results in the activation of its catalytic subunit (P110) (60). Phosphatidylinositol 3-kinase phosphorylates phosphoinositides at the 3′-position of the inositol ring, and its major lipid product is phosphatidylinositol 3,4,5-triphosphate (61). Phosphatidylinositol 3,4,5-triphosphate facilitates the recruitment of Akt to the plasma membrane through binding with the pleckstrin homology domain of Akt (61). Akt is activated by phosphoinositide-dependent kinase-1 through phosphorylation at threonine 308 and serine 473 (62). A number of pro-apoptotic proteins have been identified as direct Akt substrates, including Bad, caspase-9, and Forkhead transcription factors (63–68). The pro-apoptotic function of these molecules is suppressed upon phosphorylation by Akt. Recent studies also show that Akt induces the degradation of IκB by promoting IκBα kinase activity and subsequently stimulating the nuclear translocation of NF-κB (69). We observed that TRAIL promotes the Akt signal transduction pathway through activating (phosphorylating) Akt in attached cells but not in detached cells (Figs. 7*A* and 10*A*). Thus, we believe that inactivation of Bad and overexpression of Bcl-xL, which play an important role in acquired TRAIL resistance, occurs by activating Akt and its signal transduction pathway in attached cells. This possibility needs to be further investigated.

Results from our studies strongly suggest that Bcl-xL is involved in the development of acquired transient TRAIL resistance. Nonetheless, we may not rule out the possibility that Bcl-xL is not the only molecule that is responsible for development of acquired TRAIL resistance. One possibility is down-regulation of death receptors, DR4 and DR5, during treatment with TRAIL. Recent studies have revealed that DR5 undergoes internalization by binding with TRAIL (70). Endocytosis of death receptors may lead to reduction of cell surface DR4/DR5 and subsequently results in inhibiting the initiation of the death signal. The other possibility is that cell surface death receptors of attached cells after TRAIL treatment have an altered signaling capacity that could lead to Akt activation and up-regulation of Bcl-xL activity. Obviously, these possibilities need to be examined to understand the mechanism of development of acquired TRAIL resistance.

## REFERENCES


1. Kelley, S. K., Harris, L. A., Xie, D., Deforge, L., Totpal, K., Bussiere, J., and Fox, J. A. (2001) *J. Pharmacol. Exp. Ther.* **299,** 31–38
2. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., Deforge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z., and Schwall, R. H. (1999) *J. Clin. Investig.* **104,** 155–162
3. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H. (1999) *Nat. Med.* **5,** 157–163
4. Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., and Ashkenazi, A. (2000) *Immunity* **12,** 611–620
5. Thomas, L. R., Henson, A., Reed, J. C., Salsbury, F. R., and Thorburn, A. (2004) *J. Biol. Chem.* **279,** 32780–32785
6. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnermri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
7. Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999) *J. Cell Biol.* **144,** 281–292
8. Sun, X. M., MacFarlane, M., Zhuang, J., Wolf, B. B., Green, D. R., and Cohen, G. M. (1999) *J. Biol. Chem.* **274,** 5053–5060
9. Janicke, R. U., Ng, P., Sprengart, M. L., and Porter, A. G. (1998) *J. Biol. Chem.* **273,** 15540–15545
10. Yang, X. H., Sladek, T. L., Liu, X., Butler, B. R., Froelich, C. J., and Thor, A. D. (2001) *Cancer Res.* **61,** 348–354
11. Lee, Y. J., Froelich, C. J., Fujita, N., Tsuruo, T., and Kim, J. H. (2004) *Clin. Cancer Res.* **10,** 1894–1900
12. Morel, J., Audo, R., Hahne, M., and Combe, B. (2005) *J. Biol. Chem.* **280,**

15709–15718
13. Zauli, G., Sancilio, S., Cataldi, A., Sabatini, N., Bosco, D., and Di Pietro, R. (2005) *J. Cell. Physiol.* **202,** 900–911
14. Secchiero, P., Zerbinati, C., Rimondi, E., Corallini, F., Milani, D., Grill, V., Forti, G., Capitani, S., and Zauli, G. (2004) *Cell. Mol. Life Sci.* **61,** 1965–1974
15. Ehrhardt, H., Fulda, S., Schmid, I., Hiscott, J., Debatin, K. M., and Jeremias, I. (2003) *Oncogene* **22,** 3842–3852
16. Secchiero, P., Gonelli, A., Carnevale, E., Milani, D., Pandolfi, A., Zella, D., and Zauli, G. (2003) *Circulation* **107,** 2250–2256
17. Lin, Y., Devin, A., Cook, A., Keane, M. M., Kelliher, M., Lipkowitz, S., and Liu, Z. G. (2000) *Mol. Cell. Biol.* **20,** 6638–6645
18. Zhang, L., Gu, J., Lin, T., Huang, X., Roth, J. A., and Fang, B. (2002) *Gene Ther.* **9,** 1262–1270
19. Zhang, L., and Fang, B. (2005) *Cancer Gene Ther.* **12,** 228–237
20. Jeng, Y. M., and Hsu, H. C. (2002) *Cancer Lett.* **181,** 205–208
21. Shin, M. S., Kim, H. S., Lee, S. H., Park, W. S., Kim, S. Y., Park, J. Y., Lee, J. H., Lee, S. K., Lee, S. N., Jung, S. S., Han, J. Y., and Kim, H. (2001) *Cancer Res.* **61,** 4942–4946
22. Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Jang, J. J., Han, J. Y., Lee, J. Y., and Yoo, N. J. (2001) *Oncogene* **20,** 399–403
23. Park, W. S., Lee, J. H., Shin, M. S., Park, J. Y., Kim, H. S., Kim, Y. S., Park, C. H., Lee, S. K., Lee, S. H., Lee, S. N., Kim, H., Yoo, N. J., and Lee, J. Y. (2001) *Gastroenterology* **121,** 1219–1225
24. Lee, S. H., Shin, M. S., Kim, H. S., Lee, H. K., Park, W. S., Kim, S. Y., Lee, J. H., Han, S. Y., Park, J. Y., Oh, R. R., Jang, J. J., Han, J. Y., Lee, J. Y., and Yoo, N. J. (1999) *Cancer Res.* **59,** 5683–5686
25. Pai, S. I., Wu, G. S., Ozoren, N., Wu, L., Jen, J., Sidransky, D., and El-Deiry, W. S. (1998) *Cancer Res.* **58,** 3513–3518
26. Eggert, A., Grotzer, M. A., Zuzak, T. J., Wiewrodt, B. R., Ho, R., Ikegaki, N., and Brodeur, G. M. (2001) *Cancer Res.* **61,** 1314–1319
27. Daniel, P. T., Wieder, T., Sturm, I., and Schulze-Osthoff, K. (2001) *Leukemia* **15,** 1022–1032
28. Seol, D. W., Li, J., Seol, M. H., Park, S. Y., Talanian, R. V., and Billiar, T. R. (2001) *Cancer Res.* **61,** 1138–1143
29. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) *Genes Dev.* **13,** 1899–1911
30. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) *EMBO J.* **16,** 6914–6925
31. Tschopp, J., Irmler, M., and Thome, M. (1998) *Curr. Opin. Immunol.* **10,** 552–558
32. Shu, H. B., Halpin, D. R., and Goeddel, D. V. (1997) *Immunity* **6,** 751–763
33. Krueger, A., Schmitz, I., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001) *J. Biol. Chem.* **276,** 20633–20640
34. Burow, M. E., Weldon, C. B., Tang, Y., Navar, G. L., Krajewski, S., Reed, J. C., Hammond, T. G., Clejan, S., and Beckman, B. S. (1998) *Cancer Res.* **58,** 4940–4946
35. Laemmli, U. K. (1970) *Nature* **227,** 680–685
36. Jin, Z., Dicker, D.T., and El-Deiry, W.S. (2002) *Cell Cycle* **1,** 82–89
37. Hirai, I., and Wang, H. G. (2001) *Biochem. J.* **359,** 345–352
38. Scheid, M. P., Schubert, K. M., and Duronio, V. (1999) *J. Biol. Chem.* **274,** 31108–31113
39. Hayakawa, J., Ohmichi, M., Kurachi, H., Kanda, Y., Hisamoto, K., Nishio, Y., Adachi, K., Tasaka, K., Kanzaki, T., and Murata, Y. (2000) *Cancer Res.* **60,** 5988–5994
40. Mabuchi, S., Ohmichi, M., Kimura, A., Hisamoto, K., Hayakawa, J., Nishio, Y., Adachi, K., Takahashi, K., Arimoto-Ishida, E., Nakatsuji, Y., Tasaka, K., and Murata, Y. (2002) *J. Biol. Chem.* **277,** 33490–33500
41. Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7834–7838
42. Simonian, P. L., Grillot, D. A., Merino, R., and Nunez, G. (1996) *J. Biol. Chem.* **271,** 22764–22772
43. Zheng, T. S. (2001) *Nat. Cell Biol.* **3,** 43–46
44. Zhang, M., Liu, H., Guo, R., Ling, Y., Wu, X., Li, B., Roller, P. P., Wang, S., and Yang, D. (2003) *Biochem. Pharmacol.* **66,** 93–103
45. Chipuk, J. E., Bhat, M., Hsing, A. Y., Ma, J., and Danielpour, D. (2001) *J. Biol. Chem.* **276,** 26614–26621
46. Kondo, S., Shinomura, Y., Kanayama, S., Higashimoto, Y., Kiyohara, T., Zushi, S., Kitamura, S., Ueyama, H., and Matsuzawa, Y. (1998) *Oncogene* **17,** 2585–2591
47. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L., Ng, S. L., and Fesik, S. W. (1996) *Nature* **381,** 335–341
48. Oliver, C. L., Bauer, J. A., Wolter, K. G., Ubell, M. L., Narayan, A., O'Connell, K. M., Fisher, S. G., Wang, S., Wu, X., Ji, M., Carey, T. E., and Bradford, C. R. (2004) *Clin. Cancer Res.* **10,** 7757–7763
49. Dairaku, N., Kato, K., Honda, K., Koike, T., Iijima, K., Imatani, A., Sekine H., Ohara, S., Matsui, H., and Shimosegawa, T. (2004) *J. Lab. Clin. Med.* **143,** 143–151
50. Ray, S., Bucur, O., and Almasan, A. (2005) *Apoptosis* **10,** 1411–1418
51. Khoshnan, A., Tindell, C., Laux, I., Bae, D., Bennett, B., and Nel, A. E. (2000) *J. Immunol.* **165,** 1743–1754
52. Hettmann, T., DiDonato, J., Karin, M., and Leiden, J. M. (1999) *J. Exp. Med.* **189,** 145–158
53. Dong, Q. G., Sclabas, G. M., Fujioka, S., Schmidt, C., Peng, B., Wu, T., Tsao, M. S., Evans, D. B., Abbruzzese, J. L., McDonnell, T. J., and Chiao, P. J. (2002) *Oncogene* **21,** 6510–6519
54. Glasgow, J. N., Qiu, J., Rassin, D., Grafe, M., Wood, T., and Perez-Pol, J. R. (2001) *Neurochem. Res.* **26,** 647–659
55. Thomson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995) *Cell* **80,** 573–582
56. Baeuerle, P. A., and Baltimore, D. A. (1989) *Gene Dev.* **3,** 1689–1698
57. Zandi, E., and Karin, M. (1999) *Mol. Cell. Biol.* **19,** 4547–4551
58. Marinari, B., Costanzo, A., Marzano, V., Piccolella, E., and Tuosto, L. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6098–6103
59. Sclabas, G. M., Fujioka, S., Schmidt, C., Fan, Z., Evans, D. B., and Chiao, P. J. (2003) *J. Gastrointest. Surg.* **7,** 37–43
60. Rodriguez-Viciana, P., Marte, B., Warne, P., and Downward, J. (1996) *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **351,** 225–231
61. Rameh, L. E., and Cantley, L. C. (1999) *J. Biol. Chem.* **274,** 8347–8350
62. Alessi, D., James, S., Downes, C., Holmes, A., Gaffney, P., Reese, C., and Cohen, P. (1997) *Curr. Biol.* **7,** 261–269
63. Brunet, A., Bonni, A., Zigmond, M., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857–868
64. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282,** 1318–1321
65. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* **378,** 785–789
66. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91,** 231–241
67. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) *Science* **278,** 687–689
68. Hetman, M., Cavanaugh, J. E., Kimeiman, D., and Xia, Z. (2000) *J. Neurosci.* **20,** 2567–2574
69. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* **401,** 82–85
70. Austin, C. D., Lawrence, D. A., Peden, A. A., Varfolomeev, E. E., Totpal, K., De Maziere, A. M., Klumperman, J., Arnott, D., Pham, V., Scheller, R. H., and Ashkenazi, A. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 10283–10288