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Abstract

Silibinin, a flavonoid isolated from *Silybum marianum*, has been reported to have cancer chemopreventive and therapeutic effects. Here, we show that treatment with subtoxic doses of silibinin in combination with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces rapid apoptosis in TRAIL-resistant glioma cells, but not in human astrocytes, suggesting that this combined treatment may offer an attractive strategy for safely treating gliomas. Although the proteolytic processing of procaspase-3 by TRAIL was partially blocked in glioma cells, cotreatment with silibinin efficiently recovered TRAIL-induced caspase activation in these cells. Silibinin treatment up-regulated DR5, a death receptor of TRAIL, in a transcription factor CHOP-dependent manner. Furthermore, treatment with silibinin down-regulated the protein levels of the antiapoptotic proteins $FLIP_L$, $FLIP_S$, and survivin through proteasome-mediated degradation. Taken together, our results show that the activity of silibinin to modulate multiple components in the death receptor–mediated apoptotic pathway is responsible for its ability to recover TRAIL sensitivity in TRAIL-resistant glioma cells. [Cancer Res 2007;67(17):8274–84]

Introduction

Gliomas account for >50% of all brain tumors and are by far the most common primary brain tumors in adults (1). Despite the use of conventional treatments, including surgery, γ-irradiation, and chemotherapy, the average life expectancy of glioma patients after the initial diagnosis is usually <1 year (2). Thus, the researchers are currently attempting to develop novel therapeutic strategies for malignant gliomas.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is considered to be a potential cancer therapeutic agent because it is able to induce apoptosis in various cancer cells without affecting the majority of normal human cells (3, 4). However, recent studies have shown that many types of cancer cells, including malignant gliomas, are resistant to the apoptotic effects of TRAIL (5–7), suggesting that treatment with TRAIL alone may not be sufficient for the treatment of various malignant tumor cells. Identification of sensitizers capable of overcoming TRAIL resistance in cancer cells is needed for the establishment of more effective TRAIL-based cancer therapies.

Silibinin, a flavonoid present in the widely consumed dietary supplement milk thistle extract, has shown promising efficacy in preventing and treating human cancers, including prostate and skin cancer (8, 9). Silymarin, which is composed mainly of silibinin, has been accepted for human clinical use in Europe and Asia for the treatment of liver diseases (10). Silibinin exhibits pleiotropic anticancer effects via induction of cell cycle arrest (11) and apoptosis (12) as well as prevention of angiogenesis (13) and invasion (14), showing that silibinin may prove useful as an anticancer drug either alone or as an adjunct in combination therapy. Multiple targets, including cyclin-dependent kinases (11), p53 (12), vascular endothelial growth factor (13), and mitogen-activated protein kinase (MAPK; ref. 14), have been proposed to explain the anticancer effects of silibinin, but the underlying molecular mechanisms have not yet been fully elucidated.

Here, we show for the first time that subtoxic doses of silibinin effectively sensitize glioma cells but not human astrocytes to TRAIL-induced apoptosis. These results suggest that this combined treatment may provide a safe and effective therapeutic strategy against malignant gliomas that are resistant to various conventional treatments. Furthermore, we provide novel evidence that the prominent sensitizing effect of silibinin on TRAIL-induced apoptosis is due to modulation of multiple components in the death receptor–mediated apoptotic signaling pathway, including DR5 (a TRAIL receptor), FLIP (an inhibitor of caspase-8), and survivin (an inhibitor of effector caspases).

Materials and Methods

Reagents. Recombinant human TRAIL/Apo2 ligand (the nontagged 19-kDa protein, amino acids 114–281) was from KOMA Biotech Inc. and silibinin was from Sigma. Calcein-AM and EthD-1 were from Molecular Probes. The following antibodies were used: anti-caspase-8, caspase-3, survivin, and XIAP (Stressgen); anti-caspase-9, focal adhesion kinase (FAK), DR4, c-IAP2, CHOP/GADD153, Bcl-2, and Bcl-xL (Santa Cruz Biotechnology); anti-Bid, phosphorylated extracellular signal-regulated kinase (ERK)/total ERK, phosphorylated c-Jun NH_2-terminal kinase (JNK)/total JNK, phosphorylated p38/total p38, and phosphorylated Akt/total Akt (Cell Signaling); α-tubulin (Oncogene); anti-Flag M2 and FITC-conjugated anti-goat IgG (Sigma); anti-DR5 antibody for fluorescence-activated cell sorting (FACS) analysis, anti-DcR1, and DcR2 antibody (R&D Systems); anti-DR5 for Western blotting (KOMA Biotech); and anti-rabbit IgG horseradish peroxidase, mouse IgG, and goat IgG (Zymed Laboratories, Inc.).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Cells and culture conditions. The human glioma cells U251MG, U87MG, A172, and U251N were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies). The primary cultures of normal human astrocytes were prepared from 14-week-gestation fetal cerebrum tissues as described previously (15). Human astrocyte cultures were grown in DMEM with high glucose supplemented with 10% FBS and 20 μg/mL gentamicin and subcultured every 2 weeks, and cell culture passage number less than five was used in the present study. Immunofluorescence study indicated that >99% of cells expressed glial fibrillary acidic protein, a cell type–specific marker for astrocytes. Permission to use human brain tissues for research was granted by the clinical screening committee involving human subjects of the University.

Measurement of cellular viability. Cell viability was assessed by double labeling of cells with 2 μmol/L calcein-AM and 4 μmol/L EthD-1. The calcein-positive cells and Etd-1–positive dead cells were visualized using fluorescence microscope (Zeiss).

Construction of the expression vectors for c-FLIP$_L$ and c-FLIP$_S$. The cDNAs encoding human c-FLIP$_L$ and c-FLIP$_S$ were PCR amplified from plasmids (pCA-FLAG-hFLIP$_L$ and pCA-FLAG-hFLIP$_S$; kindly provided by Dr. S.I. Park, Korea Centers for Disease Control and Prevention, Seoul, Korea) to contain these sequences with the specific primers. c-FLIP$_L$ and c-FLIP$_S$ cDNA fragments were digested with *Kpn*I and *Xho*I and subcloned into the pcDNA 3.1(+) vector (Invitrogen), and the resulting constructs were confirmed by nucleotide sequencing.

Establishment of the stable cell lines overexpressing CrmA, Akt, survivin, c-FLIP$_L$, or c-FLIP$_S$. U87MG cells were transfected with the following: a mammalian expression vector containing CrmA cDNA, a vector containing Myc-tagged active Akt (Upstate Biotechnology), a vector containing Flag-tagged survivin, and a vector encoding Flag-tagged c-FLIP$_L$ or c-FLIP$_S$. Stable cell lines overexpressing CrmA, active Akt, survivin, c-FLIP$_L$, or c-FLIP$_S$ were selected with fresh medium containing 500 μg/mL G418 (Calbiochem). Overexpression of CrmA, active Akt, survivin, c-FLIP$_L$, or c-FLIP$_S$ was analyzed by Western blotting using anti-CrmA (BD PharMingen), anti-Myc (Covance Research Products), anti-Flag (Sigma), or anti-c-FLIP (Alexis) antibody, respectively.

Reverse transcription-PCR. Total RNA was extracted from U251MG cells using the Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) was done following the manufacturer's protocol (TaKaRa Shuzo Co. Ltd.). Conditions for final analysis were chosen when amplification of mRNA was in the middle of the exponential amplification phase for 150 μmol/L silibinin. Human DR5 mRNA was amplified using the sense primer 5′-GTCTGCTCTGATCACCCAAC-3′ and the antisense primer 5′-CTGCAAACTGTGACTCCTATG-3′ (corresponding to a 424-bp region of DR5). For CHOP, the sense primer 5′-CAACTGCAGAGATGGCAGCTGA-3′ and the antisense primer 5′-CTGATGCTCCCAATTGTTCAT-3′ (corresponding to a 536-bp region of CHOP) were used. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the sense primer 5′-CGGCCATCACGCCCACAGTTT-3′ and the antisense primer 5′-CGGCCATCACGCCCACAGTTT-3′ were used (corresponding to a 310-bp region of GAPDH). The PCR cycling conditions (30 cycles) were chosen as follows: (*a*) 30 s at 94°C for GAPDH and DR5, 45 s at 94°C for CHOP; (*b*) 30 s at 68°C for DR5, 30 s at 60°C for GAPDH, 45 s at 52.5°C for CHOP; and (*c*) 1 min and 30 s at 72°C for DR5 and GAPDH, 45 s at 72°C for CHOP, with a subsequent 10-min extension at 72°C. Reaction products were analyzed on 1.5% agarose gels. The bands were visualized by ethidium bromide.

Flow cytometry of death receptors. Cells were analyzed for the surface expression of DR5 by indirect staining with primary goat anti-human DR5 antibody (R&D Systems) followed by FITC-conjugated rabbit anti-goat IgG. Briefly, 5×10^5 cells were incubated with 200 μL PBS containing saturating amounts of anti-DR5 antibody on ice for 30 min. As a negative control, cells were incubated with a goat isotype antibody at the same condition. After incubation, cells were washed with PBS twice and reacted with FITC-conjugated rabbit anti-goat IgG on ice for 30 min. After washing with PBS, the expressions of these death receptors were analyzed by FACS sorter (Becton Dickinson and Co.).

Small interfering RNAs. The 25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and have the following sequences: DR5 (F01), UUUAGCCACCUUUAUCUCAUUGUCC; DR5 (E11), UACAAUCACCGACCUUGACCAUCCC; and CHOP, AAGACCCGCGCCGAGGUGAAG. Cells were transfected with siRNA oligonucleotides using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. Cells were fixed with fresh 4% (v/v) paraformaldehyde in PBS. Then, DNA strand breaks were detected using the APO-BrdU terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit (Molecular Probes) following the manufacturer's instructions and pictures were taken on an inverted fluorescence microscope (filter set 10: excitation, 450–490 nm bandpass; emission, 515–565 nm bandpass; filter set 20: excitation, 546/12 nm bandpass; emission, 575–640 nm bandpass; Zeiss).

Plasmids, transfection, and luciferase assay. The DR5-*Sac*I plasmid [containing DR5 promoter sequence (−2,500/+3)] and pDR5-605 [containing DR5 promoter sequence (−605/+3)] were gifts from Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Point mutations of the CHOP-binding sites to the DR5-605 promoter were generated by a two-step PCR method using the following primers: mCHOP (5′-CTTGCGGAGGA*TTGC*GTTGACGA-3′ to 5′-CTTGCGGAGGA*GGTA*GTTGACGA-3′). Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure. For transfection, in brief, cells were plated onto 60-mm dish at a density of 5×10^5 per dish and grown overnight. Cells were cotransfected with 2 μg of various plasmid constructs and 0.2 μg of the pCMV-β-galactosidase plasmid for 3 h using LipofectAMINE Plus reagent (Invitrogen) following the manufacturer's instructions. After incubation for 24 h, transfected cells were further treated with or without silibinin. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysates and expressed as an average of three independent experiments.

Statistical analysis. All data are presented as mean ± SE of at least three independent experiments. The statistical significance of differences was assessed using ANOVA (GraphPad software, GraphPad) followed by Student-Newman-Keuls multiple comparison tests. $P < 0.05$ was considered significant.

Results

Subtoxic doses of silibinin effectively sensitize TRAIL-resistant glioma cells, but not normal astrocytes, to TRAIL-mediated apoptosis. We previously reported that glioma cells are generally resistant to TRAIL-induced apoptosis (6, 7). Here, we examined whether silibinin could sensitize various resistant glioma cells to the apoptotic effects of TRAIL. Treatment of U251MG, U87MG, A172, and U251N cells with 50 to 100 ng/mL TRAIL alone or 50 to 150 μmol/L silibinin alone induced a limited cell death over 24 h (Fig. 1*A*). In contrast, glioma cell viability was significantly reduced by combined treatment under conditions of a fixed TRAIL concentration and varied silibinin concentrations as well as the reverse. However, combined treatment with silibinin and TRAIL of various concentrations had no significant effect on the viability of normal astrocytes (Fig. 1*B*). These results suggest that sensitizing regimens using silibinin and TRAIL together may be preferentially toxic to glioma cells over normal astrocytes.

Critical role of caspases in apoptosis induced by combined treatment with silibinin and TRAIL. In U251MG glioma cells cotreated with silibinin and TRAIL, typical apoptotic morphologies were observed, including cellular shrinkage, blebbing, and nuclear fragmentation (Fig. 2*A*). To examine whether silibinin enhances TRAIL-mediated cell death via caspase-dependent apoptosis, we analyzed caspase cleavage in U251MG and U87MG cells treated

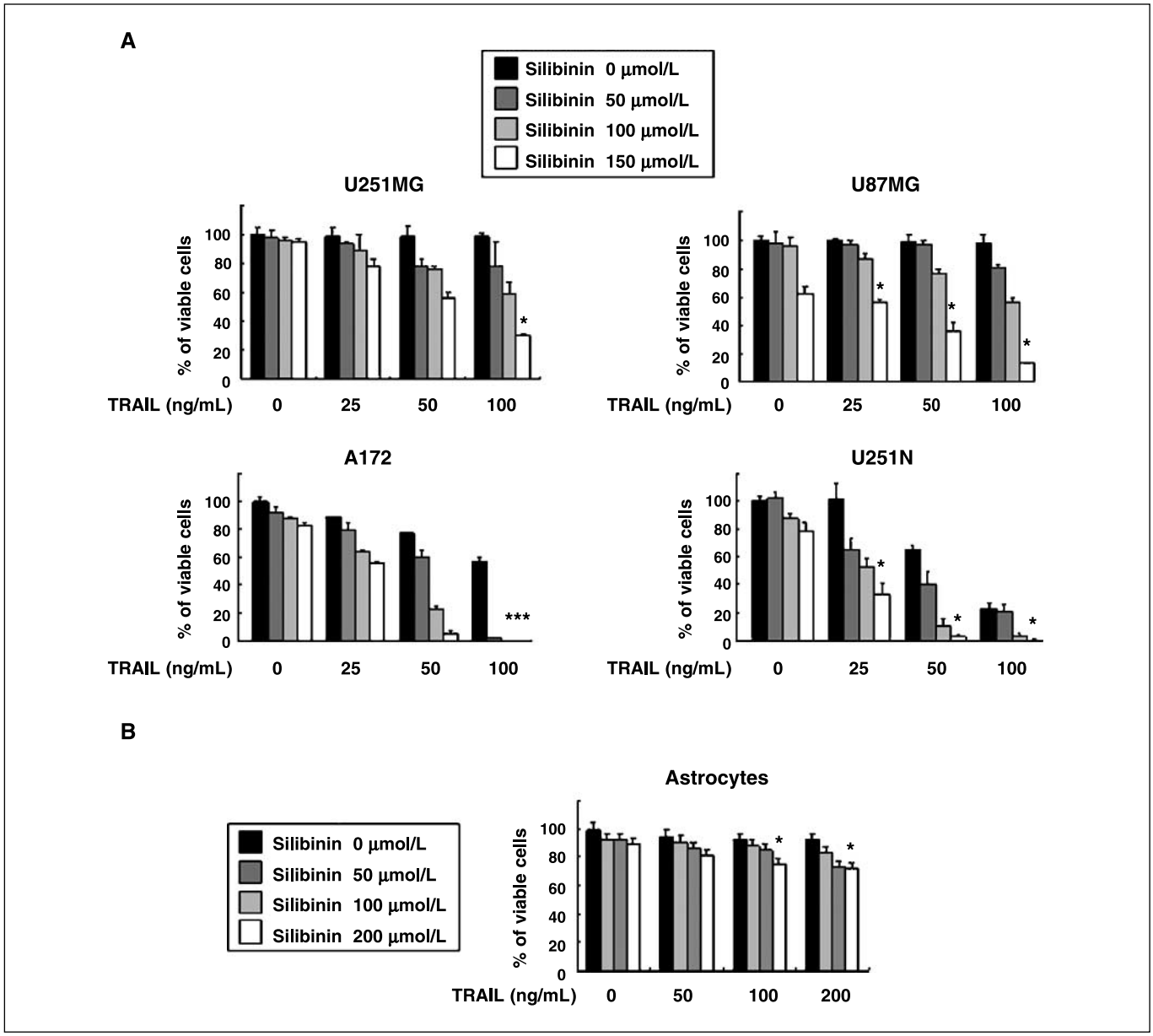

Figure 1. Subtoxic doses of silibinin significantly sensitize TRAIL-resistant glioma cells, but not normal astrocytes, to TRAIL-mediated apoptosis. Four glioma cell lines (*A*) and human astrocytes (*B*) were treated with silibinin for 30 min and further treated with or without TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and EthD-1 to detect live and dead cells, respectively. *Columns,* average of three independent experiments; *bars,* SE. *, $P < 0.05$, compared with untreated cells; ***, $P < 0.001$, compared with untreated cells.

with 150 μmol/L silibinin alone, 100 ng/mL TRAIL alone, or 150 μmol/L silibinin plus 100 ng/mL TRAIL. Our results revealed that treatment with 150 μmol/L silibinin alone did not induce the proteolytic processing of any caspase in the tested cell lines (Fig. 2*B*). In glioma cells treated with TRAIL alone for 24 h, the 32-kDa procaspase-3 was partially cleaved to a 20-kDa intermediate form, but further cleavage into the active p17 and p12 subunit was not detected. However, combined treatment with silibinin and TRAIL induced the complete processing of caspase-3 into p17/p12 (Fig. 2*B*). The precursor protein levels of caspase-8 and caspase-9 were also significantly decreased in cells exposed to combined treatment with silibinin and TRAIL but not in those treated with either agent alone. Activation of these caspases by cotreatment with silibinin and TRAIL was further confirmed by Western blotting of their substrate proteins. Both FAK, the substrate protein of caspase-3 (16), and Bid, a substrate protein of caspase-8 (17), were proteolytically processed in response to the combined treatment but not in response to either agent alone. We then examined the importance of caspases in silibinin-sensitized TRAIL-induced apoptosis. Pretreatment with the pan-caspase inhibitor z-VAD-fmk significantly blocked the cell death induced by the combined treatment (Fig. 2*C*), as did overexpression of CrmA, a viral inhibitor of caspase-8 (Fig. 2*D*; ref. 18). These results suggest that caspases play a critical role in the apoptosis induced by combined treatment with silibinin and TRAIL.

CHOP-mediated DR5 up-regulation is important for silibinin-stimulated TRAIL-induced apoptosis. To examine the underlying mechanisms by which silibinin sensitizes glioma cells

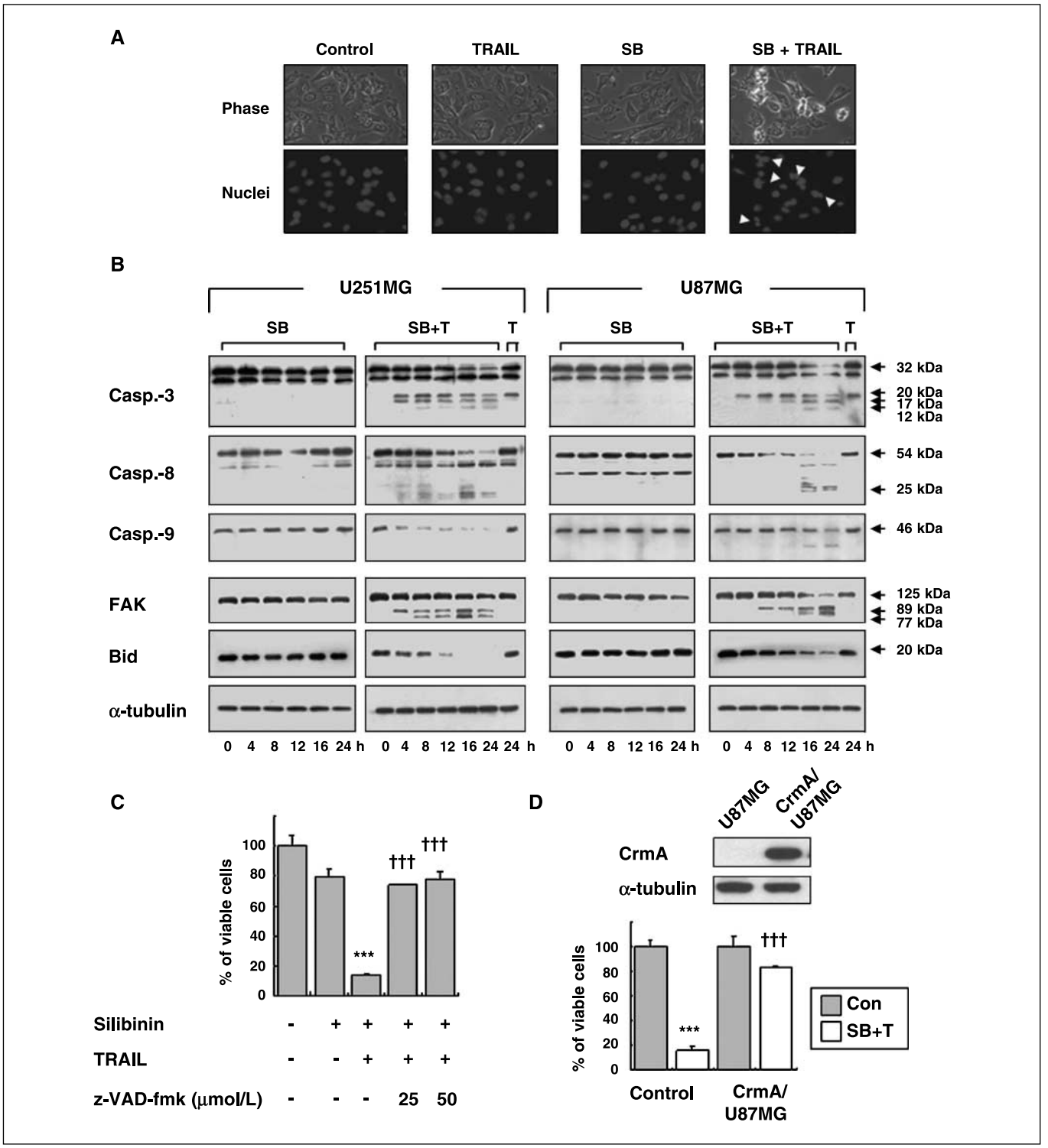

Figure 2. Silibinin-stimulated TRAIL-mediated apoptosis requires activation of caspases. *A,* DNA fragmentation of U251MG cells treated with silibinin (*SB*) plus TRAIL. U251MG cells were stained with 4′,6-diamidino-2-phenylindole after treatment with 150 μmol/L silibinin and/or 100 ng/mL TRAIL for 16 h and observed under a phase-contrast microscope and fluorescent microscope. *B,* activation of caspases during apoptosis induced by combined treatment with silibinin and TRAIL (*T*). U251MG or U87MG cells were treated with 150 μmol/L silibinin alone, 100 ng/mL TRAIL alone, or a combination of both for the indicated time points. Cell extracts were prepared for Western blotting to detect the changes in the expression of caspases. Activation of caspases was confirmed by Western blotting of the substrate proteins. α-Tubulin was used to show equal loading of protein samples. *C,* effect of the inhibition of the caspases in the silibinin-enhanced TRAIL-mediated apoptosis. U251MG cells were treated with 25 or 50 μmol/L z-VAD-fmk for 30 min and then further treated with 150 μmol/L silibinin and 100 ng/mL TRAIL. Cellular viability was measured using calcein-AM and EthD-1. *Columns,* average of three independent experiments; *bars,* SE. ***, $P < 0.001$, compared with untreated cells; †††, $P < 0.001$, compared with cells treated with silibinin plus TRAIL. *D,* overexpression of CrmA blocks silibinin-facilitated TRAIL-induced apoptosis. Control U87MG cells (transfected with vector) and the sublines overexpressing CrmA were treated with 150 μmol/L silibinin plus 100 ng/mL TRAIL for 24 h, and the cellular viability was measured using calcein-AM and EthD-1. *Columns,* average of three independent experiments; *bars,* SE. ***, $P < 0.001$, compared with untreated cells; †††, $P < 0.001$, compared with cells treated with silibinin plus TRAIL.

to TRAIL-induced apoptosis, we first examined whether silibinin affects the expression levels of the death receptors of TRAIL, DR4 and DR5. A time course experiment to determine the changes in DR5 protein levels following treatment with 150 μmol/L silibinin showed that DR5 levels progressively increased up to 16 h and declined at 24 h in both U251MG and U87MG cells (Fig. 3*A*). In contrast, DR4 protein levels were not altered by silibinin treatment. To confirm whether silibinin-induced DR5 up-regulation is restricted to particular glioma cell lines, U251N and A172 cells were treated with 100 or 150 μmol/L silibinin for 16 h and subjected to Western blot analysis, together with U251MG and U87MG cells. Dose-dependent elevation of DR5 protein levels was observed in all the glioma cells tested (Fig. 3*B*). These results show that DR5 up-regulation is a common response of various glioma cells to silibinin. When we examined the changes in DcR1 and DcR2 protein levels, neither DcR1 nor DcR2 protein levels were altered by silibinin in U87MG and U251N cells. In contrast, both DcR1 and DcR2 were too low in U251MG and A172 cells for detection by Western blotting. These results show that silibinin selectively up-regulates DR5 among the receptors of TRAIL. Flow cytometric analysis showed that silibinin treatment slightly affected the background signal of the goat isotype control antibody. However, the surface expression of DR5 was further enhanced in the presence of silibinin. In response to cotreatment with silibinin plus TRAIL, the surface expression of DR5 was also increased (Fig. 3*C*). To clarify the functional role of DR5 up-regulation in apoptosis induced by combined treatment with silibinin and TRAIL, we used siRNAs against DR5. Suppression of DR5 expression by transfection of U251MG cells with two kinds of siRNAs directed against DR5 also significantly blocked the apoptosis induced by the combined treatment when we did TUNEL assay (Fig. 3*D*). These results collectively indicate that DR5 up-regulation is important for silibinin-facilitated TRAIL-mediated apoptosis. Next, we further investigated the possible upstream signals responsible for silibinin-induced DR5 up-regulation. Recently, CHOP/GADD153 was shown to be involved in MG132-mediated (19) and tunicamycin-mediated (20) DR5 up-regulation, contributing to the sensitization of TRAIL-mediated apoptosis. We found that the protein levels of CHOP were significantly increased by silibinin treatment, preceding silibinin-induced DR5 up-regulation (Fig. 4*A*). RT-PCR analysis showed that the mRNA levels of both CHOP and DR5 were dose dependently increased by silibinin treatment (Fig. 4*B*). To investigate whether CHOP is associated with silibinin-mediated transcriptional activation of DR5, we mutated the potential CHOP site (−281 to −261) of the *DR5* gene (20). U87MG cells were transfected with pDR-*Sac*I, pDR-605, and pDR-605-mCHOP plasmids and treated with or without silibinin. The DR5-*Sac*I and pDR5-605 plasmid contain DR5 promoter sequence −2,500/+3 and sequence −605/+3, respectively. pDR5-605-mCHOP plasmid contains the mutation at CHOP-binding sites of the DR5 promoter. Whereas the promoter activities of pDR5-*Sac*I and pDR5-605 were increased by silibinin in a dose-dependent manner, the promoter activity of pDR5-605-mCHOP was not enhanced by silibinin treatment. Because mutation at the CHOP-binding site significantly abrogated the activation of the DR5 promoter by silibinin, these results suggest that CHOP is involved in the silibinin-mediated up-regulation of DR5 (Fig. 4*C*). Furthermore, suppression of CHOP by siRNA knockdown significantly inhibited silibinin-induced DR5 up-regulation, showing again that CHOP induction is required for silibinin-induced DR5 up-regulation (Fig. 4*D*). Moreover, we found that siRNA knockdown of CHOP significantly blocked the apoptosis induced by combined treatment with silibinin and TRAIL. Taken together, these results suggest that CHOP induction plays a critical role in both silibinin-induced DR5 up-regulation and silibinin-facilitated TRAIL-induced apoptosis.

Down-regulation of c-FLIP and survivin is also involved in silibinin-facilitated TRAIL-induced apoptosis. We further investigated the possible involvement of various regulators of apoptotic signaling in silibinin-stimulated TRAIL-induced apoptosis. We first examined whether silibinin affects the protein levels of caspase inhibitors, such as FLIP, a homologue of caspase-8 that is recruited to the death-inducing signaling complex, thereby inhibiting the activation and cleavage of caspase-8 (21), and the inhibitor of apoptosis proteins (IAP; e.g., XIAP, survivin, and c-IAP), which suppress apoptosis by inhibiting caspase-3, caspase-7, and/or caspase-9 (22–24). We found that the protein levels of both $FLIP_L$ and $FLIP_S$, the major splice forms of FLIP (21), were down-regulated in response to treatment with silibinin alone or silibinin plus TRAIL (Fig. 5*A*). Among the tested IAPs, we found that the protein levels of survivin significantly decreased in response to treatment with silibinin alone or silibinin plus TRAIL. In addition, the activity of Akt, an antiapoptotic protein, was decreased by silibinin alone or silibinin plus TRAIL, suggesting that the silibinin-mediated down-regulation of these antiapoptotic proteins may play a role in the enhancement of TRAIL-mediated cell death. In contrast, the protein levels of antiapoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL, were not affected by treatment with silibinin alone or silibinin plus TRAIL (Supplementary Fig. S1). When we tested for changes in members of the MAPK signaling pathways following treatment with silibinin or silibinin plus TRAIL, the activities of JNK and p38 were rather slightly reduced and ERK activities were not significantly altered (Supplementary Fig. S1). These results suggest that these antiapoptotic Bcl-2 family proteins and MAPKs are not involved in silibinin-enhanced TRAIL-induced apoptosis.

Based on the above results, we investigated whether down-regulation of c-FLIP, survivin, or Akt actually mediates silibinin-facilitated TRAIL-induced apoptosis using cell lines overexpressing these antiapoptotic proteins. In U87MG cell lines established to stably overexpress $c\text{-}FLIP_S$ or $c\text{-}FLIP_L$, we observed significant inhibition of cell death induced by silibinin plus TRAIL (Fig. 5*B*). Similarly, survivin overexpression in U87MG cells also significantly attenuated silibinin-enhanced TRAIL-induced apoptosis (Fig. 5*C*). In contrast, the cell death induced by the combined treatment with silibinin and TRAIL was not affected in the stable cell lines overexpressing active Akt (Supplementary Fig. S2*A*) or in U87MG cells transiently transfected with plasmids encoding active Akt (Supplementary Fig. S2*B*), indicating that silibinin-mediated Akt down-regulation is not responsible for the accelerated cell death in glioma cells cotreated with TRAIL. Taken together, these results indicate that silibinin treatment contributes to the recovery of TRAIL sensitivity in glioma cells by inducing down-regulation of the antiapoptotic proteins c-FLIP and survivin.

Finally, we investigated the molecular basis of silibinin-mediated down-regulation of survivin and c-FLIP. RT-PCR analysis showed that the mRNA levels of $FLIP_S$, $FLIP_L$, and survivin were not affected by silibinin treatment (Fig. 6*A*). In contrast, pretreatment with the proteasome inhibitor MG132 attenuated the silibinin-induced down-regulation of these proteins (Fig. 6*B*), suggesting that silibinin may reduce the protein levels of $FLIP_S$, $FLIP_L$, and

survivin via proteasome-mediated degradation rather than through transcriptional control. Next, we examined whether silibinin-induced up-regulation of DR5 and down-regulation of $FLIP_S$, $FLIP_L$, and survivin were dependent on one another. Suppression of DR5 or CHOP by siRNA-mediated knockdown did not affect silibinin-mediated down-regulation of c-$FLIP_S$, c-$FLIP_L$, or survivin (Fig. 6*C*). Western blotting analysis of the cell lines stably overexpressing c-$FLIP_S$, c-$FLIP_L$, and survivin also showed that silibinin-induced modulation of the other nonoverexpressed apoptotic regulators (including DR5) was similar between overexpressing and control

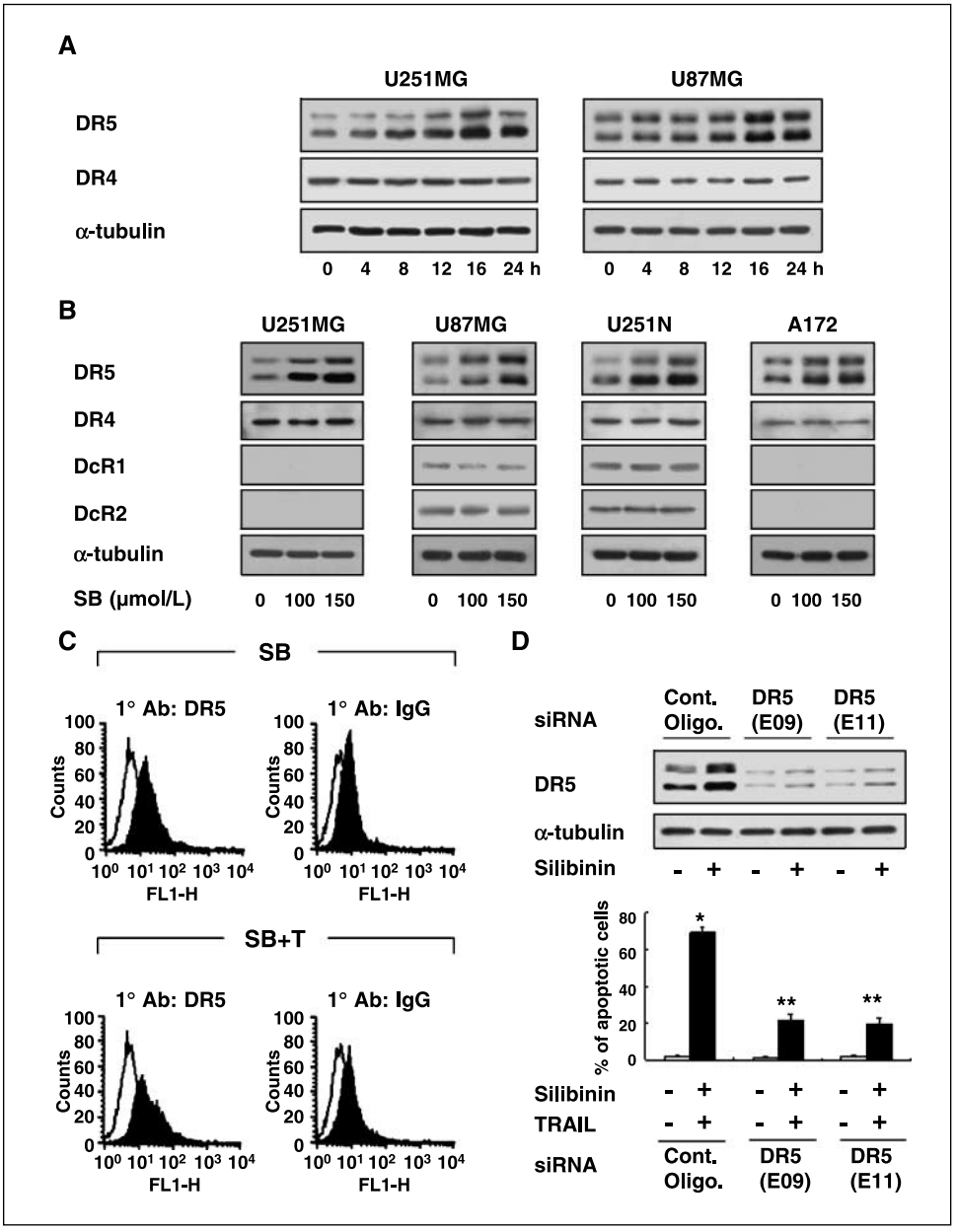

Figure 3. Silibinin-induced DR5 up-regulation is critical for sensitization of TRAIL-mediated apoptosis. *A,* silibinin up-regulates DR5 but not DR4 in U251MG and U87MG cells. U251MG and U87MG cells were treated with 150 μmol/L silibinin for the indicated time points. Cell extracts were prepared for Western blotting of DR5 and DR4. Western blotting of α-tubulin was used as a loading control. *B,* silibinin dose dependently up-regulates DR5 protein in various glioma cells. Four different glioma cells were treated with silibinin at the indicated concentrations for 16 h. Cell extracts were prepared for Western blotting of DR5, DR4, DcR1, DcR2, and α-tubulin. *C,* effect of silibinin alone or silibinin plus TRAIL on the surface expression of DR5. After treatment with 150 μmol/L silibinin alone or 150 μmol/L silibinin plus 100 ng/mL TRAIL for 16 h, the surface protein levels of DR5 were analyzed by flow cytometry as described in Materials and Methods. To confirm whether the increase in the staining patterns is specific for DR5, cells were incubated with goat isotype control antibody (*Ab*), instead of goat anti-DR5 antibody, and further incubated with FITC-conjugated rabbit antigoat IgG. *X axis,* fluorescence intensity; *Y axis,* relative number of cells. *Black histograms,* treated cells with silibinin alone or silibinin plus TRAIL; *white histograms,* untreated cells. *D,* suppression of DR5 expression by siRNAs reduces silibinin-stimulated TRAIL-induced apoptosis in U251MG cells. U251MG cells were transfected with scrambled negative control RNA or two kinds of siRNA duplexes against DR5 mRNA. Twenty-four hours after transfection, cells were treated with or without 150 μmol/L silibinin for 16 h. *Top*, down-regulation of DR5 by its siRNA transfection was analyzed by Western blotting of DR5. α-Tubulin levels were assessed to show equal gel loading. Twenty-four hours after transfection, cells were treated with or without 150 μmol/L silibinin plus 100 ng/mL TRAIL for 16 h. After fixation, DNA fragmentation was detected using APO-BrdU TUNEL assay kit following the manufacturer's instruction. Images of the cells were taken on a fluorescence microscope and the number of TUNEL-positive cells was counted; a minimum 100 cells was counted per sample. *Bottom, columns,* average of three independent experiments; *bars,* SE. *, $P < 0.005$, compared with untreated cells; **, $P < 0.05$, compared with cells treated with silibinin plus TRAIL.

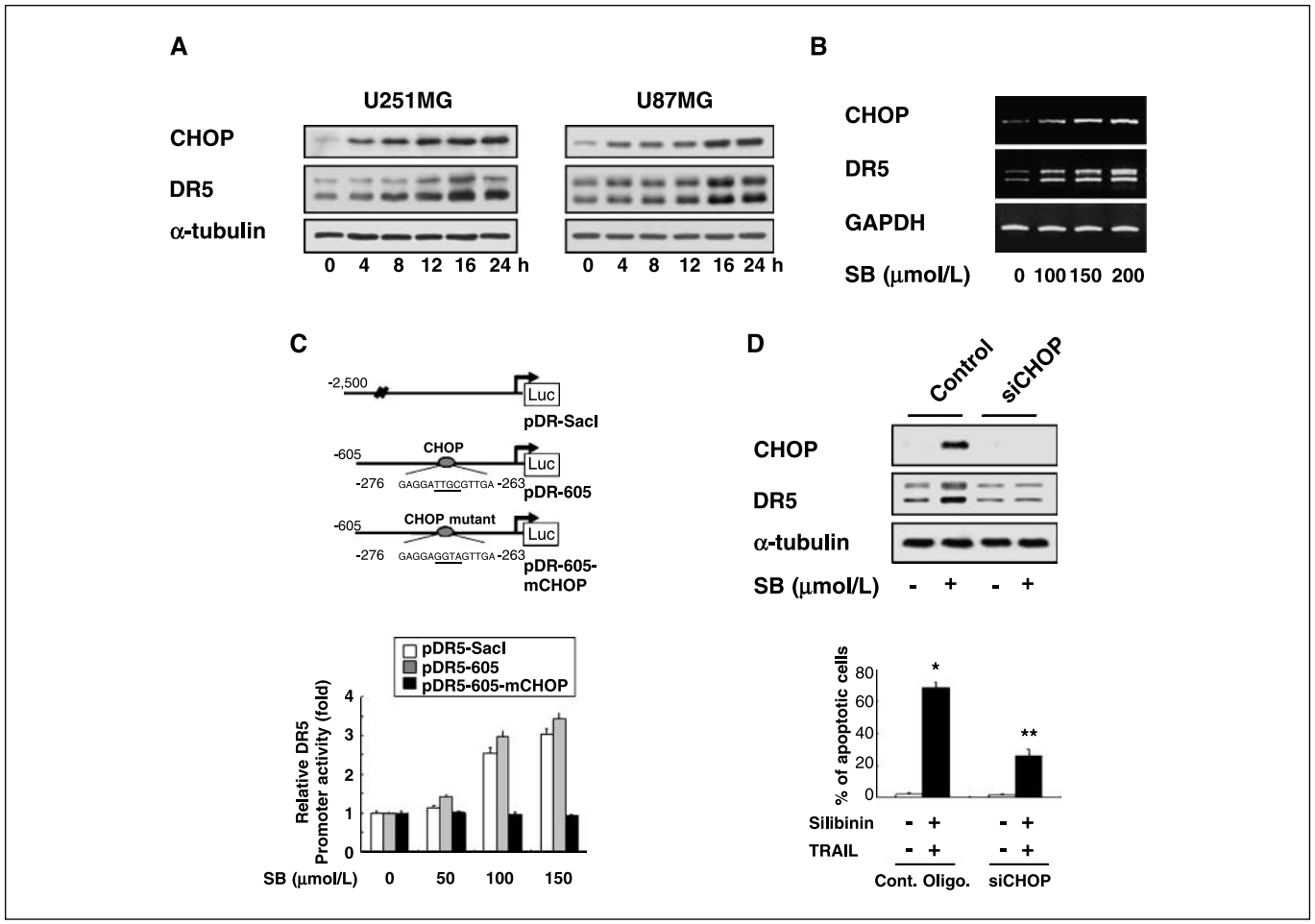

Figure 4. CHOP mediates silibinin-induced DR5 up-regulation and the apoptosis induced by silibinin plus TRAIL. *A,* silibinin-induced CHOP up-regulation. U251MG or U87MG cells were treated with 150 μmol/L silibinin and/or 100 ng/mL TRAIL for indicated times. Cell extracts were prepared for Western blotting of CHOP. α-Tubulin was used for a loading control of Western blotting. *B,* effect of silibinin on the mRNA levels of CHOP and DR5. U251MG cells were treated with various doses of silibinin for 10 h. After isolation of total RNA, RT-PCR of CHOP, DR5, and GAPDH was done. *C,* CHOP-binding site on the DR5 promoter is important for silibinin-induced DR5 up-regulation. U87MG cells were transfected with pDR-*Sac*I, pDR-605, and pDR-605-mCHOP plasmids and treated with or without silibinin. Cells were lysed and luciferase activity was measured. *Columns,* mean of at least three independent experiments; *bars,* SD. *D,* suppression of CHOP expression by siRNA-mediated knockdown reduces silibinin-induced DR5 up-regulation and silibinin-stimulated TRAIL-induced apoptosis. U251MG cells were transfected with the control scrambled nucleotide or siRNA duplexes against CHOP mRNA. Twenty-four hours after transfection, cells were treated with 150 μmol/L silibinin for 16 h. Down-regulation of CHOP by its siRNA transfection and the effect of CHOP down-regulation on DR5 protein levels were analyzed by Western blotting of CHOP and DR5. α-Tubulin levels were assessed to show equal gel loading. Twenty-four hours after transfection, cells were treated with or without 150 μmol/L silibinin plus 100 ng/mL TRAIL for 16 h. After fixation, DNA fragmentation was detected using APO-BrdU TUNEL assay kit following the manufacturer's instruction. Images of the cells were taken on a fluorescence microscope and the number of TUNEL-positive cells was counted; a minimum 100 cells was counted per sample. Percentages of apoptotic cells are depicted in graph. *Columns,* average of three independent experiments; *bars,* SE. *, $P < 0.005$, compared with untreated cells; **, $P < 0.05$, compared with cells transfected with control oligonucleotide and further treated with silibinin plus TRAIL.

cells. These results suggest that the expression levels of multiple components of the death receptor–mediated apoptotic pathway are independently controlled by silibinin rather than via upstream or downstream relationships.

Discussion

The effectiveness of chemotherapeutic drugs and radiotherapy in cancer treatment has been limited by systemic toxicity. Thus, a major goal for the development of effective cancer therapeutics has been to identify agents capable of selectively inducing cell death in cancer cells without damaging normal cells. TRAIL seems to be a good candidate because it shows selective toxicity to some tumor cells with minimal toxicity to normal cells (3, 4). However, various cancer cells reportedly show resistance to the cytotoxic effects of TRAIL (5, 6). These include various glioma cells, although they express the TRAIL receptor DR5 (7). Therefore, researchers are currently seeking to identify effective sensitizers for TRAIL-induced apoptosis that may allow cancer cells to recover TRAIL sensitivity.

Silibinin is a polyphenolic flavonoid isolated from the seeds of milk thistle (*Silybum marianum*; ref. 25). For more than 25 years, silibinin and its crude form, silymarin, have been used clinically and as dietary supplements against liver toxicity (26). Silibinin has proven nontoxic in various animal models with different modes of administration (8, 26, 27). Accumulating experimental and clinical evidence indicates that consumption of silibinin as a drug or dietary supplement seems to be safe and does not exert adverse health effects (26). Recent studies have shown that silibinin shows anticancer efficacy against a broad range of epithelial cancers in cell culture studies (28) and dietary silibinin was shown to inhibit

the growth and progression of lung (29) and prostate cancers (30) in rodent models. These findings suggest that silibinin may be useful as an anticancer drug or as an adjunct in combination therapy. Here, we show for the first time that silibinin effectively sensitizes human glioma cells, but not normal astrocytes, to TRAIL-induced apoptosis. In our study, 100 to 150 μmol/L silibinin was required for effective enhancement of TRAIL-induced apoptosis. Agarwal et al. (31) recently have reported that up to 165 μmol/L total silibinin levels can be achieved in the plasma without any signs of gross toxicity in terms of diet, water consumption, and weight gain profiles in silibinin-treated groups of animals versus controls. Furthermore, in recent clinical trial with escalating doses of silibinin in prostate cancer patients, a peak level of >100 μmol/L silibinin in plasma has been observed (32). Therefore, the silibinin concentrations used in the present study may be pharmacologically achievable *in vivo* in mice as well as humans. In both U87MG and U251MG cells, which are resistant to TRAIL-induced apoptosis, TRAIL treatment triggered partial processing of procaspase-3 into the p20 fragment but did not induce any further processing into the active subunits. In contrast, cells cotreated with silibinin plus TRAIL showed full processing from the p20 intermediate form to the active p17 and p12 forms of caspase-3. Our results collectively revealed that the molecular basis of the sensitizing effect of silibinin on TRAIL-induced apoptosis may be summarized as follows: (*a*) silibinin increases expression of the TRAIL receptor DR5, thereby amplifying TRAIL-induced death receptor–mediated apoptotic signaling; (*b*) silibinin reduces the protein levels of c-FLIP, an inhibitor of initiator caspase-8; and (*c*) silibinin reduces the protein levels of survivin, an inhibitor of effector caspases. The latter two functions act to reduce the apoptotic threshold by removing the barriers of caspase activation in this system.

TRAIL is known to trigger apoptosis through binding to its death receptors, DR4 (33) and DR5 (33, 34). The expression level of these death receptors may play a critical role in determining the intensity and/or duration of death receptor–mediated apoptotic signaling in response to TRAIL. Here, we show that silibinin-facilitated TRAIL-induced apoptosis was accompanied by up-regulation of DR5, but not DR4, in various glioma cells. Administration of siRNA against DR5 efficiently reduced the cell death induced by the combined treatment, showing the involvement of DR5. We recently showed

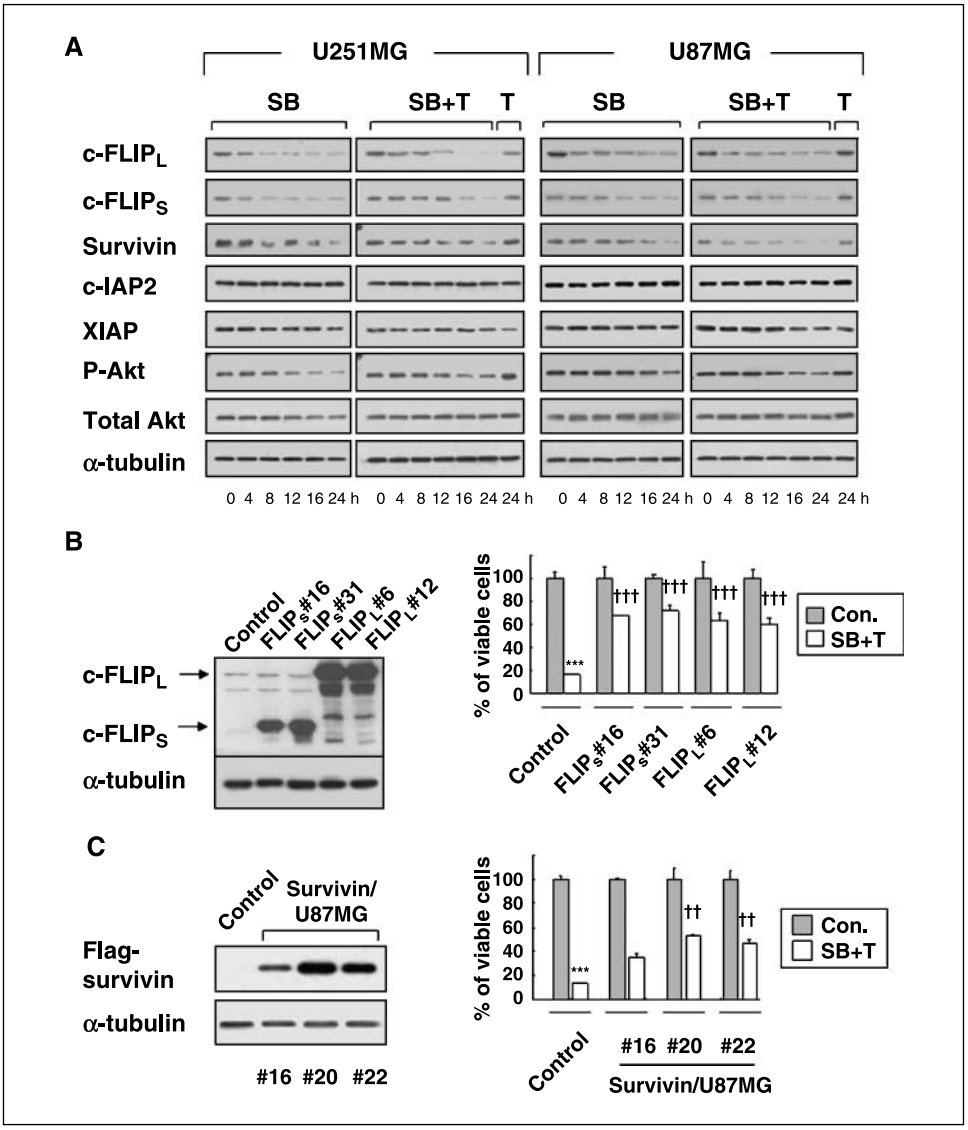

Figure 5. Silibinin-mediated down-regulation of FLIP and survivin is important for silibinin-facilitated TRAIL-induced apoptosis. *A,* effect of the combined treatment with silibinin and TRAIL on the expressions of various regulators of apoptosis. Cell extracts were prepared from U251MG or U87MG cells treated with 150 μmol/L silibinin and/or 100 ng/mL TRAIL for the indicated time points. Western blotting was done to detect the changes in the protein levels of c-FLIP and IAPs and the activity and total expressions of Akt. α-Tubulin levels were assessed to show equal gel loading. *B,* silibinin-enhanced TRAIL-mediated cell death was attenuated by overexpression of c-FLIP$_S$ or c-FLIP$_L$. *Left,* stable U87MG sublines overexpressing c-FLIP$_S$ or c-FLIP$_L$ were established, and their overexpression was confirmed by Western blotting using anti-c-FLIP antibody. Control U87MG (transfected with vector) or U87MG sublines overexpressing c-FLIP$_S$ or c-FLIP$_L$ were treated with silibinin plus TRAIL for 24 h, and cellular viability was measured with calcein-AM and EthD-1. *Right, columns,* average of three independent experiments; *bars,* SE. ***, $P < 0.001$, compared with untreated cells; †††, $P < 0.001$, compared with cells treated with silibinin plus TRAIL. *C,* forced expression of survivin significantly attenuates silibinin-facilitated TRAIL-induced apoptosis. *Left,* U87MG cells were stably transfected with plasmids encoding Flag-tagged survivin, and overexpression of survivin in these cells was confirmed by Western blotting using anti-Flag antibody. Control cells or U87MG sublines overexpressing survivin were treated with 150 μmol/L silibinin plus 100 ng/mL TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. *Right, columns,* average of three independent experiments; *bars,* SE. ***, $P < 0.001$, compared with untreated cells; ††, $P < 0.01$, compared with cells treated with silibinin plus TRAIL.

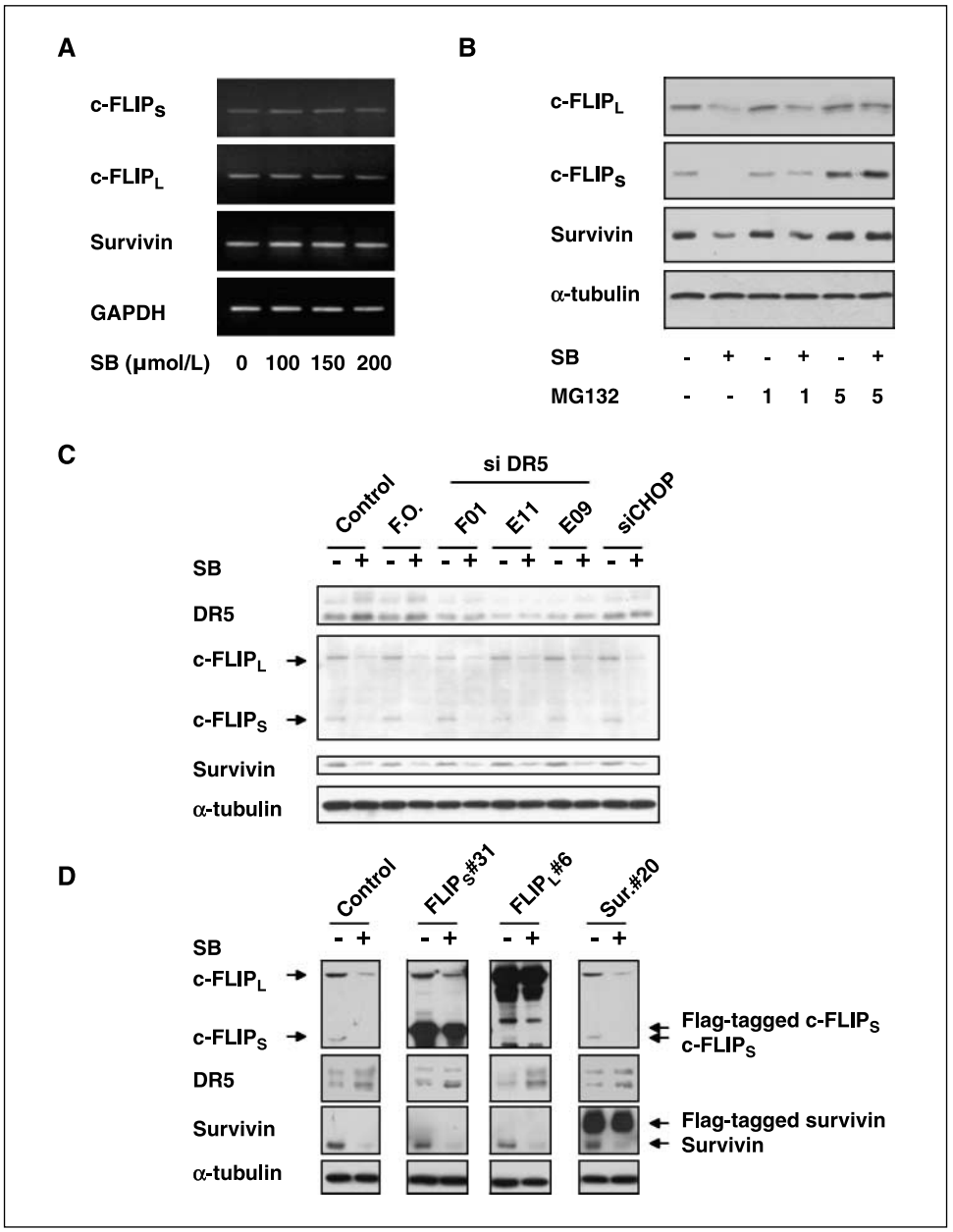

Figure 6. Silibinin-induced down-regulation of c-FLIP and survivin is controlled at the post-translational levels via proteasome-mediated degradation. *A,* effect of silibinin on the mRNA levels of c-FLIP$_S$, c-FLIP$_L$, and survivin. U251MG cells were treated with silibinin at the indicated concentrations for 12 h. Total RNA was prepared from the treated cells and RT-PCR of the indicated gene products was done. *B,* effect of MG132 on the protein levels of c-FLIP and survivin in cells treated with silibinin. U251MG cells were pretreated with or without MG132 at the indicated concentrations and further treated with 150 μmol/L silibinin for 16 h. Western blotting was done to detect c-FLIP$_L$, c-FLIP$_S$, and survivin. Western blotting of α-tubulin was done to show equal loading of protein samples. *C,* effect of suppression of DR5 or CHOP expression by its siRNA on silibinin-mediated down-regulation of c-FLIP and survivin. U87MG cells transfected with the control fluorescent oligonucleotide (*F.O.*), three different kinds of DR5 siRNAs, or CHOP siRNA were treated with or without 150 μmol/L silibinin, and the protein levels of c-FLIP and survivin were analyzed by Western blotting. Suppression of DR5 expression by siRNA transfection was analyzed by Western blotting, and α-tubulin was used as a loading control. *D,* effect of overexpression of survivin, FLIP$_S$, or FLIP$_L$ on silibinin-induced DR5 up-regulation. Control U87MG cells (transfected with vector), FLIP-overexpressing cells (short form #31 and long form #6), and survivin-overexpressing cells (#20) were treated with or without 150 μmol/L silibinin for 24 h, and the changes in DR5 protein levels were analyzed by Western blotting of DR5. In addition, changes in FLIP protein levels in survivin-overexpressing cells treated with silibinin and changes in survivin protein levels in FLIP$_S$- or FLIP$_L$-overexpressing cells were examined by Western blotting using the respective antibodies.

that reactive oxygen species (ROS)-mediated DR5 up-regulation is critical for sulforaphane-stimulated TRAIL-induced apoptosis (35). In the present study, however, we found that pretreatments with the antioxidant *N*-acetylcysteine or overexpression of catalase failed to block silibinin-induced DR5 up-regulation and apoptosis induced by combined treatment with silibinin and TRAIL in glioma cells (Supplementary Fig. S3), suggesting that ROS are not associated with silibinin-induced DR5 up-regulation in this system. CHOP/GADD153 has been reported to be involved in the up-regulation of DR5 and the sensitization of TRAIL-mediated apoptosis by MG132 (19) and tunicamycin (20). Consistent with these previous reports, we found that silibinin treatment increased the mRNA and protein levels of CHOP, and siRNA-mediated CHOP knockdown significantly blocked the cell death induced by silibinin plus TRAIL, indicating that CHOP seems to be mechanistically involved in this effect.

Another mechanism potentially involved in the regulation of TRAIL sensitivity is expressional modulation of the death receptor inhibitors c-FLIP$_L$ and c-FLIP$_S$ (21), two proteins with very short half-lives (36). Previous studies have shown that different treatments, such as synthetic triterpenoids and flavopiridol, can induce down-regulation of c-FLIP and subsequent sensitization to TRAIL-induced apoptosis in breast cancer cells (37, 38). In the present study, we found that treatment of different glioma cells with silibinin induced significant down-regulation of both c-FLIP$_L$, and c-FLIP$_S$, suggesting that silibinin-induced loss of c-FLIP expression may underlie the observed sensitization to TRAIL. Supporting this idea, overexpression of both c-FLIP$_L$ and c-FLIP$_S$ reduced the cell death induced by combined treatment with silibinin and TRAIL. Although c-FLIP can be transcriptionally regulated through the nuclear factor-κB pathway (39), we did not observe any significant decrease in c-FLIP mRNA in silibinin-treated U251MG cells,

indicating that the inhibitory action of silibinin is not mediated through transcriptional regulation of c-FLIP. Previous reports have indicated that c-FLIP protein levels can be down-regulated by proteasomal degradation (36, 38), and this modulation may lead to TRAIL sensitization (38). Consistent with this notion, pretreatment of U251MG cells with the proteasome inhibitor MG132 attenuated silibinin-induced down-regulation of c-FLIP$_S$ and c-FLIP$_L$, suggesting that silibinin treatment triggers proteasome-mediated degradation of the c-FLIP isoforms. Recently, JNK activation by TNF-α was shown to reduce c-FLIP$_L$ stability via JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase Itch, which ubiquitinates c-FLIP$_L$ and induces its proteasomal degradation (40). However, we found that silibinin treatment did not activate JNK in our system; indeed, it slightly reduced JNK activity (Supplementary Fig. S1), indicating that JNK-dependent phosphorylation is not involved in the silibinin-induced proteasomal degradation of c-FLIP in glioma cells. Further work will be required to fully clarify the molecular mechanism responsible for the silibinin-induced proteasomal degradation of c-FLIP in glioma cells.

In addition to DR5 up-regulation and c-FLIP down-regulation by silibinin, we found that down-regulation of survivin also contributed to silibinin-facilitated TRAIL-induced apoptosis. Survivin, a member of the IAP family, is overexpressed in many glioma cells and has been associated with poor prognosis (41). Survivin prevents apoptosis by direct or indirect inhibition of downstream effector caspases (42), and siRNA-mediated survivin knockdown has been reported to sensitize human melanoma cells to TRAIL-mediated apoptosis (43). Here, we found that overexpression of survivin in glioma cells significantly inhibited silibinin-enhanced TRAIL-induced apoptosis. Polyubiquitination and proteasome-dependent destruction of survivin have been shown in interphase cells, and mitotic phosphorylation of survivin Thr^{34} by Cdc2-cyclin B1 has been associated with increased protein stability at metaphase (44, 45). However, we failed to observe significant down-regulation of Cdc2, cyclin A, and cyclin B protein levels (Supplementary Fig. S4) or down-regulation of XIAP, another target protein of Cdc2 inhibition (6, 7), excluding the possible involvement of Cdc2 as an upstream signaling partner during silibinin-induced down-regulation of survivin. However, although the mRNA levels of survivin were not affected by silibinin treatment, pretreatment of glioma cells with MG132 significantly inhibited silibinin-induced down-regulation of survivin, suggesting that silibinin may promote proteasome-mediated degradation of survivin in a Cdc2-independent fashion.

In conclusion, we herein show for the first time that silibinin effectively recovers TRAIL sensitivity in glioma cells via modulation of multiple components of the death receptor–mediated signaling pathway, including DR5, c-FLIP, and survivin. Combined treatment with silibinin and TRAIL was strongly cytotoxic to various glioma cells but did not affect the viability of normal astrocytes. Therefore, combined treatment with silibinin and TRAIL seems to warrant additional study as a potential new strategy for treating TRAIL-resistant gliomas.

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