Involvement of c-FLIP and survivin down-regulation in flexible heteroarotinoid-induced apoptosis and enhancement of TRAIL-initiated apoptosis in lung cancer cells

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Abstract

The flexible heteroarotinoid, SHetA2, is a novel compound with apoptosis-inducing and anticancer activities *in vitro* and *in vivo*. Our previous research showed that up-regulation of death receptor 5 plays a critical role in the mechanism of SHetA2-induced apoptosis in human lung cancer cells. The hypothesis of this study was that the mechanism of SHetA2-induced apoptosis requires modulation of additional proteins critical for regulation of apoptosis, including cellular FLICE-inhibitory protein (c-FLIP), survivin, X-linked inhibitor of apoptosis, Bcl-2, Bcl-X_L, Bax, and Bim. Western blot analysis showed that c-FLIP and survivin were substantially reduced in all of the tested cell lines exposed to SHetA2 compared with other proteins that were reduced only in a subset of the cell lines tested. Strikingly, overexpression of c-FLIP, but not survivin, protected cells from SHetA2-induced apoptosis and enhancement of TRAIL-initiated apoptosis, although knockdown of endogenous survivin did slightly sensitize cells to SHetA2-induced apoptosis. Consistent with these results, small interfering RNA-mediated reduction of c-FLIP was more effective than survivin down-regulation in triggering apoptosis in these cell lines. SHetA2 increased ubiquitination of c-FLIP and the consequent degradation was abrogated by the proteasome inhibitor MG132. Although SHetA2 treatment led to increased c-Jun phosphorylation, the JNK inhibitor SP600125 did not prevent c-FLIP down-regulation by SHetA2. Thus, it appears that SHetA2 down-regulates c-FLIP levels by facilitating its ubiquitin/proteasome-mediated degradation independent of JNK activation. Collectively, the present study indicates that, in addition to death receptor 5 up-regulation, c-FLIP down-regulation is another important component of flexible heteroarotinoid (SHetA2)-induced apoptosis as well as enhancement of TRAIL-induced apoptosis. [Mol Cancer Ther 2008;7(11):3556–65]

Introduction

Apoptosis is a genetically well-controlled mechanism essential for the maintenance of tissue homeostasis and proper development through the elimination of unwanted cells. Thus, it represents a universal and exquisitely efficient endogenous or induced cellular suicide pathway (1, 2). It is well known that cells can die of apoptosis primarily through the extrinsic death receptor-induced pathway and/or the intrinsic mitochondria-mediated pathway. Cross-talk between these two pathways is mediated by the truncated proapoptotic protein Bid (2). A central step in the execution of apoptosis is the activation of an unusual class of cysteine proteases, termed caspases, which are widely expressed as inactive forms (2). Thus, this step is negatively regulated by multiple antiapoptotic proteins to prevent unnecessary activation of these caspases.

Caspase-8 activation is a critical step in initiating the extrinsic apoptotic pathway (3). Cellular FLICE-inhibitory protein (c-FLIP) is the major protein that prevents caspase-8 from activation by death receptors through binding to Fas-associated death domain and caspase-8 at the death-inducing signaling complex (4). Although multiple splicing isoforms of c-FLIP mRNA have been described, only two of them, $FLIP_S$ and $FLIP_L$, have been significantly studied at the protein level (5). Both proteins can be recruited to the death-inducing signaling complex and inhibit death receptor-mediated apoptosis (6). Both $FLIP_L$ and $FLIP_S$ are quick turnover proteins; thus, their levels are subject to regulation by ubiquitin/proteasome-mediated degradation (7–9). Generally speaking, c-FLIP expression correlates with resistance against death receptor-induced apoptosis. Accordingly, down-regulation of c-FLIP confers sensitivity to death receptor-induced apoptosis (5). Additionally, c-FLIP expression is associated with chemoresistance. Thus, down-regulation of c-FLIP using antisense oligonucleotides

Received 5/5/08; revised 7/11/08; accepted 8/28/08.

Grant support: Georgia Cancer Coalition Distinguished Cancer Scholar award (S-Y. Sun) and Department of Defense VITAL award W81XWH-04-1-0142 (S-Y. Sun and F.R. Khuri for Project 4).

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Note: Y. Lin and X. Liu share first authorship. F.R. Khuri and S-Y. Sun are Georgia Cancer Coalition Distinguished Cancer Scholars.

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doi:10.1158/1535-7163.MCT-08-0648

or small interfering RNA (siRNA) sensitizes cancer cells to chemotherapeutic agent-induced apoptosis (10, 11), whereas overexpression of c-FLIP protects cells from apoptosis induced by certain cancer therapeutic agents such as etoposide and cisplatin (10–17).

Caspase-9 activation is an essential step in the intrinsic apoptotic pathway (3). Survivin is a protein belonging to the inhibitor of apoptosis (IAP) gene family. Like other IAPs, survivin acts downstream of mitochondria to prevent processing or activation of initiator caspase-9 in the apoptosome, leading to inhibition of the activity of the effector caspases. Thus, survivin modulates both extrinsic and intrinsic apoptotic pathways (18). Genetic studies have shown that survivin transgenic mice exhibit apoptotic resistance (19), whereas survivin knockout mice show increased sensitivity to apoptosis (20). Moreover, many studies have shown that induction of survivin expression causes cell resistance to drug-induced apoptosis (21), whereas down-regulation of survivin using various means such as siRNA either induces apoptosis or sensitizes cells to undergo drug-induced or death ligand/receptor-induced apoptosis (22–25).

Although heteroarotinoids were initially developed as retinoids, the flexible heteroarotinoids act independently of the retinoic acid receptors to regulate growth, differentiation, and apoptosis (26–29). Moreover, SHetA2 induces apoptosis in cancer cells while sparing normal cells (30, 31). Importantly, SHetA2 effectively inhibits tumor growth *in vivo* without evidence of toxicity (26). Because of these encouraging results, SHetA2 was chosen for evaluation in the National Cancer Institute Rapid Access to Intervention Development program (application 196, compound NSC 726189) and now is in the Rapid Access to Preventive Intervention Development program, showing a potential as a cancer chemopreventive and therapeutic agent.

The mechanism of SHetA2-induced apoptosis has been shown to occur through the intrinsic mitochondrial pathway associated with loss of mitochondrial membrane integrity, generation of reactive oxygen species, release of cytochrome *c* from the mitochondria, and activation of caspase-3 in head and neck cancer cell lines (31). Likewise, SHetA2 induces apoptosis in human ovarian cancer cells through targeting the mitochondria, which is associated with alterations in the balance of Bcl-2 proteins, independent of generation of reactive oxygen species (30). It appears that the mechanisms of SHetA2 may vary in different types of cancer cells. We have shown recently that SHetA2 effectively inhibits the growth and induces apoptosis of human non-small cell lung cancer (NSCLC; ref. 32). Moreover, SHetA2 cooperates with the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to augment induction of apoptosis (32). These effects are tightly associated with death receptor 5 (DR5) up-regulation (32), implying the importance of activation of the extrinsic apoptotic pathway in SHetA2-induced apoptosis. The present study further determined whether SHetA2 modulates levels or expression of other proteins critical for regulation of apoptosis, including regulation of both intrinsic and extrinsic apoptotic pathways. In this study, we found that, of the critical apoptosis-regulating proteins,

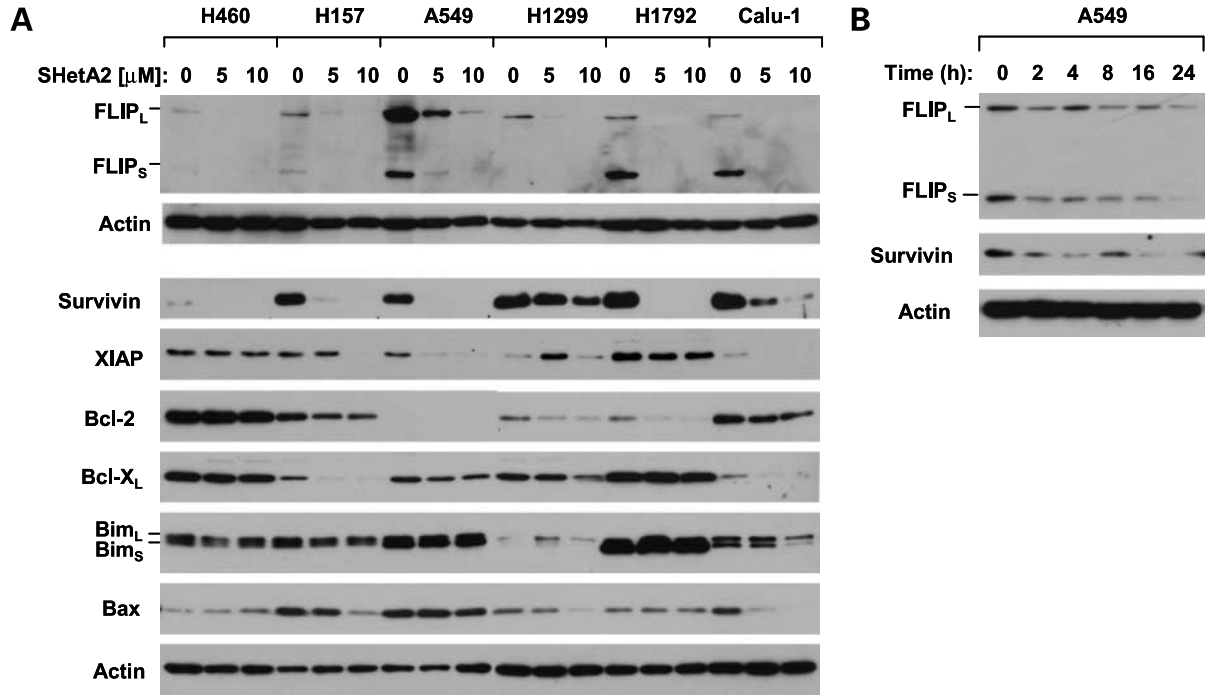

Figure 1. Modulatory effects of SHetA2 on c-FLIP, survivin, XIAP, Bcl-2, Bcl-X_L, Bim, and Bax in human NSCLC cell lines. The indicated cell lines were treated with the indicated concentrations of SHetA2 for 16 h (**A**) or with 5 μmol/L SHetA2 for the given time as indicated (**B**). The cells were then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis.

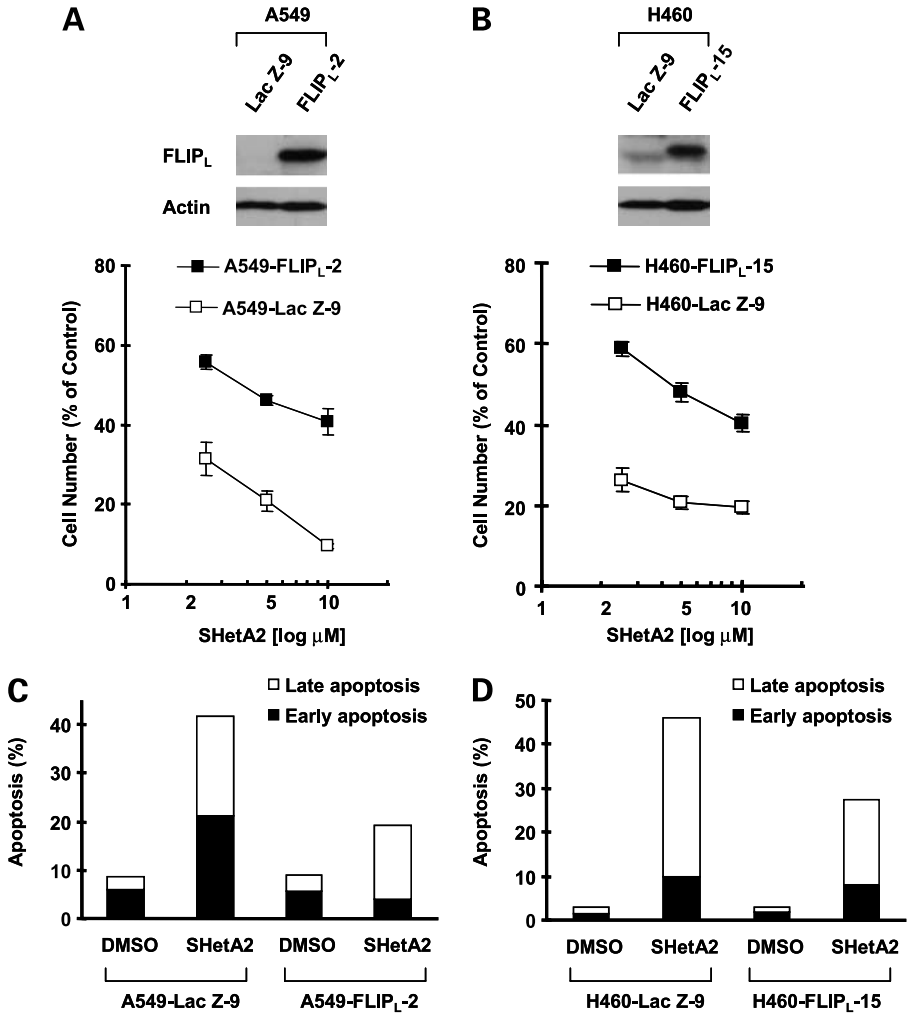

Figure 2. Enforced expression of ectopic c-FLIP protects cells from SHetA2-induced cell number decrease (**A** and **B**) and apoptosis (**C** and **D**). **A** and **B,** indicated transfectants were seeded in 96-well plates and treated with the indicated concentrations of SHetA2 ranging from 2.5 to 10 μmol/L. After 48 h, the cells were subjected to the sulforhodamine B assay for measurement of cell number. Mean of four replicate determinations. *Bars,* SD. **C** and **D,** indicated transfectants were treated with 10 μmol/L SHetA2 for 48 h and then harvested for detection of apoptotic cells using Annexin V staining. The percent positive cells in the top right and bottom right quadrants were added to yield the total of apoptotic cells.

c-FLIP was down-regulated consistently in all of the tested cell lines exposed to SHetA2 and contributed to SHetA2-induced apoptosis and enhancement of TRAIL-induced apoptosis. These results complement our previous findings to further support the importance of the activation of the extrinsic apoptotic pathway in SHetA2-induced apoptosis.

Materials and Methods

Reagents

SHetA2 was synthesized as described previously (27) and dissolved in DMSO at a concentration of 10 mmol/L, and aliquots were stored at −80°C. Stock solution was diluted to the appropriate concentrations with growth medium immediately before use. Human recombinant TRAIL was purchased from PeproTech. The specific JNK inhibitor SP600125 was purchased from Biomol. The proteasome inhibitor MG132 was purchased from Sigma. Mouse monoclonal anti-FLIP antibody (NF6) was purchased from Alexis Biochemicals. Rabbit polyclonal anti-X-linked IAP (XIAP), anti-Bim, anti-c-Jun, and anti-phospho-c-Jun (Ser^{63}) antibodies and mouse monoclonal anti-survivin antibody were purchased from Cell Signaling Technology. Mouse anti-Bax monoclonal antibody was purchased from Trevigen. Mouse anti-Bcl-2 and rabbit anti-Bcl-X_L antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-β-actin antibody was purchased from Sigma.

Cell Lines and Cell Culture

The human NSCLC cell lines used in this study were described previously (33). A549-Lac Z-9, A549-FLIP$_L$-2, H460-Lac Z-9, and H460-FLIP$_L$-15 were described previously (15, 16). These cell lines were grown in monolayer culture in RPMI 1640 supplemented with glutamine and 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Construction of Lentiviral Survivin Expression Vector and Establishment of Lung Cancer Cell Lines That Overexpress Survivin

Survivin cDNA was amplified by standard reverse transcription-PCR from RNA extracted from normal human bronchial epithelial cells using the following primers: sense 5′-CACTAGTGCCGCCACCATGGGTG-CCCCGACGTTGCCCCCTG-3′ and antisense 5′-CGGG-CCCTCAATCCATGGCAGCCAGCTGCTCG-3′. Following the amplification, the survivin cDNA was cloned into a

pT-easy vector (Promega) following the manufacturer's protocol as pT-easy-survivin. The pT-easy-survivin was then cut and cloned into a lentiviral vector using two restriction enzyme sites *Spe*I and *Apa*I as described previously (34). The newly generated plasmid was named lenti-survivin. In this study, we used pLenti-Lac Z as a vector control, which was included in the pLenti6/V5 Directional TOPO Cloning kit (Invitrogen). Lentiviruses were produced using ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's instructions. The viral titers were determined following the manufacturer's manual. For infection, the viruses were added to the cells at the multiplicity of infection of 10. For stable expression, cells were infected and then selected in the presence of 50 μg/mL blasticidin for 1 week. The individual clones and pool were expanded and screened for survivin expression using Western blot analysis.

Cell Growth Assay

Cells were cultured in 96-well cell culture plates and treated the next day with the agents indicated. Viable cell number was estimated using the sulforhodamine B assay as described previously (33).

Apoptosis Assays

Apoptosis was detected either by analysis of caspase activation using Western blot analysis as described below or by Annexin V staining using Annexin V-PE apoptosis detection kit (BD Bioscience) following the manufacturer's instructions and analyzed by flow cytometry using FACScan (Becton Dickinson). In the Annexin V assay, the percent positive cells for Annexin V-PE staining only in the bottom right quadrant and for both Annexin V and DNA (7-amino-actinomycin D) staining in the top right quadrant represent the early and late apoptotic populations, respectively. The percent positive cells for DNA staining only in the top left quadrant represent the necrotic population.

Western Blot Analysis

Preparation of whole-cell protein lysates and Western blot analysis were described previously (35).

Silencing of c-FLIP and Survivin

c-FLIP and survivin gene silencing was achieved by transfecting the given cell lines with siRNA oligonucleotides using the HiperFect transfection reagent (Qiagen) following the manufacturer's instructions. The control (nonsilencing), c-FLIP, and survivin siRNA oligonucleotide duplexes that target the sequences 5′-AATTCTCCGAACGTGTCACGT-3′, 5′-AAGCAGTCTGTTCAAGGAGCA-3′ (12), and 5′-AAGCATTCGTCCGGTTGCGCT-3′ (36), respectively, were synthesized by Qiagen. The transfection was conducted in 6-well plates. Forty-eight hours after the transfection, the cells were harvested and subjected to Annexin V staining/flow cytometric analysis. The knockdown efficiency was evaluated by Western blotting.

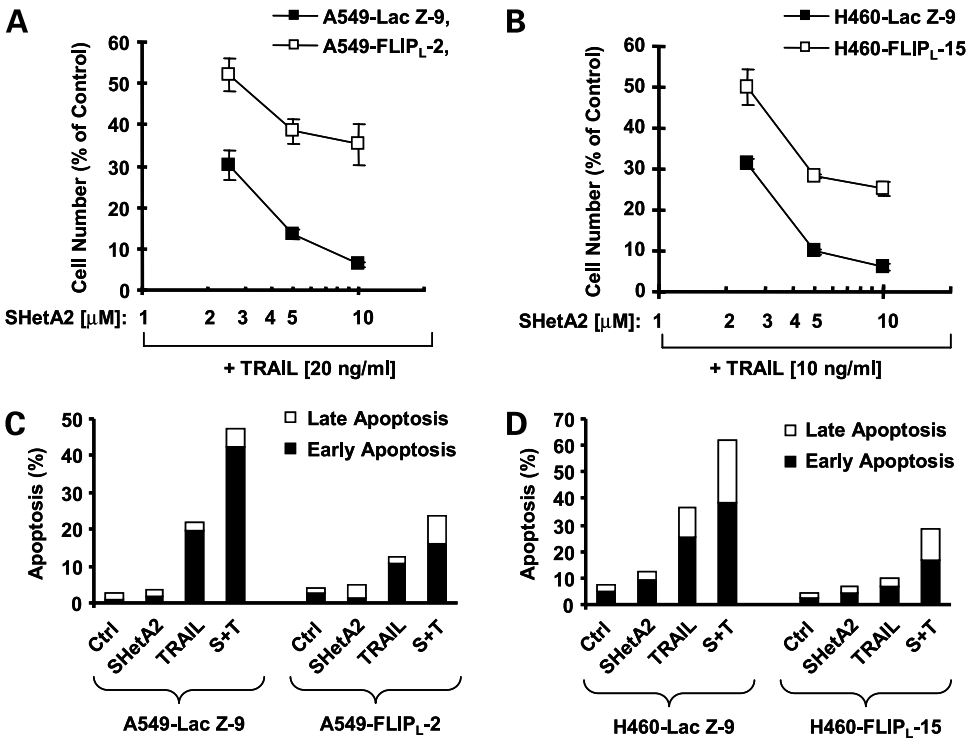

Figure 3. Enforced expression of ectopic c-FLIP confers resistance to induction of apoptosis by the combination of SHetA2 and TRAIL. **A** and **B,** indicated transfectants were seeded in 96-well plates and treated with the indicated concentrations of SHetA2 combined with 10 ng/mL TRAIL (H460) or 20 ng/mL TRAIL (A549). After 24 h, cells were subjected to the sulforhodamine B assay for measurement of cell survival. Mean of four replicate determinations. *Bars,* SD. **C** and **D,** indicated transfectants were treated with DMSO, 5 μmol/L SHetA2 alone, 10 ng/mL (H460) or 20 ng/mL (A549) TRAIL alone, or SHetA2 plus TRAIL for 24 h and then subjected to detection of apoptotic cells using Annexin V staining. The percent positive cells in the top right and bottom right quadrants were added to yield the total of apoptotic cells.

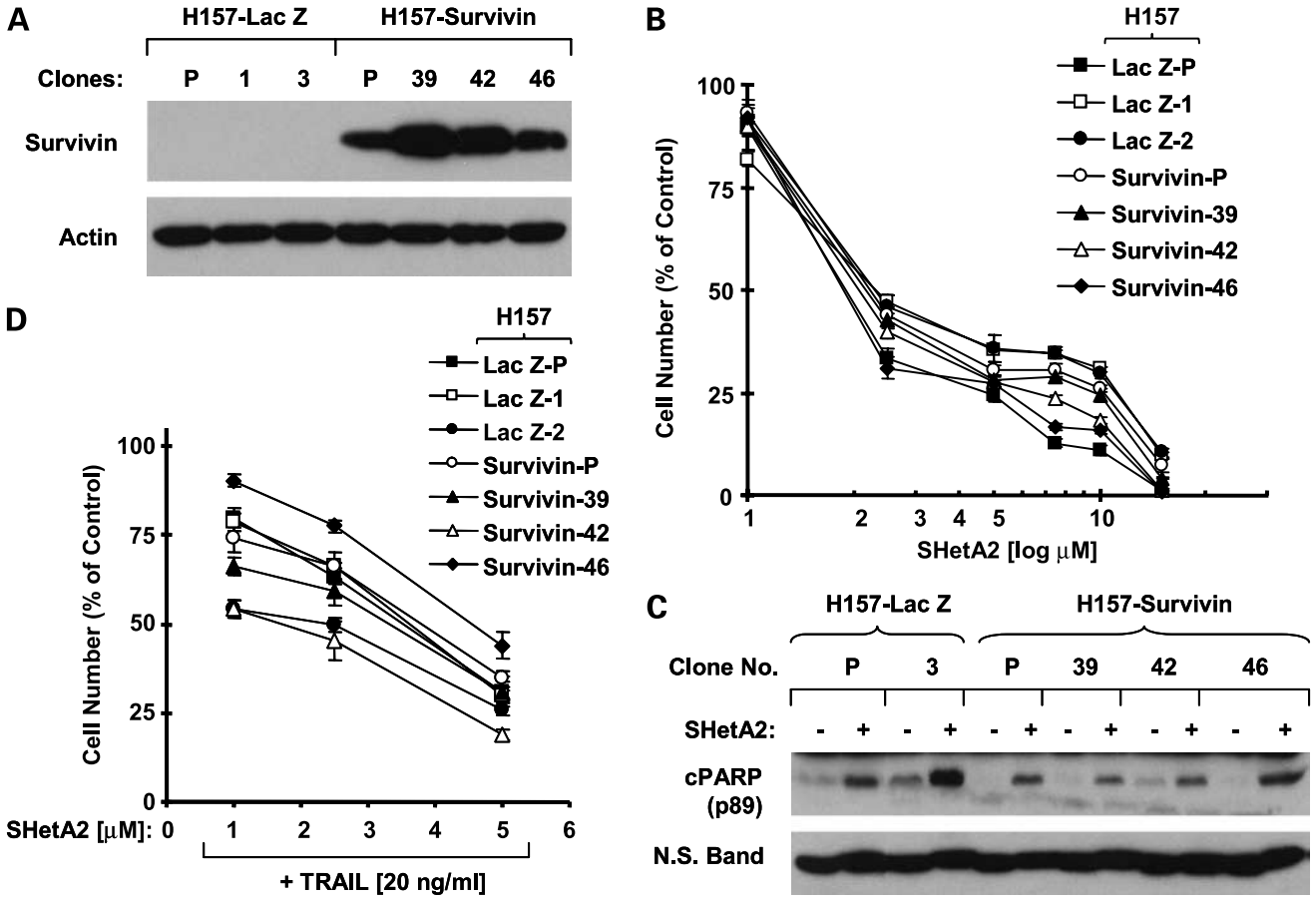

Figure 4. Enforced expression of ectopic survivin (**A**) does not confer resistance to ShetA2 (**B** and **C**) or the combination of SHetA2 and TRAIL (**D**). **A,** confirmation of ectopic survivin expression in H157 survivin transfectants by Western blotting using survivin antibody. *P,* pool. **B** and **D,** indicated transfectants from H157 cells were seeded in 96-well plates and treated with the given concentrations of SHetA2 (**B**) or the individual combination of SHetA2 and TRAIL as indicated (**D**) the next day. After 48 h (**B**) or 24 h (**D**), the cells were subjected to the sulforhodamine B assay for measurement of cell survival. Mean of four replicate determinations. *Bars,* SD. **C,** indicated transfectants were treated with 10 μmol/L SHetA2 for 48 h and then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of cleaved poly(ADP-ribose) polymerase (*cPARP*). *N.S.,* nonspecific.

Immunoprecipitation for Detection of Ubiquitinated c-FLIP

A549-FLIP$_L$-2 cells, which stably express FLIP$_L$, were transfected with HA-ubiquitin plasmid using the FuGENE 6 transfection reagent (Roche Diagnostics) following the manufacturer's instruction. After 24 h, the cells were treated with SHetA2 or MG132 plus SHetA2 for 4 h and then were lysed for immunoprecipitation of Flag-FLIP$_L$ using Flag M2 monoclonal antibody (Sigma), as described previously (37), followed by the detection of ubiquitinated FLIP$_L$ with Western blotting using anti-HA antibody (Abgent).

Results

SHetA2 Modulates the Levels of c-FLIP, Survivin, and Other Apoptosis-Related Proteins in Human NSCLC Cells

To better understand the mechanisms by which SHetA2 induces apoptosis, we examined the effects of SHetA2 on modulation of several important proteins involved in regulation of both extrinsic and intrinsic apoptotic pathways in a panel of human NSCLC cell lines. As presented in Fig. 1A, SHetA2 at 5 or 10 μmol/L effectively reduced the levels of c-FLIP (both FLIP$_L$ and FLIP$_S$) and survivin in all of the 6 tested cell lines. Compared with other cell lines, H1299 cells exhibited the least decrease in survivin levels on treatment with SHetA2. We also noted that the basal levels of FLIP$_S$ in H460, H157, and H1299 cells and the basal levels of survivin in H460 cells were very low or undetectable. The down-regulation of both c-FLIP and survivin occurred rapidly within 2 h and was sustained up to 24 h post-SHetA2 treatment (Fig. 1B).

SHetA2 also reduced the levels of XIAP, Bcl-2, and Bcl-X$_L$; however, these modulations occurred only in some of the tested cell lines (Fig. 1A). For example, SHetA2 decreased the levels of XIAP and Bcl-X$_L$ in H157, A549, and Calu-1 cell lines but not in H460 and H1792 cell lines. Bcl-2 levels were reduced in 4 of the 5 tested cell lines that

express detectable Bcl-2, except for H460 cells on treatment with SHetA2. SHetA2 did not modulate the levels of Bim in all of the tested cell lines. Bax levels were either not altered (e.g., H460 and A549) or even decreased (e.g., H157 and Calu-1) particularly by the high dose of SHetA2 (e.g., 10 μmol/L; Fig. 1A).

Collectively, it appears that down-regulation of c-FLIP and survivin is likely to be the common and important mechanism contributing to SHetA2-induced apoptosis. Therefore, the subsequent experiments focused on showing the importance of c-FLIP and survivin down-regulation in SHetA2-induced apoptosis as well as enhancement of TRAIL-induced apoptosis.

Enforced Expression of Ectopic c-FLIP Protects NSCLC Cells from Induction of Apoptosis by SHetA2 Alone or When Combined with TRAIL

To test the importance of c-FLIP in SHetA2-induced apoptosis, the effect of c-FLIP overexpression on induction of apoptosis by SHetA2 alone or combined with TRAIL was examined. As presented in Fig. 2, in both A549 and H460 cells, SHetA2 effectively decreased the numbers of control cells that expressed the control protein Lac Z (A549-Lac Z-9

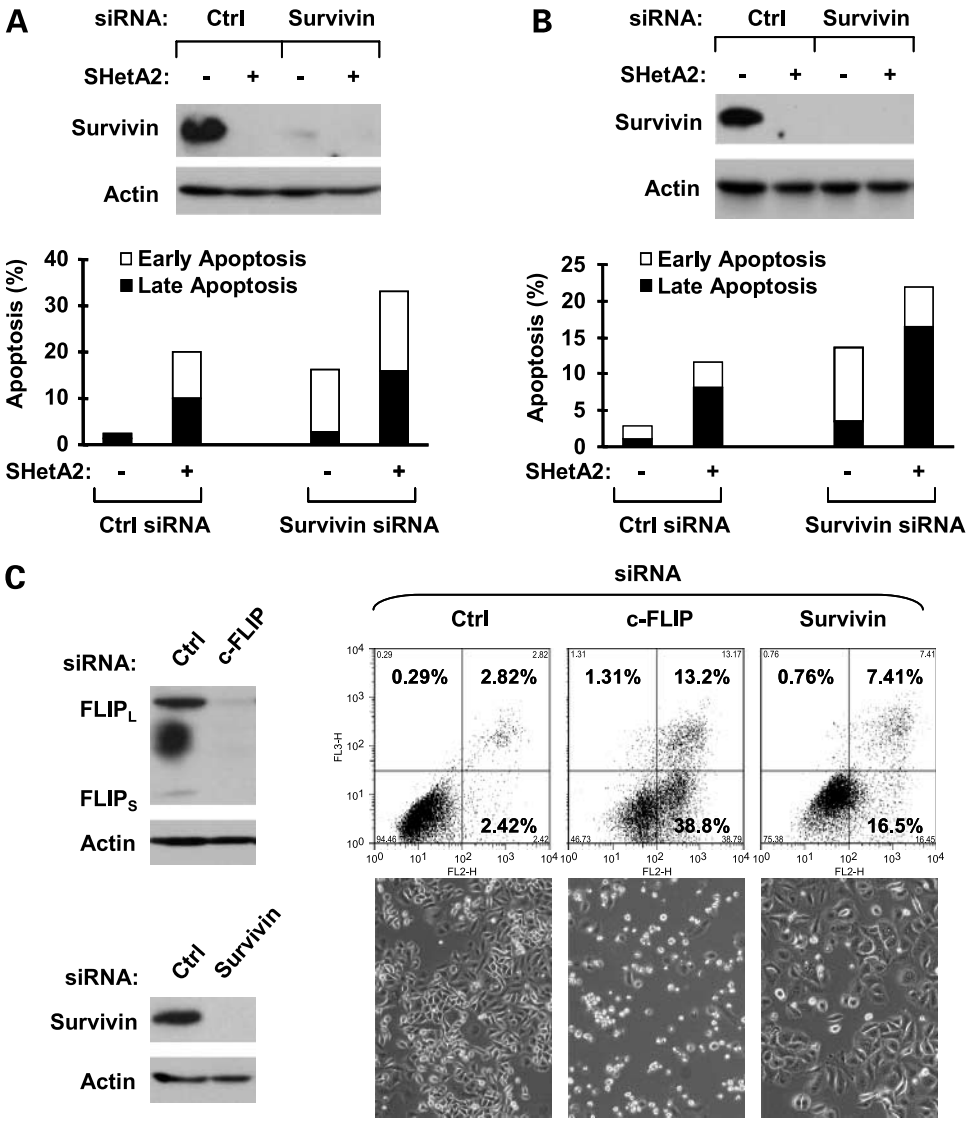

Figure 5. Effects of siRNA-mediated reduction of endogenous survivin on SHetA2-induced apoptosis (**A** and **B**) and comparison of the potencies of siRNA-mediated down-regulation of c-FLIP and survivin on triggering apoptosis (**C**). A549 (**A**) or H1299 (**B**) cells were seeded in 6-well plates and the next day transfected with 60 nmol/L control (*Ctrl*) and survivin siRNAs, respectively. Twenty-four hours later, the cells were treated with 10 μmol/L SHetA2. After an additional 48 h, the cells were harvested for evaluation of survivin knockdown efficiency by Western blotting (*top*) and apoptosis using Annexin V staining/flow cytometry (*bottom*), respectively. **C,** A549 cells were seeded in 6-well plates and the next day subjected to transfection with 60 nmol/L control, c-FLIP, and survivin siRNAs, respectively. After 48 h, the cells were harvested for evaluation of c-FLIP or survivin knockdown efficiency by Western blot analysis (*left*) and for detection of apoptotic cells using Annexin V staining/flow cytometry (*right*). In addition, the morphologic changes of the transfected cells were also documented (*right*).

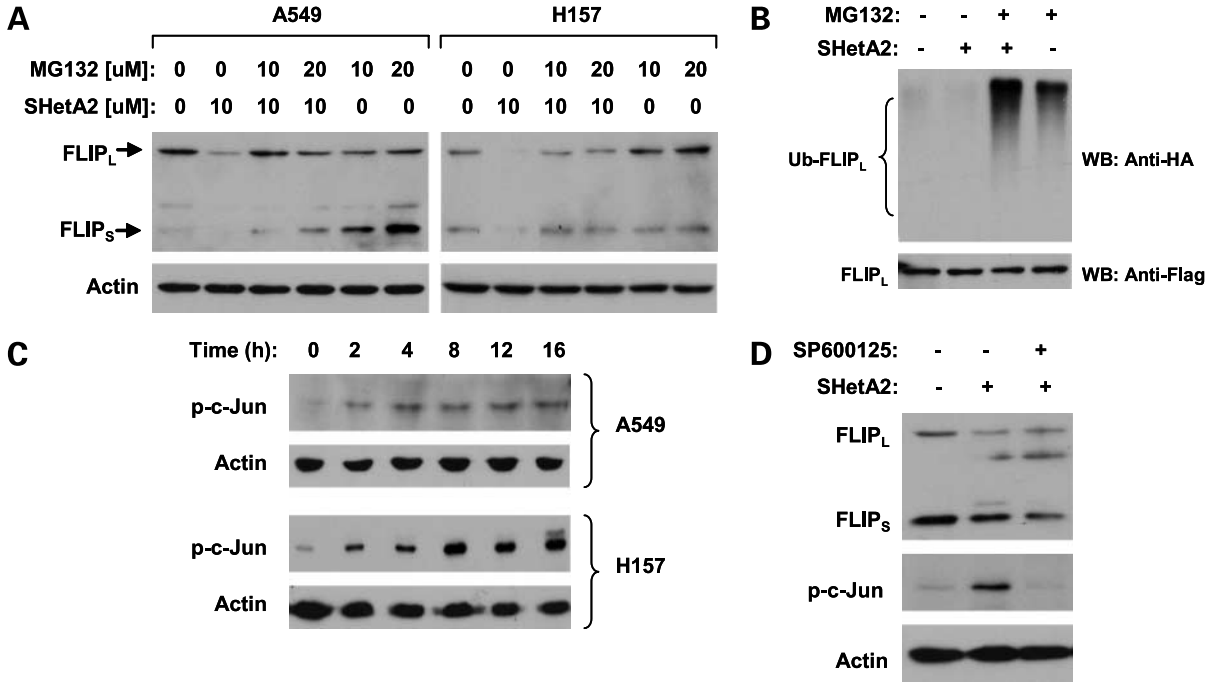

Figure 6. SHetA2 down-regulates c-FLIP levels through ubiquitin/proteasome-mediated protein degradation (**A** and **B**) independent of JNK (**C** and **D**). **A,** given cell lines were pretreated with 10 or 20 μmol/L MG132 for 30 min before the addition of 10 μmol/L SHetA2. After cotreatment for 4 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. **B,** A549-FLIP$_L$-2 cells that stably express ectopic flag-FLIP$_L$ were transfected with HA-ubiquitin plasmid using FuGENE 6 transfection reagent for 24 h. The cells were then pretreated with 20 μmol/L MG132 for 30 min and then cotreated with 10 μmol/L SHetA2 for 4 h. Whole-cell protein lysates were then prepared for immunoprecipitation using anti-Flag antibody followed by Western blotting (*WB*) using anti-HA antibody for detection of ubiquitinated FLIP$_L$ (*Ub-FLIP$_L$*) and anti-Flag antibody for detection of ectopic FLIP$_L$. FLIP$_L$. **C,** indicated cell lines were treated with 10 μmol/L SHetA2 for the given times. **D,** A549 cells were pretreated with 20 μmol/L SP600125 for 30 min and then cotreated with 10 μmol/L SHetA2 for another 12 h. After the aforementioned treatments (**C** and **D**), the cells were then subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of the indicated proteins.

and H460-Lac Z-9); however, this effect was substantially diminished in cell lines that overexpressed ectopic FLIP$_L$ (A549-FLIP$_L$-2 and H460-FLIP$_L$-15; Fig. 2A and B). Consistently, SHetA2 caused ~42% and 46% apoptotic cells in A549-Lac Z-9 and H460-Lac Z-9 cells, respectively, but only 19% and 27% apoptosis in A549-FLIP$_L$-2 and H460-FLIP$_L$-15 cells, respectively (Fig. 2C and D), indicating that enforced overexpression of c-FLIP confers cell resistance to SHetA2-induced apoptosis. Taken together, we conclude that c-FLIP overexpression partially protects NSCLC cells from SHetA2-induced apoptosis.

Moreover, we examined the effects of c-FLIP overexpression on SHetA2/TRAIL-induced apoptosis. As shown in Fig. 3A and B, SHetA2/TRAIL had much less effects on decreasing the numbers of A549-FLIP$_L$-2 and H460-FLIP$_L$-15 cells than those of A549-Lac Z-9 and H460-Lac Z-9 cells, indicating that the cell-killing effects of the SHetA2 and TRAIL combination were substantially inhibited in NSCLC cells that overexpress c-FLIP. In agreement, we detected ~62% and 48% apoptosis, respectively, in H460-Lac Z-9 and A549-Lac Z-9 cells but only 27% and 24% apoptosis, respectively, in H460-FLIP$_L$-15 and A549-FLIP$_L$-2 cells (Fig. 3C and D), showing that enforced expression of ectopic c-FLIP partially protected NSCLC cells from cooperative induction of apoptosis by the SHetA2 and TRAIL combination. Thus, c-FLIP overexpression decreases the sensitivity of NSCLC cells to SHetA2/TRAIL-induced apoptosis as well.

Enforced Overexpression of Ectopic Survivin Does Not Protect NSCLC Cells from SHetA2- or SHetA2/TRAIL-induced Apoptosis

Given that SHetA2 also effectively reduced the levels of survivin in human NSCLC cells, we further determined whether survivin reduction plays a role in regulation of SHetA2-induced apoptosis or SHetA2-mediated enhancement of TRAIL-induced apoptosis. To this end, we infected H157 cells, which have a high lentiviral infection efficiency, with lentiviral Lac Z (as a control) or survivin and established several sublines that stably express Lac Z or survivin as presented in Fig. 4A. After obtaining these cell lines, we then examined their sensitivity to SHetA2 or SHetA2/TRAIL. Unexpectedly, we found that the H157-survivin sublines, despite having high levels of survivin, were not different in their sensitivity to either SHetA2 or SHetA2/TRAIL compared with the Lac Z sublines albeit their sublines exhibited various sensitivity (Fig. 4B and D). Overall, it appears that survivin overexpression does not alter cell sensitivity to either SHetA2 or SHetA2/TRAIL. Moreover, we looked at poly(ADP-ribose) polymerase cleavage in these cell lines exposed to SHetA2 and found

that induction of poly(ADP-ribose) polymerase cleavage by SHetA2 between Lac Z and survivin sublines were overall comparable (Fig. 4C). These results further indicate that overexpression of survivin does not alter cell sensitivity to SHetA2-induced apoptosis.

Silencing of Endogenous Survivin Expression Slightly Enhances SHetA2-Induced Apoptosis

We also determined whether enforced reduction of endogenous survivin expression with transfection of survivin siRNA alters cell sensitivity to SHetA2. As presented in Fig. 5A and B, transfection of survivin siRNA in either A549 or H1299 cells substantially reduced basal levels of survivin. In A549 cells, SHetA2-induced apoptosis was increased from 20% in control siRNA-transfected cells to 33% in survivin siRNA-transfected cells (Fig. 5A). In H1299 cells, SHetA2-induced apoptosis was increased from 12% in control siRNA-transfected cells to 22% in survivin siRNA-transfected cells (Fig. 5B). Overall, data from both cell lines indicate that siRNA-mediated reduction of endogenous survivin results in ~10% increase in SHetA2-induced apoptosis. Thus, we conclude that enforced down-regulation of endogenous survivin slightly enhances SHetA2-induced apoptosis.

siRNA-Mediated Down-regulation of c-FLIP More Effectively Initiates Apoptosis Than Survivin Reduction

To further decipher the involvement or importance of c-FLIP and survivin in regulation of apoptosis, we used siRNA to silence c-FLIP or survivin and then examined their respective effect on induction of apoptosis. As presented in Fig. 5C, transfection of c-FLIP and survivin siRNA into A549 cells efficiently reduced the levels of c-FLIP and survivin proteins, respectively (*left*). However, c-FLIP down-regulation induced much more apoptotic cells than survivin reduction (~44% compared with 24%; *right*). These results again suggest that c-FLIP plays a more important role than survivin in regulation of apoptosis.

SHetA2 Down-regulates c-FLIP through Promoting Ubiquitin/Proteasome-Mediated Degradation Independent of JNK

Considering the importance of c-FLIP down-regulation in SHetA2-induced and SHetA2/TRAIL-induced apoptosis as shown above, we also questioned how SHetA2 reduced c-FLIP levels. Because c-FLIP proteins are known to be regulated by ubiquitin/proteasome-mediated degradation (7, 9), we then determined whether the observed down-regulation of c-FLIP by SHetA2 would be mediated via this process. To this end, we first treated cells with SHetA2 in the absence and presence of the proteasome inhibitor MG132 and then compared c-FLIP modulation under these conditions. In both A549 and H157 cells, SHetA2-induced down-regulation of c-FLIP was inhibited by MG132 (Fig. 6A), indicating that SHetA2-induced c-FLIP degradation is proteasome dependent. By immunoprecipitation/Western blotting, we detected the highest levels of ubiquitinated $FLIP_L$ in cells treated with SHetA2 plus MG132 compared with cells exposed to SHetA2 alone or MG132 alone (Fig. 6B), indicating that SHetA2 increases c-FLIP ubiquitination. Taken together, we conclude that SHetA2 initiates ubiquitin/proteasome-mediated c-FLIP degradation, leading to down-regulation of c-FLIP in human NSCLC cells.

Recently, JNK has been linked to tumor necrosis factor-induced, ubiquitin/proteasome-mediated $FLIP_L$ degradation (9). Therefore, we determined whether JNK activation is involved in mediating SHetA2-induced c-FLIP degradation. To this end, we first looked at whether SHetA2 increases JNK activity. As presented in Fig. 6C, SHetA2 caused a time-dependent increase in the levels of phospho-c-Jun, a well-known readout of JNK activity, indicating that SHetA2 treatment leads to activation of JNK. Following this study, we examined the effects of SHetA2 on c-FLIP down-regulation in the presence of the JNK-specific inhibitor SP600125. SP600125 at the concentration of 20 μmol/L blocked SHetA2-induced c-Jun phosphorylation, confirming that SP600125 worked as expected in our cell system. However, SP600125 did not block SHetA2-induced c-FLIP (both $FLIP_L$ and $FLIP_S$) down-regulation (Fig. 6D). Collectively, we suggest that SHetA2 down-regulates c-FLIP independent of JNK activation.

Discussion

By examining the modulatory effects of SHetA2 on the levels of several proteins involved in the regulation of both extrinsic and intrinsic apoptotic pathways, including c-FLIP, survivin, XIAP, Bcl-2, Bcl-X_L, Bim, and Bax, we noted that c-FLIP, a major inhibitor of the extrinsic apoptotic pathway, and survivin, a major inhibitor of the intrinsic apoptotic pathway, are the only proteins that were down-regulated in all of the tested NSCLC cell lines (Fig. 1). Thus, down-regulation of c-FLIP and survivin appears important for SHetA2 regulation of apoptosis.

It has been documented that modulation of either c-FLIP or survivin levels alters cell sensitivity to drug-induced or death receptor-mediated apoptosis (5–7, 38–40). In this study, enforced expression of ectopic $FLIP_L$ attenuated induction of apoptosis by SHetA2 or its combination with TRAIL (Figs. 2 and 3), suggesting a critical role of c-FLIP down-regulation in mediating SHetA2-induced apoptosis and the augmentation of TRAIL-induced apoptosis. In contrast, enforced expression of ectopic survivin did not protect cancer cells from either SHetA2-induced apoptosis or SHetA2/TRAIL-induced apoptosis (Fig. 4). Interestingly, siRNA-mediated reduction of endogenous survivin did slightly enhance SHetA2-induced apoptosis (Fig. 5A and B). Thus, it seems that survivin reduction plays a role, albeit limited, in this process. In our previous study, the proteasome inhibitor PS-341 induced apoptosis and augmented TRAIL-induced apoptosis despite up-regulation of survivin (41). In this study, we also showed that siRNA-mediated silencing of c-FLIP induced massive apoptosis, whereas silencing of survivin initiated limited apoptosis (Fig. 5C). These results suggest that cancer cells are more subject to undergo apoptosis by modulation of c-FLIP levels than by alteration of survivin levels, although both c-FLIP and survivin are involved in the regulation of apoptosis.

Our previous study showed that SHetA2 up-regulates DR5 expression, which contributes to SHetA2-induced apoptosis and enhancement of TRAIL-induced apoptosis in human NSCLC cells (32). The present findings on modulation of c-FLIP and its involvement in SHetA2-induced apoptosis alone and in combination with TRAIL further highlight the importance of the activation of the DR5-mediated extrinsic apoptotic pathway in SHetA2 mechanisms at least in NSCLC cells. Based on these previous (32) and current findings, we propose that SHetA2 induces apoptosis and enhances TRAIL-induced apoptosis primarily through activation of the extrinsic apoptotic pathway by induction of DR5 expression and down-regulation of c-FLIP in human NSCLC cells. In addition, down-regulation of survivin can further amplify death signaling from activation of the extrinsic apoptotic pathway.

It has been documented that both $FLIP_L$ and $FLIP_S$ are quick turnover proteins regulated by ubiquitin/proteasome-mediated degradation (7, 9). In the current study, SHetA2 down-regulated c-FLIP levels by facilitating ubiquitin/proteasome-mediated degradation of c-FLIP, as SHetA2 increased ubiquitinated c-FLIP levels and lost activity in reducing c-FLIP levels in the presence of the proteasome inhibitor MG132 (Fig. 6). JNK activation has been recently suggested to regulate ubiquitin/proteasome-dependent degradation of $FLIP_L$ (9). Although SHetA2 treatment leads to activation of the JNK pathway, the JNK inhibitor SP600125 did not prevent SHetA2-induced down-regulation of c-FLIP. Moreover, SHetA2 decreased both forms of c-FLIP ($FLIP_L$ and $FLIP_S$), whereas JNK regulates the degradation of only the long form of c-FLIP ($FLIP_L$; ref. 9). Thus, our results suggest that SHetA2 down-regulates c-FLIP independent of JNK.

We noted only in some of the tested cell lines that SHetA2 did down-regulate the expression of Bcl-2, Bcl-X_L, and XIAP, all of which are antiapoptotic proteins involved in regulation of the intrinsic apoptotic pathway (2). Thus, it appears that the modulation of these proteins may not be as important as c-FLIP down-regulation in mediating SHetA2-induced apoptosis. However, it is still possible that the modulation of these proteins contributes to SHetA2-induced apoptosis or augmentation of TRAIL-induced apoptosis, to a certain extent, in a specific cell line. It is likely that SHetA2-mediated induction of DR5 and down-regulation of c-FLIP initiates apoptosis, whereas down-regulation of other antiapoptotic proteins such as survivin and Bcl-2 lowers the cellular apoptotic threshold to further sensitize cancer cells to SHetA2-induced apoptosis. Nonetheless, the roles of these antiapoptotic proteins in regulating SHetA2-induced apoptosis including sensitization of TRAIL-induced apoptosis need further investigation.

In summary, the present study shows for the first time that SHetA2 down-regulates c-FLIP in human NSCLC cells; this down-regulation contributes to SHetA2-mediated induction of apoptosis and enhancement of TRAIL-induced apoptosis. The current results complement our previous finding that the DR5-mediated extrinsic apoptotic pathway plays a critical role in SHetA2-induced apoptosis in human NSCLC cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. H.A. Elrod in our laboratory for editing the article.

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