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CHOP Down-Regulates cFLIP$_L$ Expression by Promoting Ubiquitin/Proteasome-Mediated cFLIP$_L$ Degradation

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ABSTRACT

The transcription factor CHOP/GADD153 is induced during the unfolded protein response and is related to the induction of ER stress-mediated apoptosis. However, how CHOP is organized between the pro-survival and pro-apoptotic roles of ER stress remains largely undefined. In this study, we identified the apoptosis regulating protein suppressed by CHOP. We found that treatment of Caki cells with CHOP-inducing drugs including withaferin A, thapsigargin, brefeldin A, and silybin led to a strong reduction in cFLIP$_L$ protein levels together with a concomitant increase in the CHOP protein. Interestingly, Wit A down-regulated cFLIP$_L$ expression via both suppressing mRNA transcription and increasing cFLIPL protein instability. We also found that forced expression of CHOP dose-dependently led to a decrease of cFLIP$_L$ protein expression but did not alter cFLIP$_L$ mRNA levels. Additionally, we observed that siRNA-mediated CHOP silencing recovered the cFLIP$_L$ expression decreased by CHOP-inducing agents in Caki cells. Finally, we showed that CHOP facilitates ubiquitin/proteasome-mediated cFLIP$_L$ degradation, leading to down-regulation of cFLIP$_L$. Finally, cFLIP$_L$ over-expression reduced cell death induced by treatment with brefeldin A, thapsigargin, and silybin. Taken together, our results provide novel evidence that cFLIP$_L$ is a CHOP control target and that CHOP-induced down-regulation of cFLIP$_L$ is due to activation of the ubiquitin/proteasome pathways. J. Cell. Biochem. 113: 3692–3700, 2012.

KEY WORDS: CHOP; cFLIP$_L$; ER STRESS; UBIQUITIN/PROTEASOME

cFLIP$_L$ is catalytically inactive homolog of caspase-8, which inhibits caspase-8 recruitment and processing at the DISC [Krueger et al., 2001]. cFLIP$_L$ consists of the N-terminal death effector domains and a C-terminal caspase-like domain that does not possess enzymatic activity [Goltsev et al., 1997]. cFLIP$_L$ is first processed to a truncated p43 form by caspase 8, which inhibits complete processing of caspase-8 to its active subunits. cFLIP$_L$ over-expression protects against apoptosis mediated by the death receptors including FasL and TRAIL in several cancer cells in vitro [Irmler et al., 1997; Shu et al., 1997; Medema and Borst, 1999]. cFLIP$_L$ over-expression potently inhibits chemotherapeutic drug-induced cell death in solid tumor-derived cancer cell lines [Matta et al., 2002; Longley et al., 2006]. These reports suggest that cFLIP$_L$ is responsible for protecting against various anti-cancer drugs, inducing death receptor- or mitochondrial-mediated apoptosis. Previous reports have indicated that cFLIP protein levels are transcriptionally regulated through the nuclear factor-κB or c-fos pathways [Micheau et al., 2001; Ichikawa et al., 2006; Zhang et al., 2007] or by ubiquitin/proteasome- and caspase-mediated degradation [Fukazawa et al., 2001; Lee et al., 2006; Palacios et al., 2006].

CHOP (CCAAT-binding homologous protein; GADD 153) was originally identified as a gene induced in response to genotoxic stress and growth arrest signals [Fornace et al., 1988]. CHOP is also been involved in the critical cellular response for the transcriptional

H.-J. Noh and S.-J. Lee contributed equally to this work.

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control of endoplasmic reticulum (ER) stress-mediated apoptosis. Upon nuclear translocation, CHOP up-regulates transcription of proapoptotic factors and down-regulates antiapoptotic genes [Wang et al., 1998; Zinszner et al., 1998]. For example, (i) CHOP up-regulates expression of carbonic anhydrase VI, which is predicted to increase proton concentration and decrease intracellular pH; (ii) CHOP increases expression of a homolog of Tenm/Odz that might function in signaling at compartment boundaries; (iii) CHOP induces a homolog of the actin-binding proteins, which is implicated in changes in the actin cytoskeleton during apoptosis; and (iv) CHOP leads to enhanced expression of Death Receptor 5 (DR5), which activates the TRAIL-mediated apoptotic pathway. In contrast, CHOP overexpression decreases in the Bcl-2 protein and Bcl-2 over-expression blocks CHOP-induced apoptosis [McCullough et al., 2001]. Although several studies have correlated CHOP expression with cell death, the mechanistic link between CHOP and apoptosis remains poorly understood. To address this issue, we define what role, if any, CHOP plays in the regulation of cell death by modulating apoptosis regulating proteins.

In our study, we demonstrated that elevated CHOP expression by introducing a CHOP expression vector or treatment with CHOP inducing agents resulted in a decrease in $cFLIP_L$ expression in renal cancer cells. We also provide evidence that CHOP-induced down-regulation of $cFLIP_L$ is due to activation of the ubiquitin/proteasome pathways.

MATERIALS AND METHODS

CELLS AND MATERIALS

Caki cells, human renal cancer cell, were obtained from the American Type Culture Collection (Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Lipofectamine2000 was obtained from Life Technologies, Inc. (Rockville, MD). Anti-CHOP and anti-actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-cFLIP antibody was purchased from Alexis Corp. (San Diego, CA). Withaferin A (Wit A), Tapsigargin, brefeldin A, Cyclohexamide, MG132 were obtained from Sigma Chemical Co. (St. Louis, MO). Sylibin was purchased from Cayman Chemical (Ann Arbor, MI). Chop siRNA and Grp78 siRNA were obtained from Santa Cruz Biotechnology Inc.

WESTERN BLOTTING

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA). Specific proteins were detected with an ECL Western blotting kit according to the manufacturer's instructions.

PLASMIDS AND TRANSFECTION

The pcDNA 3.1-CHOP expression construct was kindly provided by Dr. TK Kwon (School of Medicine, Keimyung University, Korea). Briefly, cells were plated onto six-well plates at a density of 5×10^5 cells/well and grown overnight. The cells were transfected with the CHOP expression vector for 5 h using the Lipofectamine2000 method (Invitrogen, Calsbad, CA). After transfection, the cells were cultured in 10% FCS medium for 24 h. Caki/vector and Caki/$cFLIP_L$ overexpressed cells were kindly provided by Dr. TK Kwon.

SMALL INTERFERING RNAs (siRNAs)

The siRNA duplexes used were a pool of three target-specific 19–25 nt siRNAs designed to knockdown CHOP expression (cat no. sc-35437, Santa Cruz Biotechnology). The control siRNA duplex was green fluorescent protein (GFP) with the following sequence: AAG ACC CGC GCC GAG GUG AAG. Cells were transfected with siRNA oligonucleotides using Lipofectamine2000 according to the manufacturer's recommendations.

RNA ISOLATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was extracted from cells using TRIzol reagent (Life Technologies). cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The cDNA for c-$FLIP_L$, CHOP, and actin were amplified by PCR with specific primers. For CHOP, the sense primer was 5′-CAACTGCA-GAGATGGCAGCTGA-3′ and the antisense primer was 5′-CTG-ATGCTCCCAATTGTTCAT-3′ (corresponding to a 536-bp region of CHOP) were used. The sequences of the sense for c-$FLIP_L$ were 5′-CGGACTATAGAGTGCTGATGG-3′ and the antisense primer was 5′-GATTATCAGGCAGATTCCTAG-3′. PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide.

REAL-TIME RT-PCR

Equal amounts of cDNA were submitted to PCR, in the presence of SYBR green dye with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) and the ABI PRISM 6700 Real Time PCR Detection machine (Applied Biosystems, Foster City, CA). The samples quantified using LightCycler Relative Quantification Software version 3.3 (Roche Molecular Systems, Alameda, CA). In each system, the GAPDH housekeeping gene was amplified as a reference standard. $cFLIP_L$ and GAPDH were amplified using primers as follows. For human c-$FLIP_L$, the following primers were used: 5′-GCTGAAGTCATCCATCAGGT-3′ (sense) and 5′-CATACTGAGATG-CAAGAATT-3′ (antisense). For human GAPDH: 5′-GTCAACGGA-TTTGGTCGTATTG-3′ (sense) and 5′-TGGAGGGATCTCGCTCCTGG AAGA. T-3′ (antisense). Reactions were prepared in duplicate and heated to 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 20 s. Standard curves (cycle threshold values vs. template concentration) were prepared for each target gene and for the endogenous reference (GAPDH) in each sample.

IMMUNOPRECIPITATION TO DETECT UBIQUITINATED $cFLIP_L$

Caki cells were transfected with various plasmid DNAs using the Lipofectamine2000 transfection reagent following the

manufacturer's instructions. After 24 h, the cells were treated with MG132 for 4 h and were then lysed with a buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail for 1 h followed by centrifugation. Equal amounts of proteins were immunoprecipitated with anti-FLAG M2 coupled agarose beads (Sigma–Aldrich) for at least 4 h at 4°C. The beads were washed with cell lysis buffer 4 times and then subjected to Western blot to detect ubiquitinated $FLIP_L$ using anti-HA antibody (Abgent; San Diego, CA).

STATISTICAL ANALYSIS

Three or more separate experiments were performed. The statistical analysis was conducted with the paired Student's *t*-test. A *P*-value < 0.05 was considered a significant difference between the experimental and control groups.

RESULTS

CHOP-INDUCING AGENTS DOWN-REGULATED $cFLIP_L$ PROTEIN EXPRESSION

To identify the co-relationship between CHOP and $cFLIP_L$, we first investigated the effect of the CHOP-inducing drug, Wit A, on $cFLIP_L$ expression in Caki cells. As shown in Figure 1A, Wit A treatment induced CHOP expression in a dose-dependent manner in Caki cells. In contrast, treatments with Wit A dose-dependently down-regulated $cFLIP_L$ expression. Next, we examined whether other CHOP-inducing drugs such as silybin, thapsigargin (TG), and brefeldin A (BFA) could suppress the $cFLIP_L$ protein expression in Caki cells. Treatment of Caki cells with these CHOP-inducing drugs led to a reduction in $cFLIP_L$ protein levels together with a concomitant increase in the CHOP protein (Fig. 1B–D). These results suggest that CHOP might repress $cFLIP_L$ expression in Caki cells. To prove the major contribution of CHOP in down-regulating $cFLIP_L$ during the unfolded protein responses and linked ER-stress, we analyzed the expression of GRP78 in response to Wit A, silybin, TG, and BFA treatments. However, treatment with silybin, TG, and BFA slightly increased the expression of GRP78 protein, Grp78 protein level was almost constant in Wit A-treated cells (Fig. 1). Next, suppression of Grp78 expression by siRNA transfection failed to inhibit TG- or BFA-induced $cFLIP_L$ down-regulation, showing that GRP78 induction is not required for TG- or BFA-induced $cFLIP_L$ down-regulation (Supplement data #1). These data seem that ER stress is not commonly contributed to CHOP-triggered $cFLIP_L$ down-regulation.

WIT A DOWN-REGULATED $cFLIP_L$ PROTEIN LEVEL

As the $cFLIP_L$ protein was degraded by activated caspases, we examined whether the decreased $cFLIP_L$ protein expression was related to caspase activation. Pre-treatment with z-VAD-fmk, a potent pancaspase inhibitor, failed to recover $cFLIP_L$ protein to the

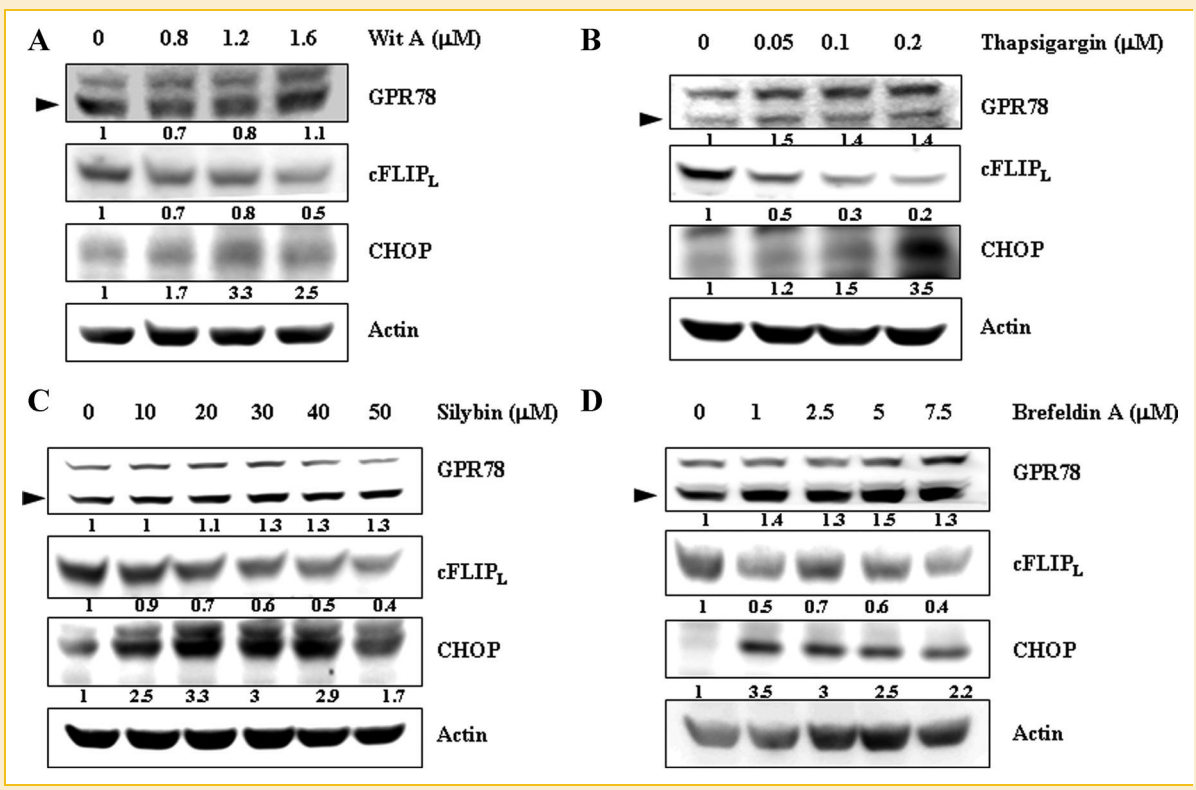

Fig. 1. CHOP-inducing agents down-regulate $cFLIP_L$ protein expression. Caki cells were treated with several doses of withaferin A (Wit A) (A), thapsigargin (B), silybin (C), and brefeldin A (D) for 24 h. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for $cFLIP_L$, CHOP, Grp78, and actin for normalization. Relative levels of each protein in drug-treated cells were expressed as a ratio of the densitometric value of each protein to that of Actin using the Bio-Rad Gel Doc System.

basal level, indicating that the decreased $cFLIP_L$ protein levels were mediated by caspase-independent pathways (Fig. 2A). To further elucidate the mechanism responsible for the changes in amounts of $cFLIP_L$ protein, we determined $cFLIP_L$ mRNA levels by RT-PCR. $cFLIP_L$ mRNA level remained constant in Caki cells throughout TG treatments at different doses (Fig. 2B), suggesting that TG-mediated degradation of total $cFLIP_L$ protein may be regulated post-transcriptionally. It has been reported that Wit A down-regulated $cFLIP_L$ mRNA expression via inhibiting NF-κB pathways [Lee et al., 2009]. In this study, treatment of Caki cells with Wit A down-regulated $cFLIP_L$ mRNA expression (Fig. 2B). $cFLIP_L$ mRNA level remained also constant in Caki cells throughout BFA, another CHOP-inducing drug, treatments at different doses (Supplement data #2).

CHOP DOWN-REGULATED $cFLIP_L$ EXPRESSION AT THE POST-TRANSCRIPTIONAL LEVEL

We employed Caki cells transiently transfected with CHOP expression vectors or empty vector to determine whether CHOP over-expression could suppress $cFLIP_L$ expression. Ectopic expression of CHOP dose-dependently decreased $cFLIP_L$ protein levels but did not alter $cFLIP_L$ mRNA levels in Caki cells (Fig. 3A,B), suggesting that CHOP down-regulated $cFLIP_L$ expression post-transcriptionally. Next, suppression of CHOP expression by siRNA transfection significantly inhibited Wit A- or TG-induced $cFLIP_L$ down-regulation, showing that CHOP induction is required for Wit A- or TG-induced $cFLIP_L$ down-regulation (Fig. 3C,D). As shown in Figure 3C,D, basal expression levels of the $cFLIP_L$ protein were higher in CHOP siRNA-transfected cells than those in control siRNA-transfected cells. Taken together, these results suggest that CHOP induction plays a critical role in both Wit A- and TG-induced $cFLIP_L$ down-regulation.

CHOP INCREASED $cFLIP_L$ PROTEIN INSTABILITY AND PROMOTED UBIQUITIN/PROTEASOME-MEDIATED $cFLIP_L$ DEGRADATION

We analyzed $cFLIP_L$ protein stability to further clarify the underlying mechanisms of the decreased $cFLIP_L$ protein level in Wit A- or TG-treated cells. Caki cells were treated with cyclohexamide (CHX) plus Wit A or TG for different time periods. We found that $cFLIP_L$ protein degradation was facilitated by Wit A or TG treatment (Fig. 4A,B), implying that Wit A or TG treatment reduced $cFLIP_L$ protein stability. Based on these results, we hypothesized that the $cFLIP_L$ protein would be degraded by the proteasome-dependent pathway. To confirm this hypothesis, we treated the cells with the proteasome inhibitor MG132. Caki cells were treated with Wit A plus CHX in the absence or presence of MG132 (0.25 μM). As expected, the decreased $cFLIP_L$ protein levels in Wit A- or TG-treated Caki cells almost recovered to basal levels following MG132 treatment (Fig. 5A,B). Immunoprecipitation/Western blotting revealed the highest levels of ubiquitinated $FLIP_L$

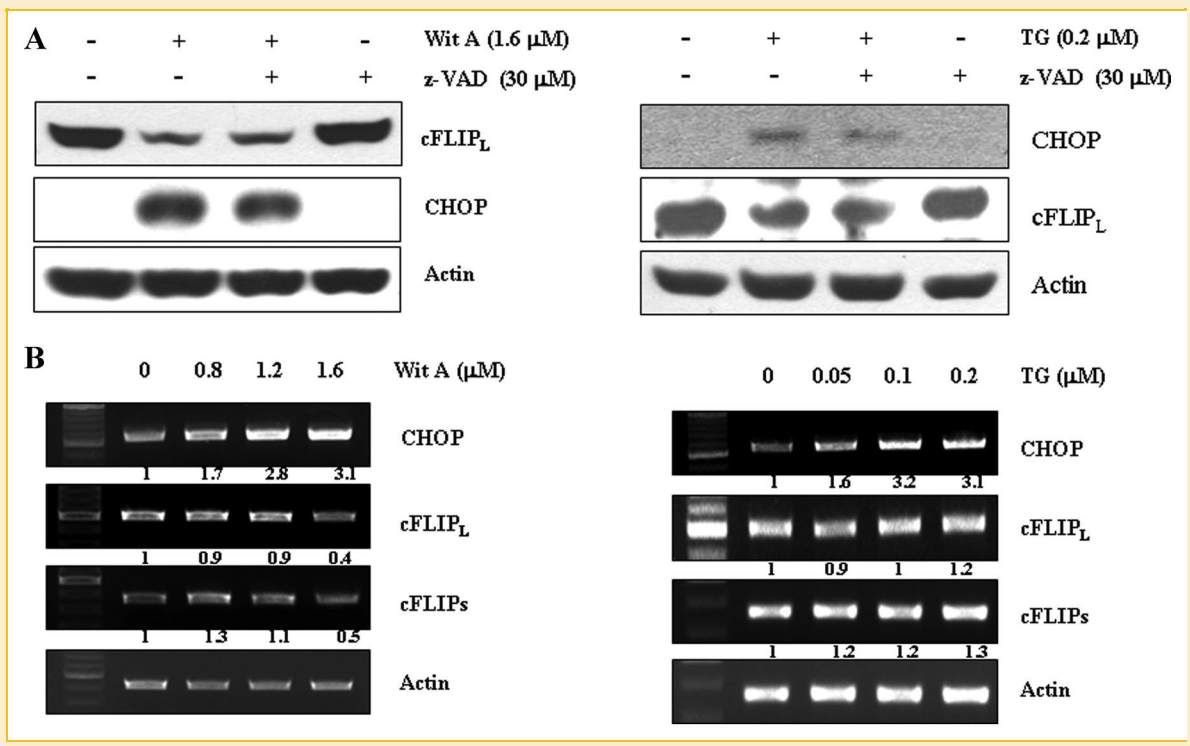

Fig. 2. Withaferin A (Wit A)-induced down-regulation of $cFLIP_L$ is regulated at the post-transcriptional level. A: Caki cells were treated with 1.6 μM withaferin A (Wit A) in the presence or absence of z-VAD (50 μM). Western blotting was performed using anti-$cFLIP_L$, anti-CHOP, and anti-actin antibodies to serve as control for protein loading. B: Caki cells were treated with the indicated concentrations of Wit A (left panel) or TG (right panel). Total RNA was isolated and RT-PCR analysis was performed. A representative experiment is shown; two additional experiments yielded similar results.

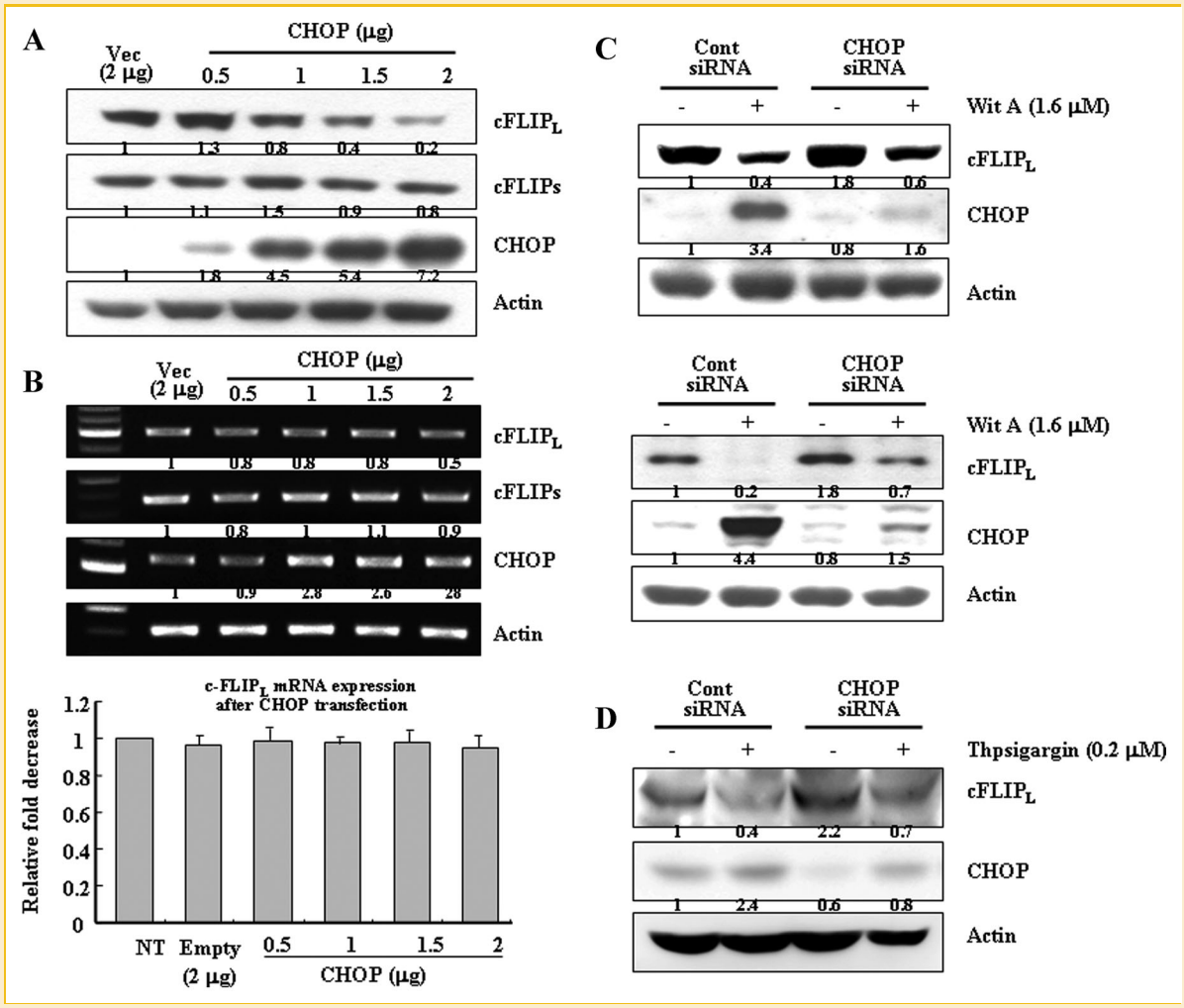

Fig. 3. CHOP down-regulates cFLIP$_L$ protein levels. A and B: CHOP overexpression dose-dependently decreased cFLIP$_L$ protein levels. Caki cells were transfected with CHOP expression vectors at the indicated concentrations for 24 h. After isolating cell lysates or total RNA, RT-PCR, or Western blotting were performed. Relative levels of each protein in drug-treated cells were expressed as a ratio of the densitometric value of each protein to that of actin using the Bio-Rad Gel Doc System. cFLIP$_L$ mRNA expression was detected by real-time RT-PCR (Bottom panel). Data are representative of three experiments. mRNA quantity of each gene was calculated from a standard curve made by amplifying different amounts of GAPDH first-strand cDNA, and normalized by considering the average of independent basal mRNA quantity of non-transfected cells as 1. C and D: CHOP down-regulation by siRNA recovers cFLIP$_L$ protein levels, which were lessened by withaferin A (Wit A) (C) or thapsigargin (D) treatment. Caki cells were transfected with control or CHOP siRNAs and then treated with 1.6 μM Wit A or 0.2 μM thapsigargin. After 24 h, cell extracts were prepared for Western blotting.

in cells transfected with the CHOP expression vector plus MG132 compared with cells transfected with an empty vector plus MG132 or MG132 alone (Fig. 5C), indicating that CHOP increases cFLIP$_L$ ubiquitination. Taken together, we conclude that CHOP facilitated ubiquitin/proteasome-mediated cFLIP$_L$ degradation, leading to cFLIP$_L$ down-regulation in Caki cells.

OVERREGULATION OF cFLIP$_L$ REDUCED CHOP INDUCING AGENT-MEDIATED APOPTOSIS

Next, we examined whether down-regulation of c-FLIP$_L$ by CHOP is critical to stimulate CHOP inducing agent-mediated apoptosis. Overexpression of c-FLIP$_L$ in Caki cells significantly attenuated TG-or-BFA-mediated apoptosis, whereas treatment with TG-or-BFA induced significant apoptosis in Caki/vector cells (Fig. 6A,B). Furthermore, CHOP expression levels by BFA, TG, or silybin treatment were slightly higher in Caki/vector cells than in Caki/cFLIP$_L$ cells. In addition, cFLIP$_L$ levels were down-regulated by these drugs (Fig. 6C). These data demonstrate that cFLIP$_L$ down-regulation contributed to CHOP-facilitated drug-induced apoptosis.

DISCUSSION

In the present study, we report that treatment with Wit A, TG, BFA, and silybin suppressed cFLIP$_L$ levels. cFLIP$_L$ protein production was repressed concomitant with CHOP expression. Using a CHOP overexpression system, we demonstrated that elevated CHOP expression resulted in decreased cFLIP$_L$ expression in renal cancer cells. In contrast, siRNA-mediated inhibition of CHOP restored cFLIP$_L$ expression that was prevented by CHOP-inducing agents.

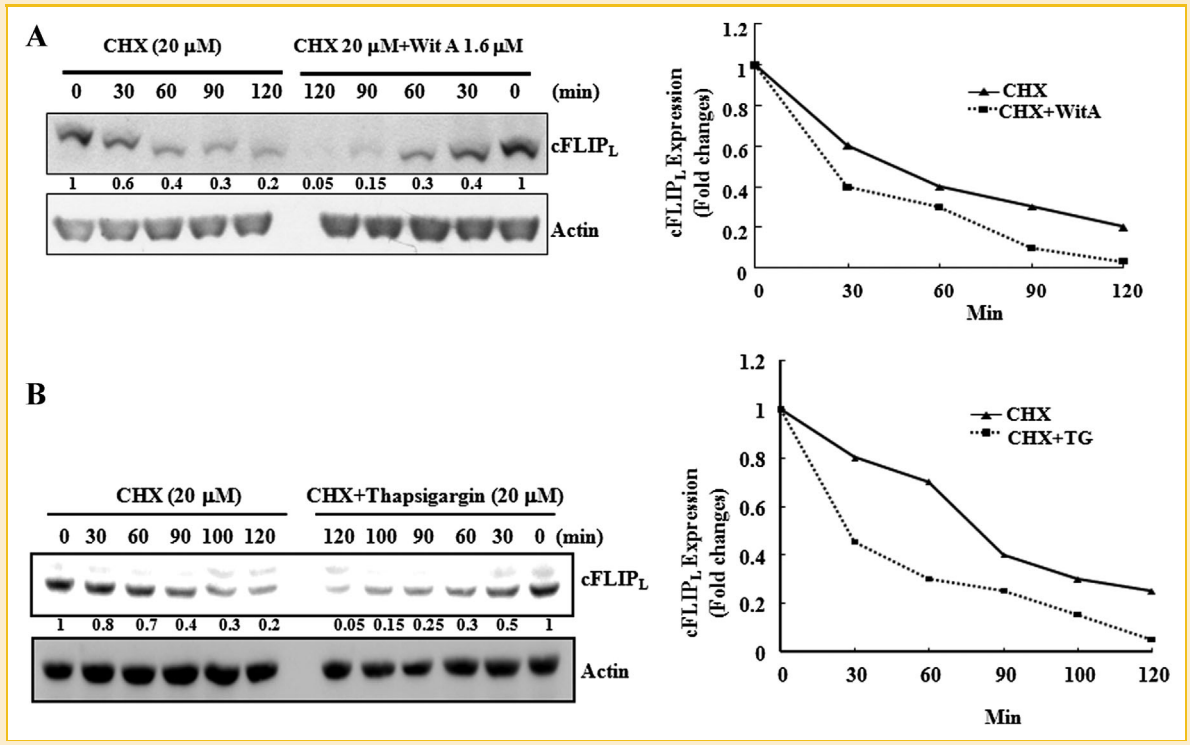

Fig. 4. CHOP increases cFLIP$_L$ instability. A and B: Caki cells were treated with 1.6 μM withaferin A (Wit A) (A) or 20 μM thapsigargin (B) in the presence or absence of cyclohexamide (CHX) for the indicated times. Western blotting was performed using anti-cFLIP$_L$ and anti-actin antibodies. Relative levels of each protein in Wit A- or thapsigargin-treated cells were expressed as a fold of the densitometric value of each protein to that of the control.

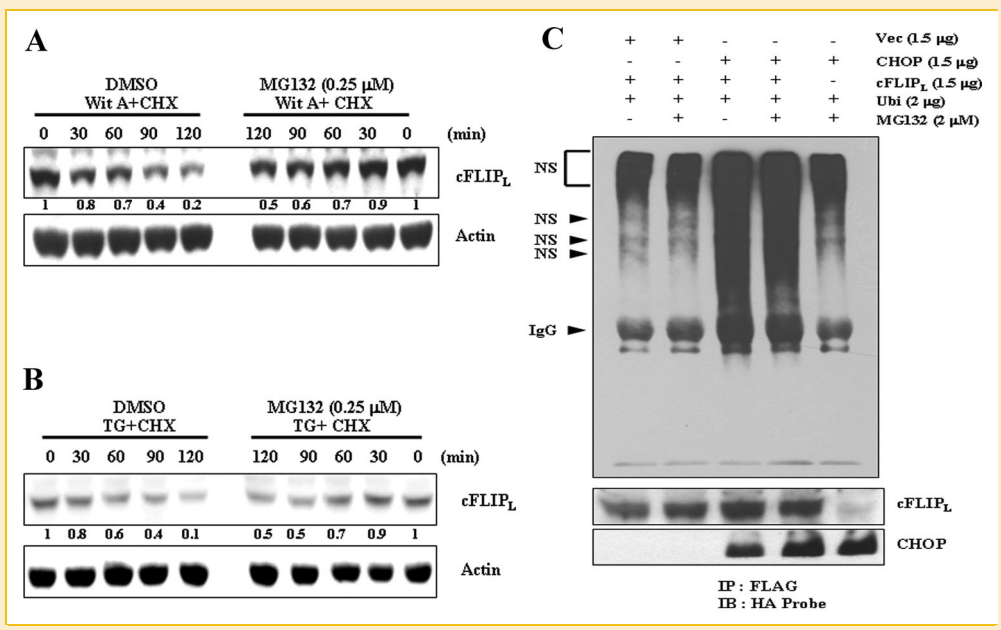

Fig. 5. CHOP promotes ubiquitin/proteasome-mediated cFLIP$_L$ degradation. A and B: Caki cells were treated with 1.6 μM withaferin A (Wit A) (A) or 0.2 μM thapsigargin (B) in the presence or absence of 0.25 μM MG132 for the indicated times. Western blotting was performed using the anti-cFLIP$_L$ and anti-actin antibodies. Relative levels of each protein in drug-treated cells were expressed as a ratio of the densitometric value of each protein to that of actin using the Bio-Rad Gel Doc System. C: Caki cells were transfected with various plasmids as indicated for 24 h. Then, the cells were treated with 2 μM MG132 for 4 h. Whole-cell protein lysates were prepared for immunoprecipitation using anti-Flag antibody followed by Western blotting using the anti-HA antibody to detect ubiquitinated FLIP$_L$ and anti-Flag antibody to detect ectopic FLIP$_L$. NS indicate non-specific band.

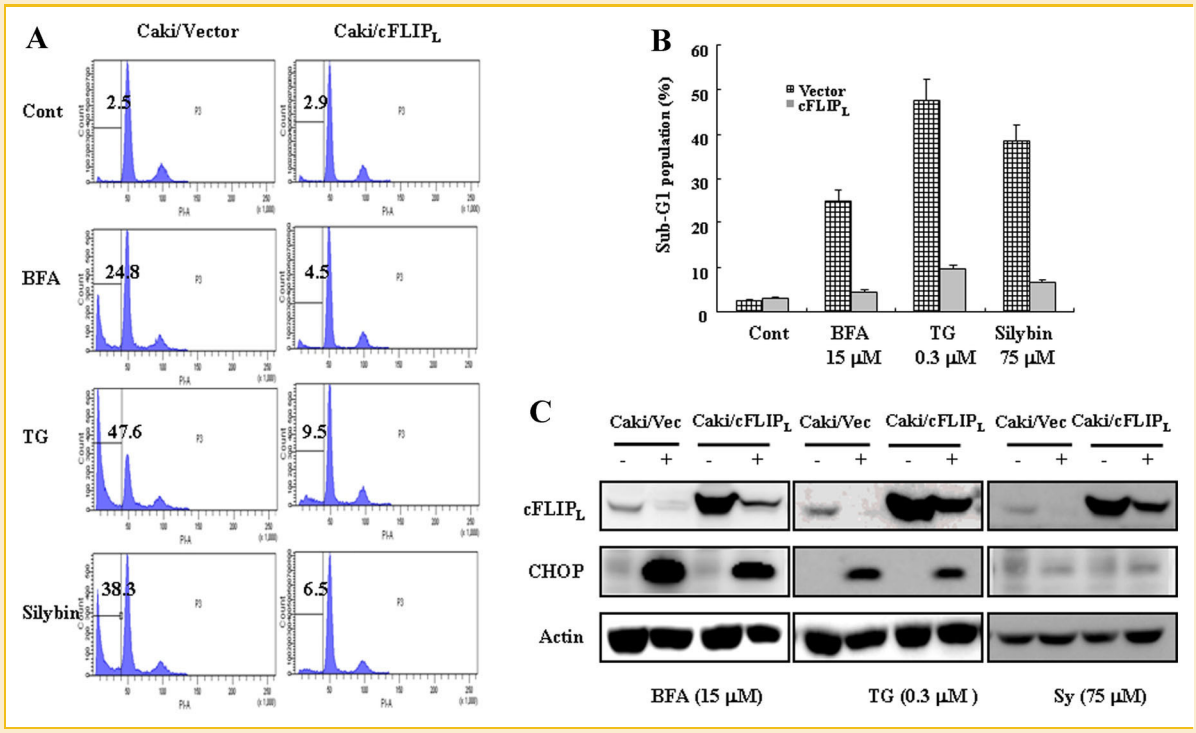

Fig. 6. CHOP-mediates down-regulation of cFLIP$_L$ contributes to CHOP-stimulated drug-induced apoptosis. A: Caki/vector and Caki/cFLIP$_L$ cells were treated for 24 h with brefeldin A (15 μM), thapsigargin (0.3 μM), or silybin (75 μM). Apoptosis was analyzed as a sub-G1 fraction by flow cytometry. B: Graph of sub-G1 population. Data shown are mean ± SD (n = 3). $^aP < 0.05$ compared to drug-treated pcDNA cells. C: Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for CHOP, and cFLIP$_L$.

Finally, we also provided evidence that these events were due to activation of the ubiquitin/proteasome pathways.

Numerous studies have demonstrated that CHOP is induced during the unfolded protein response, which leads to a critical cellular response for the transcriptional control of ER stress-induced apoptosis [Friedman, 1996; Matsumoto et al., 1996; Eymin et al., 1997; Halleck et al., 1997]. Although all reported types of ER stress induce CHOP expression, a mechanistic link between CHOP and apoptosis has remained largely undefined. Several reports have revealed that CHOP acts as transcriptional repressor. For example, CHOP prevented Bcl-2 expression via suppressing bcl-2 transcription by interacting with C/EBP, which mediates their proapoptotic functions [Matsumoto et al., 1996; McCullough et al., 2001]. TG-mediated CHOP induction suppresses PPARγ transcription by sequestering C/EBPβ and limiting the availability of C/EBPβ binding to the PPARγ promoter [Park et al., 2010]. In our study, we found that ectopic expression of c/EBP family such as c/EBPα, c/EBPβ, and c/EBPδ did not affect on cFLIP$_L$ protein expression (Supplement data #6). This result might rule out the possibility that CHOP down-regulated cFLIP$_L$ expression via suppressing cFLIP$_L$ transcription by interacting with C/EBP. It has also been reported that pharmacological induction of CHOP by tunicamycin suppresses p21 expression [Mihailidou et al., 2010]. In contrast, CHOP directly interacts with the DR5 promoter to increase DR5 transcription, which enhances sensitivity to TRAIL [Shiraishi et al., 2005; Yoshida et al., 2005]. Thus, CHOP up-regulates the transcription of proapoptotic factors and down-regulates antiapoptotic genes. However, it is not well known how CHOP regulates apoptosis regulating proteins at the post-transcriptional level.

A major post-transcriptional mechanism controlling protein levels in eukaryotic cells is ubiquitin-dependent proteasomal turnover. cFLIP expression is strongly regulated at the protein level by altered proteasomal degradation rates [Fukazawa et al., 2001]. In this study, we found that ectopic expression of CHOP dose-dependently led to a decrease in cFLIP$_L$ protein expression but did not alter cFLIP$_L$ mRNA levels in Caki cells, suggesting that CHOP down-regulated cFLIP$_L$ expression post-transcriptionally. Furthermore, we found that CHOP inducing-drugs caused increased cFLIP$_L$ instability and that forced expression of CHOP enhanced levels of ubiquitinated cFLIP$_L$. JNK activation by tumor necrosis factor (TNF)-α reduces cFLIP$_L$ stability via JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase Itch/AIP4, which promotes cFLIP ubiquitination and degradation through proteasomes [Chang et al., 2006]. It has been reported recently that α-tocopherol ether-linked acetic acid (α-TEA) down-regulation of cFLIP$_L$ protein levels is mediated by the JNK/CHOP/DR5 loop via a JNK-dependent Itch E3 ligase-mediated ubiquitination [Tiwary et al., 2010]. We found that CHOP down-regulated cFLIP$_L$ levels by facilitating ubiquitin/proteasome-mediated cFLIP$_L$ degradation. Our result and that of the previous paper suggested the possibility that CHOP might function as a repressor for cFLIP$_L$ due to cFLIP$_L$ protein depletion by inducing E3 ligase expression, which resulted in activating proteasomal pathway. However, in our system, we failed to detect a role for JNK in mediating CHOP-induced cFLIP$_L$ degradation (data

not shown). In addition, we found that treatment with CHOP inducing-drugs did not affect on Itch expression (Supplement data #5). Thus, Itch is unlikely to be involved in CHOP-mediated $cFLIP_L$ down-regulation in Caki cells. Although, very little information is available concerning the intracellular pathways involved in the effects of CHOP on $cFLIP_L$ expression, further studies are needed to show how CHOP down-regulates $cFLIP_L$ expression by activating the proteasomal pathway or searching for an apoptosis effector molecule that interacts with CHOP.

It has been reported that Wit A down-regulated $cFLIP_L$ mRNA expression via inhibiting NF-κB pathways [Lee et al., 2009]. In this study, we found that Wit A down-regulated $cFLIP_L$ expression via both suppressing mRNA transcription and increasing $cFLIP_L$ protein instability. Our result and that of the previous paper suggested that Wit A-mediated $cFLIP_L$ down-regulation were caused by suppressing mRNA transcription via inhibiting NF-κB pathways and increasing $cFLIP_L$ protein instability mediated by CHOP induction. On the other hand, TG or BFA inhibited $cFLIP_L$ protein expression through accelerating protein degradation. Thus, the increasing $cFLIP_L$ protein instability was induced by Wit A, TG, or BFA treatment in our system.

In summary, our results demonstrate that CHOP down-regulated $cFLIP_L$ levels by facilitating ubiquitin/proteasome-mediated $cFLIP_L$ degradation. This was evidenced by the attenuation of Wit A- or TG-mediated $cFLIP_L$ reduction using CHOP siRNA, by increased instability of $cFLIP_L$, which were detected in cells co-treated with CHX and Wit A or TG by Western blotting, and by increased levels of ubiquitinated $cFLIP_L$, which were detected in cells treated with MG132 and transfected with CHOP.

For the first time, we demonstrated that CHOP down-regulated $cFLIP_L$ at the protein level, which was mediated by proteasomal degradation. Our findings are significant in that they provide a better understanding of $cFLIP_L$ regulation and provide new insights into CHOP-mediated apoptotic signaling in human cancer cells. Additionally, CHOP-mediated apoptosis will provide a basis for new therapeutic approaches to diseases associated with ER stress.

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