

# Cellular FLICE-Inhibitory Protein Down-regulation Contributes to Celecoxib-Induced Apoptosis in Human Lung Cancer Cells

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## Abstract

The cyclooxygenase-2 (COX-2) inhibitor celecoxib is an approved drug in the clinic for colon cancer chemoprevention and has been tested for its chemopreventive and therapeutic efficacy in various clinical trials. Celecoxib induces apoptosis in a variety of human cancer cells including lung cancer cells. Our previous work has shown that celecoxib induces death receptor 5 expression, resulting in induction of apoptosis and enhancement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human lung cancer cells. In the current study, we further show that celecoxib down-regulated the expression of cellular FLICE-inhibitory protein (c-FLIP), a major negative regulator of the death receptor-mediated extrinsic apoptotic pathway, through a ubiquitin/proteasome-dependent mechanism independent of COX-2 in human lung cancer cells. Over-expression of c-FLIP, particularly FLIP<sub>L</sub>, inhibited not only celecoxib-induced apoptosis but also apoptosis induced by the combination of celecoxib and TRAIL. These results thus indicate that c-FLIP down-regulation also contributes to celecoxib-induced apoptosis and enhancement of TRAIL-induced apoptosis, which complements our previous finding that the extrinsic apoptotic pathway plays a critical role in celecoxib-induced apoptosis in human lung cancer cells. Collectively, we conclude that celecoxib induces apoptosis in human lung cancer cells through activation of the extrinsic apoptotic pathway, primarily by induction of death receptor 5 and down-regulation of c-FLIP. (Cancer Res 2006; 66(23): 11115-9)

## Introduction

Celecoxib, a marketed anti-inflammatory and anti-pain drug, is being tested in clinical trials for its chemopreventive and therapeutic effects against a broad spectrum of epithelial malignancies, including lung cancers, either as a single agent or in combination with other agents. The antitumor activity of celecoxib is thought to be associated with its ability to induce apoptosis in a variety of cancer cells (1). The molecular mechanism underlying celecoxib-mediated apoptosis remains largely uncharacterized, although it seems to be associated with inactivation of Akt, induction of endoplasmic reticulum stress involving up-regulation of CCAAT/enhancer-binding protein-homologous protein (CHOP)/GADD153 and increase in Ca<sup>2+</sup> levels, or down-regulation

of the antiapoptotic protein survivin (2). There are two major apoptotic pathways: the extrinsic death receptor-mediated pathway and the intrinsic mitochondria-mediated pathway, with truncated Bid protein accounting for the cross-talk between the two pathways (3). Our previous results have shown that celecoxib induces apoptosis in non-small-cell lung cancer cell lines primarily through the activation of the extrinsic death receptor pathway (4).

The cellular FLICE-inhibitory protein (c-FLIP) plays a key role in negatively regulating the extrinsic apoptotic pathway through inhibition of caspase-8 activation (5). c-FLIP has multiple splice variants, and two main forms have been well characterized: c-FLIP short form (c-FLIP<sub>s</sub>) and long form (c-FLIP<sub>L</sub>; ref. 5). It has been well documented that elevated c-FLIP expression protects cells from death receptor-mediated apoptosis, whereas down-regulation of c-FLIP by chemicals or small interfering RNA (siRNA) sensitizes cells to death receptor-mediated apoptosis (5). Moreover, over-expression of c-FLIP also protects cells from apoptosis induced by cancer therapeutic agents such as etoposide and cisplatin (6–8). In the present study, we show for the first time that celecoxib, in addition to up-regulating death receptor 5, down-regulates c-FLIP expression, which contributes to celecoxib-induced apoptosis in non-small-cell lung cancer cells. This further confirms and expands our previous finding that celecoxib induces apoptosis in non-small-cell lung cancer cell lines primarily through the activation of the extrinsic death receptor pathway (4).

## Materials and Methods

**Reagents.** Celecoxib, other nonsteroidal anti-inflammatory drugs, and antibodies against caspases were the same as previously described (4). 2,5-Dimethyl-celecoxib was synthesized as previously described (9). Human recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was purchased from PeproTech, Inc. (Rocky Hill, NJ). Mouse monoclonal anti-FLIP antibody (NF6) was purchased from Alexis Biochemicals (San Diego, CA). MG132 and SP600125 were purchased from Sigma Chemicals (St. Louis, MO) and Biomol (Plymouth Meeting, PA), respectively.

**Cell lines and cell culture.** The human non-small-cell lung cancer cell lines used in this study were purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described (4). H157-V and H157-AS cell lines, in which retroviral vector and antisense cyclooxygenase-2 (COX-2) were stably transfected, respectively (10), were kindly provided by Dr. S.M. Dubinett.

**Western blot analysis.** Preparation of whole-cell protein lysates and the procedures for the Western blotting were previously described (4).

**Immunoprecipitation.** A549-FLIP<sub>L</sub>-2 cells, which stably express FLIP<sub>L</sub>, were transfected with hemagglutinin-ubiquitin plasmid using FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) following the manufacturer's instruction. After 24 hours, the cells were treated with celecoxib or MG132 plus celecoxib for 4 hours and then were lysed for immunoprecipitation of Flag-FLIP<sub>L</sub> with Flag M2 monoclonal antibody (Sigma Chemicals) as previously described (11), followed by the detection of ubiquitinated FLIP<sub>L</sub> by Western blotting with antihemagglutinin antibody (Abgent, San Diego, CA).

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

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**Silencing of COX-2 expression with siRNA.** Stealth COX-2 siRNA that targets the sequence 5'-GCAGGCAGATGAAATACCAGTCTTT-3' and Stealth control siRNA (12) were synthesized by Invitrogen (Carlsbad, CA). The transfection of siRNA was conducted as previously described (12).

**Generation of lentiviral c-FLIP expression constructs and establishment of stable lines that overexpress c-FLIP.** *c-FLIP<sub>L</sub>* and *c-FLIP<sub>S</sub>* coding regions were amplified by PCR using plasmids containing full-length cDNAs of *FLIP<sub>L</sub>* and *FLIP<sub>S</sub>*, respectively, which were provided by Dr. J. Tschoop. The amplified fragments were then ligated into the pT-easy vector (Promega, Madison, WI) following the manufacturer's protocol as pT-easy-*FLIP<sub>L</sub>* and pT-easy-*FLIP<sub>S</sub>*, respectively, using the following primers: *FLIP<sub>L</sub>* sense, 5'-GACTAGTCCCGCCACCATGGATTACAAAGACGATGACG-3'; *FLIP<sub>L</sub>* antisense, 5'-CGGGCCCTTATGTGTAGGAGAGGATAAGTTTC-3'; *FLIP<sub>S</sub>* sense, 5'-GACTAGTCCCGCCACCATGTCTGTGAAGTCATCCATCAGG-3'; and *FLIP<sub>S</sub>* antisense, 5'-CGGGCCCTCACATGGAACAATTTTCAAG-3'. Both pLenti-DcR1 (a lentiviral vector harboring the *DcR1* gene, which was constructed using the pLenti6/V5 Directional TOPO Cloning kit purchased from Invitrogen) and pT-easy-*FLIP<sub>L</sub>* or pT-easy-*FLIP<sub>S</sub>* were cut with *SpeI* and *ApaI* restriction enzymes. The released fragment containing *c-FLIP<sub>L</sub>* or *c-FLIP<sub>S</sub>* gene was then cloned into the digested pLenti6/V5 vector and the resultant constructs were named pLenti-Flag-*FLIP<sub>L</sub>* and pLenti-*FLIP<sub>S</sub>*, respectively. In this study, we used pLenti-LacZ as a vector control, which was included in the pLenti6/V5 Directional TOPO Cloning kit.

Lentiviral production and titer determination were previously described (12). For infection, the viruses were added to the cells at a multiplicity of infection of 10 with 10 µg/mL polybrene. For transient expression, cells were infected and then subjected to initial selection with 50 µg/mL blasticidin beginning at 24 hours after infection. Five days later, the cells were used for the given experiments. For stable expression, cell clones were picked after a 2-week selection with 50 µg/mL blasticidin postinfection and screened for FLIP expression by Western blotting with c-FLIP antibody. The clones with the highest levels of FLIP expression were used in the experiment.

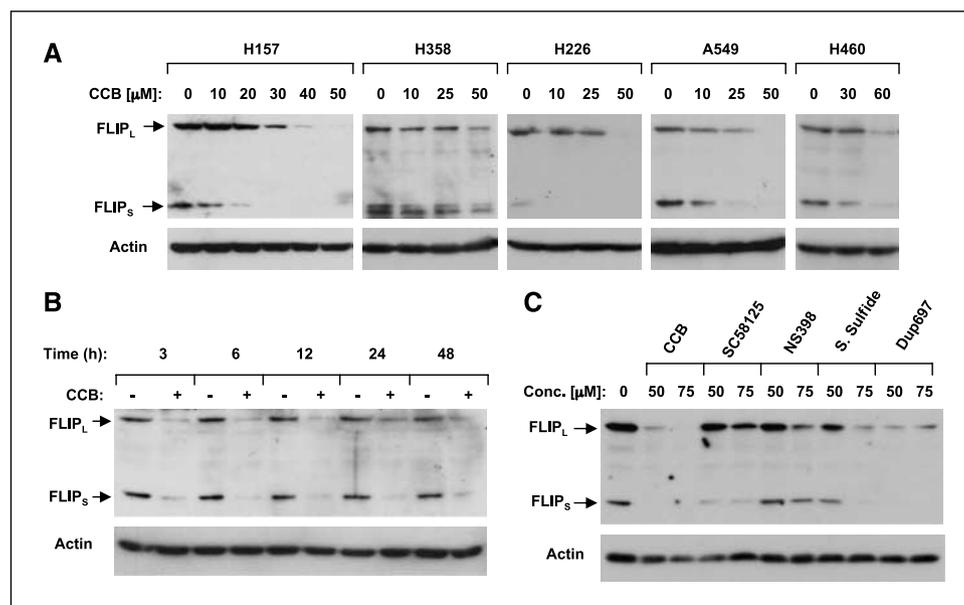
**Detection of apoptosis.** Apoptosis was evaluated by Annexin V staining using Annexin V-PE apoptosis detection kit purchased from BD Biosciences (San Jose, CA) following the manufacturer's instructions. We also detected caspase activation by Western blotting (as described above) as an additional indicator of apoptosis.

## Results and Discussion

Because c-FLIP levels are modulated by many cancer therapeutic agents, we were interested in determining whether celecoxib altered

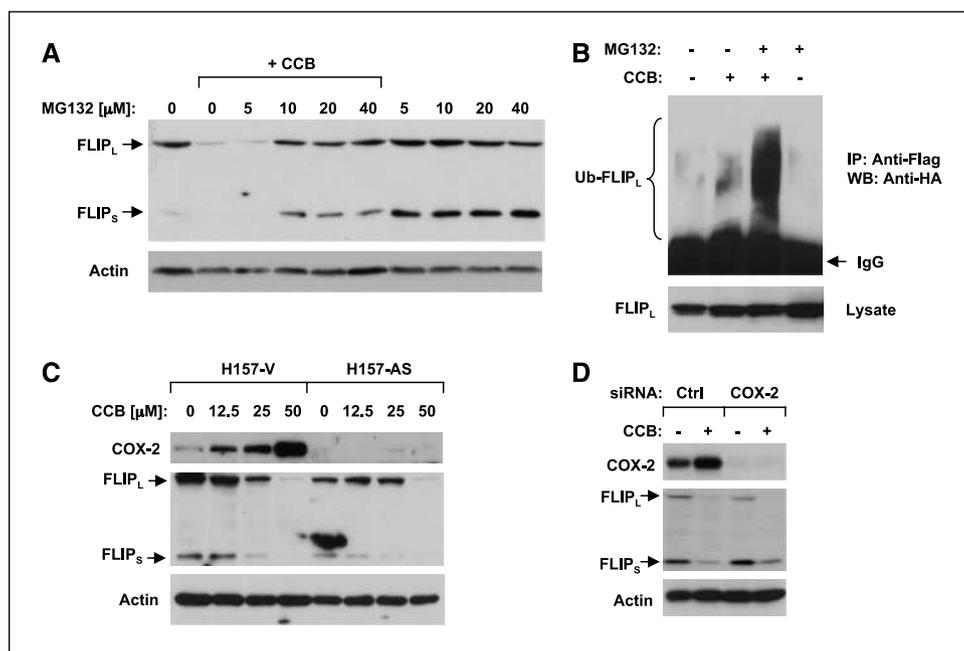
c-FLIP expression levels. Thus, we treated several non-small-cell lung cancer cell lines with increasing concentrations of celecoxib and then assessed c-FLIP levels. As presented in Fig. 1A, the expression levels of both *FLIP<sub>L</sub>* and *FLIP<sub>S</sub>* in these cell lines were reduced by celecoxib in a concentration-dependent manner after a 16-hour incubation. We noted that *FLIP<sub>S</sub>* levels were decreased after treatment with 10 µmol/L celecoxib, whereas *FLIP<sub>L</sub>* levels were reduced by relatively high concentrations of celecoxib (e.g., ≥25 µmol/L), suggesting that *FLIP<sub>S</sub>* is somewhat more sensitive to modulation by celecoxib than *FLIP<sub>L</sub>*. The down-regulation of both *FLIP<sub>L</sub>* and *FLIP<sub>S</sub>* occurred as early as 3 hours and was sustained up to 48 hours after celecoxib treatment (Fig. 1B). We noted that the reduction of c-FLIP, particularly *FLIP<sub>L</sub>*, at the late time points (e.g., 24 and 48 hours) was not as strong as that at the early time points (e.g., 3 and 6 hours). Nevertheless, these results clearly indicate that celecoxib down-regulates c-FLIP expression in human non-small-cell lung cancer cells, which represents an early event during celecoxib-induced apoptosis. We next determined whether other nonsteroidal anti-inflammatory drugs, including SC58125, NS-398, sulindac sulfide, and Dup697, down-regulated c-FLIP expression. As presented in Fig. 1C, these agents, particularly at 75 µmol/L, decreased the levels of both *FLIP<sub>L</sub>* and *FLIP<sub>S</sub>*, albeit with weaker activity than celecoxib, which, at 50 µmol/L, effectively reduced c-FLIP levels. Thus, we conclude that other COX-2 inhibitors down-regulate c-FLIP expression as well.

c-FLIP is known to be regulated by a ubiquitin-proteasome mechanism (13, 14), and certain cancer therapeutic agents stimulate down-regulation of c-FLIP expression through this mechanism (13). To determine whether celecoxib induces proteasome-mediated c-FLIP degradation, we examined the effects of celecoxib on c-FLIP expression in the absence and presence of the proteasome inhibitor MG132 in A549 cells. As shown in Fig. 2A, MG132 at concentrations of ≥10 µmol/L abrogated the ability of celecoxib to reduce both *FLIP<sub>L</sub>* and *FLIP<sub>S</sub>*, suggesting that celecoxib down-regulates c-FLIP through proteasome-mediated protein degradation. We also noted that MG132 alone did not increase the levels of *FLIP<sub>L</sub>* but strikingly increased the levels of *FLIP<sub>S</sub>*, suggesting that *FLIP<sub>S</sub>* is more prone to proteasome-mediated



**Figure 1.** Down-regulation of c-FLIP expression by celecoxib (A and B) and other nonsteroidal anti-inflammatory drugs (C) in human non-small-cell lung cancer cells. A, the indicated cell lines were treated with the given concentrations of celecoxib (CCB) for 16 hours. B, H157 cells were treated with 50 µmol/L celecoxib for the indicated times. C, H157 cells were treated with the given concentrations of the indicated nonsteroidal anti-inflammatory drugs for 7 hours. Whole-cell protein lysates were prepared from the aforementioned treatments for detection of the given proteins using Western blot analysis, with actin serving as a loading control.

**Figure 2.** Celecoxib down-regulates c-FLIP through ubiquitin/proteasome-mediated protein degradation (A and B) independent of COX-2 (C and D). A, A549 cells were pretreated with the indicated concentrations of MG132 for 30 minutes and then cotreated with 50  $\mu\text{mol/L}$  celecoxib (CCB) for another 4 hours. B, A549-FLIP<sub>L</sub>-2 cells were transfected with hemagglutinin-ubiquitin plasmid using FuGENE 6 transfection reagent for 24 hours. The cells were then pretreated with 20  $\mu\text{mol/L}$  MG132 for 30 minutes and then cotreated with 50  $\mu\text{mol/L}$  celecoxib for 4 hours. C, H157-V (vector-control) and H157-AS (antisense COX-2) paired cell lines were treated with the given concentrations of celecoxib for 16 hours. D, A549 cells were transfected with control (Ctrl) or COX-2 siRNA for 48 hours and then treated with 50  $\mu\text{mol/L}$  celecoxib for 16 hours. Whole-cell protein lysates were then prepared from the aforementioned treatments for detection of the given proteins by Western blot analysis (A, C, and D), with actin serving as the loading control, or immunoprecipitation (IP) with antihemagglutinin antibody followed by Western blotting (WB) for detection of ubiquitinated FLIP<sub>L</sub> (Ub-FLIP<sub>L</sub>; B).



degradation, which is consistent to the recent findings by Poukkula et al. (14). Moreover, we examined the effects of celecoxib on overall proteasome activity and c-FLIP ubiquitination. Celecoxib did not increase proteasome activity (data not shown). However, it increased the levels of ubiquitinated c-FLIP, particularly in the presence of MG132 (Fig. 2B), indicating that celecoxib increases c-FLIP ubiquitination. Collectively, we suggest that celecoxib down-regulates c-FLIP levels through a ubiquitin-proteasome mechanism.

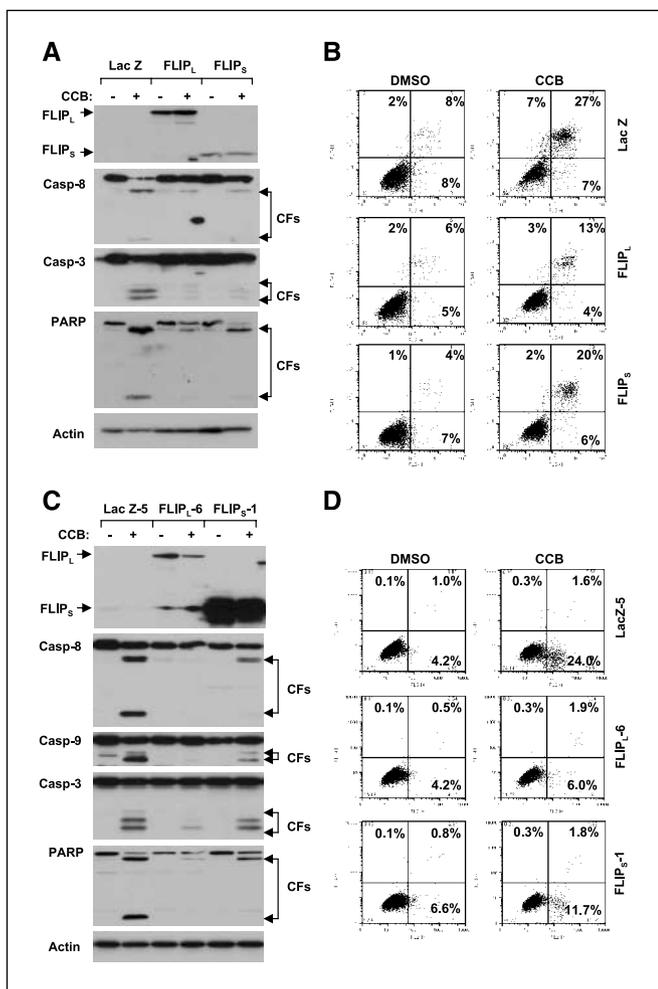
A recent study has shown that c-jun NH<sub>2</sub>-terminal kinase (JNK) activation modulates FLIP<sub>L</sub> degradation (15). We then determined whether celecoxib down-regulates c-FLIP through a JNK-dependent mechanism. Celecoxib indeed increased the levels of phosphorylated c-Jun (p-c-Jun), an indicator of JNK activation, and decreased the levels of both FLIP<sub>L</sub> and FLIP<sub>S</sub>. The JNK inhibitor SP600125 at concentrations up to 30  $\mu\text{mol/L}$  abrogated celecoxib-induced c-Jun phosphorylation, but failed to block down-regulation of either FLIP<sub>L</sub> or FLIP<sub>S</sub> by celecoxib (Supplementary Fig. S1). Considering these findings and the fact that JNK does not modulate FLIP<sub>S</sub> turnover (15) whereas celecoxib down-regulates the levels of both FLIP<sub>L</sub> and FLIP<sub>S</sub> (Fig. 1), we conclude that celecoxib induces a JNK-independent degradation of c-FLIP.

It is well known that celecoxib is a specific COX-2 inhibitor. However, many studies show that celecoxib induces apoptosis independent of COX-2 inhibitory activity (2). To determine whether celecoxib decreases c-FLIP levels through its COX-2 inhibitory activity, we compared the modulatory effects of celecoxib on c-FLIP between H157 cells stably transfected with a retroviral vector harboring antisense COX-2 (H157-AS) and vector control cells (H157-V). As shown in Fig. 2C, the basal levels of COX-2 in H157 cell were reduced compared with H157-V cells. Interestingly, we found that celecoxib, albeit a COX-2 inhibitor, actually increased COX-2 protein levels in a concentration-dependent manner in H157-V cells; this effect was abolished in H157-AS cells. Together, these results validate the cell system in which COX-2 expression is inhibited due to the expression of antisense COX-2. In both cell lines, celecoxib still effectively reduced levels of FLIP<sub>L</sub> and FLIP<sub>S</sub>,

indicating that celecoxib modulates c-FLIP levels regardless of the presence of COX-2. Some nonsteroidal anti-inflammatory drugs were reported to induce COX-2 expression (16, 17). However, the inducing effect of celecoxib on COX-2 expression has not been reported. Currently, it is unclear what the implications of COX-2 induction are in celecoxib-mediated anticancer activity.

Moreover, we used siRNA to knockdown COX-2 expression in A549 cells and then examined its effect on celecoxib-induced c-FLIP down-regulation. Transfection of COX-2 siRNA inhibited not only the basal levels of COX-2 but also celecoxib-mediated COX-2 induction. However, it did not alter the effect of celecoxib on reduction of c-FLIP (Fig. 2D), furthering the notion that celecoxib down-regulates c-FLIP expression levels irrespective of COX-2 expression. In addition, we examined the effect of 2,5-dimethyl-celecoxib, a derivative of celecoxib completely lacking COX-2 inhibitory activity (9), on c-FLIP expression and found that 2,5-dimethyl-celecoxib still decreased c-FLIP levels, albeit with more potency than celecoxib (Supplementary Fig. S2). This result again suggests that celecoxib reduces c-FLIP levels independent of its COX-2-inhibitory activity. Collectively, we conclude that celecoxib down-regulates c-FLIP expression independent of COX-2.

To determine the involvement of c-FLIP down-regulation in celecoxib-induced apoptosis, we used a lentiviral expression system to enforce expression of c-FLIP in non-small-cell lung cancer cell lines and then analyzed its effect on celecoxib-induced caspase activation and apoptosis. Taking advantage of a lentiviral expression system that can achieve both transient and stable gene expression, we first transiently expressed FLIP<sub>L</sub> or FLIP<sub>S</sub> in H1792 cells. By Western blotting, we could detect high levels of ectopic FLIP<sub>L</sub> or FLIP<sub>S</sub> (Fig. 3A). Celecoxib induced cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase in lacZ-infected control cells, evidenced by the decrease in the levels of their proforms and the increase in the amounts of their cleaved bands. However, these effects were inhibited or diminished in cells infected with c-FLIP, particularly FLIP<sub>L</sub> (Fig. 3A). Consistently, we detected 34% apoptotic cells in lacZ-infected cells, but only 17% and 26% apoptosis in cells infected with FLIP<sub>L</sub> and FLIP<sub>S</sub>,



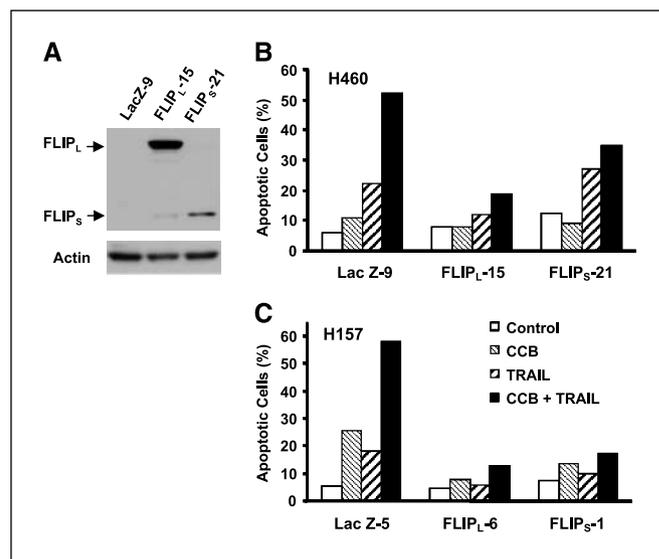
**Figure 3.** Both transient (A and B) and stable (C and D) overexpression of exogenous c-FLIP protect cells from celecoxib-induced caspase activation (A and C) and apoptosis (B and D) in human lung cancer cell lines. A and B, H1792 cells were infected with lentiviruses carrying lacZ, FLIP<sub>L</sub>, and FLIP<sub>S</sub>. After a brief selection with blasticidin for 5 days, the cells were treated with DMSO or 50 μmol/L celecoxib (CCB). Twenty-four hours later, the cells were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of caspase activation (A) or harvested for detection of apoptosis by Annexin V staining-flow cytometry (B). C and D, H157 stable transfectants expressing lacZ, FLIP<sub>L</sub>, and FLIP<sub>S</sub> were treated with DMSO or 50 μmol/L celecoxib. Twenty-four hours later, the cells were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for detection of caspase activation (C) or harvested for detection of apoptosis by Annexin V staining-flow cytometry (D). CFs, cleaved forms. Note that the endogenous levels of FLIP<sub>L</sub> and FLIP<sub>S</sub> proteins are not visible in these Western blots due to the short exposure times required to visualize the highly overexpressed exogenous forms of these proteins. In the Annexin V staining assay, the percent positive cells in the top right and bottom right quadrants were added to yield the total of apoptotic cells.

respectively, by Annexin V staining (Fig. 3B). Thus, these results show partial protective effects of c-FLIP, particularly FLIP<sub>L</sub>, on celecoxib-induced apoptosis.

Following this study, we further established stable cell lines from H157 cells that expressed ectopic FLIP<sub>L</sub> or FLIP<sub>S</sub> and examined their responses to celecoxib-induced apoptosis. By Western blot analysis, we detected the expression of ectopic FLIP<sub>L</sub> and FLIP<sub>S</sub> in H157-FLIP<sub>L</sub>-6 and H157-FLIP<sub>S</sub>-1 transfectants, respectively (Fig. 3C), indicating the successful expression of ectopic FLIP<sub>L</sub> or FLIP<sub>S</sub> in H157 cells. Celecoxib treatment strongly increased amounts of cleaved forms of caspase-8, caspase-9, caspase-3, and

poly(ADP-ribose) polymerase in the control H157-lacZ-5 cells; however, these effects were abrogated or diminished in either H157-FLIP<sub>L</sub>-6 or H157-FLIP<sub>S</sub>-1 cells. Accordingly, celecoxib caused 25.6% apoptosis in H157-lacZ-5 cells, but only 7.9% and 13.5% apoptosis in H157-FLIP<sub>L</sub>-6 and H157-FLIP<sub>S</sub>-1 transfectants, respectively (Fig. 3D). These results thus indicate that stable overexpression of c-FLIP, particularly, FLIP<sub>L</sub>, protects cells from celecoxib-induced apoptosis. Taken together, we conclude that down-regulation of c-FLIP contributes to celecoxib-induced apoptosis. Because c-FLIP is a key regulatory protein that inhibits death receptor-mediated apoptosis (5), these findings further support our notion that the extrinsic apoptotic pathway plays a important role in celecoxib-induced apoptosis, at least in human non-small-cell lung cancer cells as previously shown (4). In agreement with our finding, a recent study has shown that celecoxib activates the death receptor-mediated apoptosis in hepatocellular carcinoma cells (18).

We previously reported that celecoxib in combination with TRAIL augmented the induction of apoptosis in human non-small-cell lung cancer cells (4). To determine whether down-regulation of c-FLIP also contributes to synergy between celecoxib and TRAIL on apoptosis induction, we examined the effects of enforced overexpression of c-FLIP on celecoxib-mediated enhancement of TRAIL-induced apoptosis in two non-small-cell lung cancer cell lines. To this end, we further established H460 transfectants that overexpressed ectopic FLIP<sub>L</sub> and FLIP<sub>S</sub> (Fig. 4A), in addition to the aforementioned H157 cells. In the two tested cell lines (i.e., H157 and H460), the combination of celecoxib and TRAIL induced more than additive effects on induction of apoptosis compared with each single agent in lacZ-infected control cells evaluated by Annexin V staining; however, these effects were either abrogated or



**Figure 4.** Overexpression of exogenous c-FLIP protects cells from induction of apoptosis by the combination of celecoxib (CCB) and TRAIL in H460 (A and B) and H157 (C) human lung cancer cell lines. A and B, the indicated H460 stable transfectants were treated with DMSO, 50 μmol/L celecoxib alone, 3 ng/mL TRAIL alone, or celecoxib and TRAIL combination for 24 hours (B). The expression of ectopic FLIP<sub>L</sub> and FLIP<sub>S</sub> was detected by Western blot analysis (A). C, the indicated H157 stable transfectants expressing lacZ, FLIP<sub>L</sub>, and FLIP<sub>S</sub>, as shown in Fig. 3, were treated with DMSO, 50 μmol/L celecoxib alone, 4 ng/mL TRAIL alone, or celecoxib and TRAIL combination for 24 hours. At the end of the aforementioned treatments (B and C), the cells were harvested for detection of apoptosis by Annexin V staining-flow cytometry.

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inhibited in cells overexpressing c-FLIP, particularly FLIP<sub>L</sub> (Fig. 4B and C). For example, the combination of celecoxib and TRAIL caused ~58% apoptosis in H157-lacZ-5 cells, but only 13% apoptosis in H157-FLIP<sub>L</sub>-6 cells and 17% apoptosis in H157-FLIP<sub>S</sub>-1 cells (Fig. 4C). Thus, these results collectively show that down-regulation of c-FLIP also contributes to celecoxib-mediated enhancement of TRAIL-induced apoptosis.

We noted that the concentrations ( $\geq 10$   $\mu\text{mol/L}$ ) required for celecoxib to down-regulate c-FLIP are higher than clinically achievable peak plasma concentrations (3.2-5.6  $\mu\text{mol/L}$ ) of celecoxib in humans after oral administration of a single dose of 800 mg (19). Given that celecoxib has been developed and marketed mainly for treatment of arthritis and pain, but not primarily for anticancer purposes, it is conceivable that this drug might be suboptimal for inclusion in the therapy of advanced cancers, such as non-small-cell lung cancer cells. In this regard, it might be beneficial to consider streamlined celecoxib derivatives that are optimized for anticancer applications, and some promising efforts have indeed been made in this direction. Certain novel non-COX-2 inhibitory celecoxib derivatives show better activity than celecoxib in inducing apoptosis and inhibiting the growth of tumors (9, 20), further emphasizing the need to explore and understand the underlying molecular mechanisms by which these drugs exert their proapoptotic, antitumor potential. In this regard, our finding that celecoxib and its COX-2-inactive derivative 2,5-dimethyl-celecoxib down-regulate c-FLIP provides a novel aspect of this process and is important for understanding the molecular mechanisms by which these drugs induce apoptosis. We noted that 2,5-dimethyl-celecoxib, which is more potent than celecoxib in down-regulating c-FLIP, was also more potent than celecoxib in

decreasing cell survival and inducing apoptosis in human non-small-cell lung cancer cells,<sup>3</sup> suggesting that there is an inverse relationship between down-regulation of c-FLIP and induction of apoptosis by celecoxib and its derivatives. Therefore, we may consider modulation of c-FLIP as a screening tool for the development of novel celecoxib derivatives with better anticancer efficacy.

In conclusion, the present study shows for the first time that celecoxib down-regulates c-FLIP expression in human non-small-cell lung cancer cells; this down-regulation accounts for celecoxib-mediated induction of apoptosis and enhancement of TRAIL-induced apoptosis. These results complement our previous finding that the death receptor-mediated extrinsic apoptotic pathway plays a critical role in celecoxib-induced apoptosis in human lung cancer cells.

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<sup>3</sup> Our unpublished data.

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