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mTOR complex 2 is involved in regulation of Cbl-dependent c-FLIP degradation and sensitivity of TRAIL-induced apoptosis

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Abstract

The mammalian target of rapamycin (mTOR) positively regulates cell proliferation and survival through forming two complexes with raptor (mTOR complex 1; mTORC1) or rictor (mTOR complex 2; mTORC2). Compared with the mTORC1, relatively little is known about the biological functions of mTORC2. The current study focuses on addressing whether mTORC2 regulates apoptosis, particularly induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, TNFSF10). Using the mTOR kinase inhibitor, PP242, as a research tool, we found that it synergized with TRAIL to augment apoptosis of cancer cells. PP242 reduced the abundance of the short form of c-FLIP (FLIP\(_S\), CFLAR\(_S\)) and survivin (BIRC5). Enforced expression of ectopic FLIP\(_S\), but not survivin, attenuated augmented apoptosis induced by PP242 plus TRAIL. Thus, it is FLIP\(_S\) downregulation that contributes to synergistic induction of apoptosis by PP242 plus TRAIL. PP242 decreased FLIP\(_S\) stability, increased FLIP\(_S\) ubiquitination and facilitated FLIP\(_S\) degradation. Moreover, knockdown of the E3 ligase Cbl (CBL) abolished PP242-induced FLIP\(_S\) reduction. Thus, PP242 induces Cbl-dependent degradation of FLIP\(_S\), leading to FLIP\(_S\) downregulation. Consistently, knockdown of rictor or mTOR, but not raptor, mimicked PP242 in decreasing FLIP\(_S\) levels and sensitizing cells to TRAIL. Rictor knockdown decreased FLIP\(_S\) stability, whereas enforced expression of rictor stabilized FLIP\(_S\). Moreover, silencing of Cbl abrogated FLIP\(_S\) reduction induced by rictor knockdown. Collectively we conclude that it is mTORC2 inhibition that results in FLIP\(_S\) downregulation and subsequent sensitization of TRAIL-induced apoptosis. Our findings provide the first evidence showing that mTORC2 stabilizes FLIP\(_S\), hence connecting mTORC2 signaling to the regulation of death receptor-mediated apoptosis.

Keywords
mTOR; rictor; PP242; TRAIL; c-FLIP; apoptosis

Introduction
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called APO-2L) is a member of the tumor necrosis factor superfamily. Recombinant TRAIL is currently being tested in phase I clinical trials as a potential cancer therapeutic agent based on its unique ability to primarily trigger apoptosis in various types of cancer cells while sparing normal cells (1). However, cancer cells exhibit varied sensitivity to TRAIL and a substantial proportion of cancer cell lines are intrinsically insensitive to TRAIL (2). Thus, in these...
insensitive cancer cells, additional sensitization is needed to potentiate the killing effect of TRAIL.

TRAIL-initiated apoptosis involves the initial binding of TRAIL to death receptor 4 (DR4) or 5 (DR5) followed by oligomerization of the death receptor and formation of the death inducing-signaling complex (DISC). DISC assembly recruits the adaptor molecule FADD and pro-caspase-8, leading to autocleavage and activation of caspase-8, which further activates effector caspases (e.g., caspase-3) that eventually drive apoptotic death (1, 2). Cellular FLICE-inhibitory protein (c-FLIP) is a truncated form of caspase-8 that lacks enzymatic activity. It can also be recruited to the DISC, but suppresses apoptosis by blocking the activation of caspase-8 through competing with caspase-8 for binding to FADD (3). It has been well documented that elevated c-FLIP expression protects cells from death receptor–mediated apoptosis, whereas downregulation of c-FLIP by chemicals or small interfering RNA (siRNA) sensitizes cells to death receptor–mediated apoptosis (4). Therefore, c-FLIP acts as a key inhibitor of TRAIL/death receptor-induced apoptosis. c-FLIP has multiple isoforms; however, only two forms have been well characterized at the protein level in human cells: short form (FLIP\textsubscript{S}) and long form (FLIP\textsubscript{L}). Elevated levels of c-FLIP have been found in a number of different cancers and are often correlated with a poor prognosis in certain types of cancers (5). Both FLIP\textsubscript{L} and FLIP\textsubscript{S} are rapidly turned over proteins regulated by ubiquitination/proteasome-mediated degradation (6, 7). However, the mechanisms underlying c-FLIP degradation are largely unclear even though the E3 ubiquitin ligases, Itch and Cbl, have been suggested to be involved in c-FLIP degradation (8, 9).

The mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase related tightly to the family of the phosphatidylinositol 3-kinase-related kinases. mTOR exerts a variety of different biological functions primarily through forming two complexes characterized by the essential partner proteins raptor (mTOR complex 1; mTORC1) and rictor (mTOR complex 2; mTORC2) (10, 11). mTORC1 is deeply involved in many cellular processes critical for the maintenance of cell metabolism and growth generally via regulating cap-dependent protein translation initiation; this involves phosphorylation of two key initiation factors S6K and 4E-BP1 followed by facilitating formation of the translation initiation complex (10). While the mTORC1 is sensitive to rapamycin, the mTORC2 is generally thought to be rapamycin-insensitive (12). Moreover, relatively little is known about the biological functions of the mTORC2 other than its regulation of cytoskeleton and Akt-mediated cell survival (11). Several recent studies have suggested that mTORC2 exerts oncogenic functions in cancer development (13–15). Nonetheless, mTORC signaling is dysregulated in various types of human cancers and hence has emerged as an attractive cancer therapeutic target (16).

Rapamycin and its analogues (rapalogs) are conventional mTOR allosteric inhibitors with particular specificity to the mTORC1. Some rapalogs have shown encouraging results in improving overall survival among patients with metastatic renal cell carcinoma (17, 18) or advanced pancreatic neuroendocrine tumors (19). Consequently, these agents have been approved for clinical treatment of these indications. Despite this, the single agent activity of rapalogs in most other tumor types has been modest at best (20), likely due to the inaccessibility of the mTORC2 and their ability to activate AKT, MEK/ERK and other survival pathways (16).

The discovery of mTORC2 as an Akt S473 kinase has spurred efforts to identify novel mTOR inhibitors that inhibit both mTORC1 and mTORC2 activity. As a result, several ATP-competitive inhibitors of mTOR kinase including PP242 and INK128 have been developed and tested in clinical trials (21, 22). These inhibitors have been shown to more dramatically inhibit protein synthesis, suppress Akt phosphorylation and induce G1 arrest.
and/or apoptosis in some cancer cells than rapamycin (23–26). A robust in vivo anticancer activity of these inhibitors against certain types of cancers was also observed (24, 27, 28). Therefore, these mTOR kinase inhibitors not only represent novel potential therapeutic agents, but are also valuable research tools for understanding the biology of mTORCs.

A previous study showed that rapamycin sensitizes gliolastoma cells to TRAIL-induced apoptosis (29). However, we and others failed to show that rapalogs or mTOR knockdown can sensitize cancer cells including glioblastoma cells to TRAIL (30, 31). The current study focuses on determining whether mTOR kinase inhibitors enhance TRAIL-induced apoptosis and if so, defining the underlying mechanisms.

**Materials and Methods**

**Reagents and antibodies**

PP242 and INK128 were purchased from Active Biochem (Maplewood, NJ). Rapamycin was purchased from LC Laboratories (Woburn, MA). BEZ235 was provided by Novartis Pharmaceuticals Corporation (East Hanover, NJ). The soluble recombinant human TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ). The proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide (CHX) were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-FLIP antibody (NF6) was obtained from Alexis Biochemicals (San Diego, CA). Mouse monoclonal caspase-8, survivin and polyclonal caspase-9, PARP, p-Akt (S473), p-Akt (T308), Akt, p-GSK3α/β (S21/9), p-S6 (S235/236), S6, p-PRAS40 (T246) and PRAS40 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). p-FOXO3a (T32) and GSK3α/β antibodies were purchased from Upstate/EMD Millipore (Billerica, MA). Mouse monoclonal caspase-3 antibody was purchased from Imgenex (San Diego, CA). Rabbit polyclonal DR5 antibody was obtained from ProSci Inc. (Poway, CA). Mouse monoclonal DR4 antibody (B-N28) was purchased from Diaclone (Stamford, CT). Polyclonal rictor and raptor antibodies were purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Both polyclonal and monoclonal actin antibodies were purchased from Sigma Chemical Co.

**Cell lines and cell culture**

Human non-small cell lung carcinoma (NSCLC) cell lines used in this study were described in our previous work (32). Except for H157 and A549 cells, which were recently authenticated by Genetic DNA Laboratories, Inc. (Cincinnati, OH) through analyzing short tandem repeat DNA profile, other cell lines have not been authenticated. The stable cell lines, H157-Lac Z-5 vs. H157-FLIP<sup>S</sup>-1 and H157-Lac Z vs. H157-survivin, were described previously (33). H157-scramble, H157-shRaptor and H157-shRictor stable lines were described in our previous study (34). A549 stable lines with pLKO.1 (empty vector control), raptor small-hairpin RNA (shRaptor) or rictor shRNA (shRictor) were established as described previously (34). These cell lines were cultured in RPMI 1640 medium containing 5% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

**Cell survival and apoptosis assays**

Cells were seeded in 96-well cell culture plates and treated the next day with the given agents. The viable cell number was determined using sulforhodamine B (SRB) assay as described previously (35). Combination index (CI) for drug interaction (e.g., synergy) was calculated using the CompuSyn software (CompuSyn, Inc.; Paramus, NJ). Apoptosis was evaluated with annexin V-PE apoptosis detection kit purchased from BD Biosciences (San Jose, CA). The percent positive cells in the upper right and lower right quadrants represent the total apoptotic cell population. We also detected caspases and PARP cleavage by Western blot analysis as described below as additional indicators of apoptosis.
Western blot analysis
Preparation of whole-cell protein lysates and performance of the Western blot analysis were the same as described previously (32).

Immunoprecipitation (IP) for detection of ubiquitinated FLIPs
The given cells were co-transfected with HA-ubiquitin plus Flag-FLIPs plasmids using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) based on the manufacturer’s instructions. After 24 h, the cells were treated with PP242, PP242 plus MG132 or MG132 alone for 4 h. Cells were collected and lysed for IP using Flag M2 monoclonal antibody (Sigma) as previously described (33), followed by detection of ubiquitinated FLIPs with Western blot analysis using anti-HA antibody (Abgent, San Diego, CA).

Gene knockdown by small interfering RNA (siRNA) or short-hairpin RNA (shRNA)
Rictor #1 (5′-AAGCAGCCTTGAACTGGTAA-3′), rictor #2 (5′-AAACTTGGAAGAATCTCAGTAC-3′), raptor #1 (5′-AAGGCTAGTCTGTTTCGAAAT-3′), raptor #2 (5′-AAGGACAACGGCCAAAGTAC-3′) and Cbl (5′-AACCTCTTCAAGCCTG-3′) (36) siRNAs were synthesized by Qiagen (Valencia, CA). mTOR siRNA was purchased from Cell Signaling Technology, Inc (Cat. # 6381). Transfection of these siRNA duplexes was conducted in 6-well plates using the HiPerFect transfection reagent (Qiagen) following the manufacturer’s manual. The lentiviral Cbl shRNA set (Cat. # RHS4533), containing 7 different clones, was purchased from Thermo Scientific/Open Biosystems (Lafayette, CO). Viral preparation, cell infection and subsequent cell selection with an antibiotic were carried out following the manufacturer’s instructions and our previous description (34).

Detection of rictor and mTOR interaction
The given cells were transfected with myc-rictor expression plasmid (Addgene; Cambridge MA) and then lysed for detection of rictor and mTOR interaction with IP as described previously (34).

Adenoviral infection of cancer cells
Adenovirus harboring an empty vector (Ad-CMV) or a constitutively activated form of Akt (myristoylated Akt; Ad-myr-Akt) and cell infection were described previously (37).

Results
PP242 cooperates with TRAIL to enhance apoptosis
We first determined whether PP242 has the ability to enhance TRAIL-induced apoptosis. As presented in Fig. 1A, the combination of PP242 and TRAIL was more effective than either agent alone in decreasing the survival of the tested NSCLC cell lines including H157, H226 and H358 cells. The CIs for these combinations were < 1 (Fig. S1), indicating that the combination of PP242 and TRAIL synergistically decreases the survival of NSCLC cells. In agreement, the combination of PP242 (e.g., 1 μM) and TRAIL (e.g., 25 ng/ml) was also much more potent than either agent alone in increasing cleavage of caspase-8, caspase-9, caspase-3 and PARP in Western blot analysis (Fig. 1B) and in increasing the number of annexin V-positive cells as demonstrated by annexin V assay (Fig. 1C) in two representative cell lines, H157 and H226. Taking the H226 cell line as an example, we detected approximately 16% and 3% of apoptotic cells in cells treated with TRAIL and PP242, respectively, but 46% of apoptotic cells following exposure to the combination of PP242
and TRAIL (Fig. 1C), which is greater than the sum of apoptosis induced by both single agents, further indicating that the combination of PP242 and TRAIL exerts synergistic apoptosis-inducing activity. Taken together, we conclude that the combination of PP242 and TRAIL synergistically induces apoptosis in NSCLC cells.

**PP242 reduces the levels of FLIP<sub>S</sub> and survivin in NSCLC cells**

To reveal the mechanism by which PP242 enhances TRAIL-initiated apoptosis, we analyzed alterations of several key proteins including c-FLIP, DR4 and DR5 in the TRAIL/death receptor-mediated apoptotic pathway in cells exposed to PP242. We also looked at the levels of survivin in cells exposed to PP242, since this is an mTOR-regulated protein (38) and is involved in regulation of TRAIL-induced apoptosis (39). Within the indicated concentration range (0.25–4 μM), PP242 exerted a dose-dependent effect on reducing the levels of FLIP<sub>S</sub> and survivin, but not FLIP<sub>L</sub> (Figs. 2A and 2B). Even at 0.25 μM, PP242 effectively reduced the levels of FLIP<sub>S</sub> in H226 cells. Time course analysis showed that both FLIP<sub>S</sub> and survivin reduction occurred at about 4 h and was sustained up to 24 h in H157 cells post PP242 treatment (Fig. 2C). PP242 did not increase DR5 expression in either of the tested cell lines. We noted that it increased DR4 expression in H157 cells, but not in H226 cells, suggesting a cell line-dependent effect on DR4 expression.

**Enforced expression of ectopic FLIPs, but not survivin, abrogates synergistic induction of apoptosis by the PP242 and TRAIL combination**

To explore the roles of FLIP<sub>S</sub> and survivin downregulation in the augmentation of apoptosis by PP242 plus TRAIL, we compared the effects of PP242 plus TRAIL on apoptosis induction in H157 cell lines that express ectopic Lac Z (as a control), FLIP<sub>S</sub>, or survivin. The combination of PP242 and TRAIL, as demonstrated above, exerted augmented effects on induction of apoptosis and cleavage of caspase-8, caspase-3 and PARP in comparison with either single agent in H157-Lac Z control cells, but not in H157-FLIP<sub>S</sub> cells (Figs. 3A and 3B). In contrast, H157-Lac Z and H157-survivin cell lines were equally sensitive to PP242 plus TRAIL treatment evaluated both for caspase cleavage and for apoptosis induction (Figs. 3C and 3D). These data thus clearly indicate that downregulation of FLIP<sub>S</sub>, but not survivin, accounts for the enhancement of TRAIL-induced apoptosis by PP242.

**Inhibition of mTORC2, but not mTORC1, decreases FLIP<sub>S</sub> expression**

Given the important role of FLIP<sub>S</sub> downregulation in mediating the synergistic induction of apoptosis by PP242 and TRAIL, we focused our following studies on unraveling the mechanism by which PP242 reduces FLIP<sub>S</sub> levels. We asked whether PP242-mediated FLIP<sub>S</sub> downregulation is a consequence of mTORC2 inhibition. To this end, we evaluated the effects of PP242 on suppression of mTORC signaling pathways including Akt signaling. Two well-known events, phosphorylation of S6 and Akt, downstream of the mTORC1 and mTORC2, respectively, were effectively inhibited in cells exposed to PP242 in both dose- and time-dependent manners (Fig. 4A and supplementary Fig. S2A), indicating that PP242 indeed effectively inhibits both mTORC1 and mTORC2 signaling. Considering recent finding on dual effects of mTOR kinase inhibitors on Akt (40), we further looked at effects of PP242 on Akt signaling in our cell systems or conditions. After treatment with PP242 for 16 h, the levels of p-Akt (T308), p-GSK3 and p-FOXO3a were increased in H157 cells in a concentration-dependent manner accompanied with reduction of p-PRAS40 levels. In contrast, the levels of p-GSK3, p-FOXO3a and p-PRAS40 were concentration-dependently decreased in H226 cells (Fig. S2B). p-Akt (T308) levels in H226 cells were too low to be detected. Hence, PP242 exerts opposite effects on modulating the Akt signaling in these two cell lines.
Furthermore, we mimicked inhibition of mTORC signaling with mTOR siRNA, which is assumed to inhibit both mTORC1 and mTORC2, and then analyzed its effect on c-FLIP expression. Silencing of mTOR in both H157 and H226 cells reduced the levels of FLIP<sub>S</sub> as well as survivin (Fig. 4B), confirming that mTORC inhibition indeed causes the reduction of FLIP<sub>S</sub> and survivin. We noted that knockdown of mTOR also reduced FLIP<sub>L</sub> levels in H226 cells, but not in H157 cells (Fig. 4B).

We next addressed which of the mTORCs is involved in regulation of FLIP<sub>S</sub> levels. We knocked down rictor and raptor expression separately to mimic inhibition of the mTORC1 and mTORC2 and then examined their impact on FLIP<sub>S</sub> abundance. Our results demonstrated that knockdown of rictor, but not raptor, reduced the levels of FLIP<sub>S</sub> in both H157 and H226 cell lines (Figs. 4C and 4D). Survivin levels were not reduced in either of the rictor siRNA-transfected cell lines (Fig. 4D). We also examined c-FLIP and survivin levels in our established A549-shRaptor and A549-shRictor stable cell lines and found that FLIP<sub>S</sub> levels were reduced in A549-shRictor, but not in A549-shRaptor cells. In contrast, survivin was reduced in A549-shRaptor cells, but not in A549-shRictor cells (supplementary Fig. S3). In cells exposed to rapamycin, which is more selective for mTORC1 inhibition, we detected a dose-dependent reduction of survivin, but not of c-FLIP at all (Fig. 4E).

Moreover, two additional mTOR kinase inhibitors, INK128 and BEZ235, also reduced FLIP<sub>S</sub> levels (Figs. S4A and S4B) and synergized with TRAIL to kill cancer cells (Figs. S4C and S4D). These data together clearly indicate that inhibition of the mTORC2 downregulates FLIP<sub>S</sub> levels, whereas inhibition of the mTORC1 causes survivin reduction. Accordingly, we suggest that mTORC2 is involved in the positive regulation of FLIP<sub>S</sub> levels.

**Knockdown of rictor, but not raptor, sensitizes NSCLC cells to TRAIL-induced apoptosis**

If knockdown of rictor downregulates c-FLIP expression, we reasonably speculated that it will also enhance TRAIL-induced apoptosis. Thus, we compared the impact of raptor and rictor knockdown on cell responses to TRAIL. As presented in Fig. 5A, H157-shRictor cells, in which rictor expression is stably knocked down, were much more sensitive to TRAIL than H157-scramble control cells and H157-shRaptor cells, in which raptor expression is stably silenced, as determined by measuring cell number changes (Fig. 5A). Similarly both H157 and H226 cell lines transfected with rictor siRNA were much more sensitive than those transfected with either control or raptor siRNA to TRAIL (Figs. S5A and S5B). The annexin V assay revealed that H157-shRictor cells exhibited higher sensitivity than both H157-scramble and H157-shRaptor cells to TRAIL, evidenced by 46% annexin V-positive or apoptotic cells in H157-shRictor cells compared with 17% and 22% apoptotic cells in H157-scramble and H157-shRaptor cells, respectively (Fig. 5B). Consistently, TRAIL induced stronger cleavage of caspases (including caspase-8, -9 and -3) and PARP in H157-shRictor than in H157-scramble and H157-shRaptor cells although the cleavage of these proteins was slightly higher in H157-shRaptor than H157-scramble cells (Fig. 5C). Similar results were also generated in H226 cells transiently transfected with control, rictor or raptor siRNA. The strongest cleavage of caspase-8, caspase-3 and PARP was detected in rictor siRNA-transfected cells in comparison with those transfected with control or raptor siRNA (supplementary Fig. S5C). Taken together, these data convincingly demonstrate that inhibition of rictor or mTORC2 sensitizes cancer cells to TRAIL-induced apoptosis.

**PP242 and rictor knockdown facilitate FLIP<sub>S</sub> degradation, whereas enforced rictor expression stabilizes FLIP<sub>S</sub>**

We explored whether a proteasome degradation-mediated mechanism is involved in the reduction of FLIP<sub>S</sub> levels by PP242 and rictor knockdown, since c-FLIP is known to be regulated by an ubiquitin/proteasome-dependent mechanism (6, 7). We first determined...
whether PP242 alters FLIP<sub>S</sub> stability. To this end, CHX was added to cells 16 h after DMSO or PP242 treatment. The cells were then harvested at the indicated time post CHX for analysis of FLIP<sub>S</sub> degradation rates. Data in Fig. 6A revealed that the half-life of FLIP<sub>S</sub> in DMSO-treated cells was about 50 min; in contrast, its half-life in PP242-treated samples was < 20 min. Therefore, it is apparent that PP242 reduces FLIP<sub>S</sub> protein stability. Next, we determined whether PP242 induces FLIP<sub>S</sub> degradation. Thus, we treated H157 and H1299 cells with PP242 in the absence and presence of the proteasome inhibitor MG132 followed by detection of FLIP<sub>S</sub> with Western blot analysis. In the absence of MG132, PP242 decreased FLIP<sub>S</sub> levels as we demonstrated above. However, the presence of MG132 increased basal levels of FLIP<sub>S</sub> and prevented FLIP<sub>S</sub> from reduction by PP242 (Fig. 6B). This result suggests that PP242 induces FLIP<sub>S</sub> reduction through a proteasome-dependent mechanism. Furthermore, we determined whether PP242 increases FLIP<sub>S</sub> ubiquitination. As presented in Fig. 6C, the highest level of ubiquitinated FLIP<sub>S</sub> was detected in cells treated with PP242 plus MG132 compared with PP242 or MG132 alone, indicating that PP242 increases FLIP<sub>S</sub> ubiquitination. Our aforementioned results have demonstrated that the mTORC2 is involved in positively regulating FLIP<sub>S</sub> levels. Therefore, we wondered whether the mTORC2 does this by regulating FLIP<sub>S</sub> degradation. To test this hypothesis, we knocked down rictor in H226 cells and then conducted a CHX chase assay to analyze FLIP<sub>S</sub> protein stability. Our results showed that rictor knockdown (Fig. 6D) greatly decreased FLIP<sub>S</sub> stability, since the half-life of FLIP<sub>S</sub> was shortened from about 50 min in control siRNA-transfected H226 control cells to approximately 15 min in H226 cells transfected with rictor siRNA (Fig. 6E). Moreover, rictor knockdown also increased ubiquitination of FLIP<sub>S</sub> (Fig 6F). Complementarily, co-expression of FLIP<sub>S</sub> and rictor resulted in elevated levels of FLIP<sub>S</sub> (Fig. 6G). In agreement, enforced expression of rictor substantially slowed down FLIP<sub>S</sub> degradation rate (Fig. 6H), indicating that rictor expression stabilizes FLIP<sub>S</sub> protein. Under the tested condition, expression of the ectopic rictor interacted with endogenous mTOR since endogenous mTOR could be pulled down with anti-myc (rictor) antibody in the IP experiment and increased p-Akt (S473) levels (Fig. S6A), indicating that it does form and activate mTORC2. Besides, enforced expression of ectopic rictor protected cells from TRAIL-induced apoptosis because we detected less amounts of cleaved PARP and caspase-3 in cells transfected with rictor than in cells transfected with vector control (Fig. S6B). Collectively, these data robustly indicate that mTORC2 indeed negatively regulates FLIP<sub>S</sub> stability. Hence we concluded that PP242 facilitates ubiquitin/proteasome-mediated FLIP<sub>S</sub> degradation through inhibition of the mTORC2, leading to downregulation of FLIP<sub>S</sub>.

Cbl is involved in mTORC2-mediated regulation of FLIP<sub>S</sub> degradation

Given that Cbl has been suggested to mediate FLIP<sub>S</sub> degradation (9), we then asked whether Cbl is involved in mediating FLIP<sub>S</sub> degradation induced by mTORC2 inhibition. Knockdown of Cbl with Cbl siRNA elevated basal levels of FLIP<sub>S</sub> and also prevented PP242-induced FLIP<sub>S</sub> reduction (Fig. 7A). A similar result was also generated in H1299 cells in which Cbl expression was stably silenced with two different Cbl shRNAs (Fig. 7B). Silencing of rictor decreased FLIP<sub>S</sub> levels in control siRNA-transfected H157 and H226 cells, but failed to do so in Cbl siRNA-transfected cells (Fig. 7C). These data together clearly indicate that Cbl is the E3 ubiquitin ligase that mediates FLIP<sub>S</sub> degradation induced by mTORC2 inhibition.

Expression of an active form of Akt does not impair the ability of PP242 to reduce FLIP<sub>S</sub> levels

Considering that Akt is a major substrate of mTORC2 and has been suggested to regulate c-FLIP expression (41–43), we further asked whether mTORC2 inhibition-induced FLIP<sub>S</sub> degradation is secondary to Akt inhibition. Thus, we expressed a constitutively active form
of Akt (i.e., myr-Akt) through adenoviral infection and then examined its impact on PP2A-induced FLIP\textsubscript{S} reduction. As presented in supplementary Fig. S7, expression of myr-Akt substantially increased the levels of p-GKS3, a well known substrate of Akt, thus confirming Akt activation. However, PP242 was equally effective in reducing FLIP\textsubscript{S} levels in cells infected with either Ad-CMV or Ad-myr-Akt, indicating that enforced activation of Akt does not impair the ability of PP242 to induce FLIP\textsubscript{S} degradation.

Discussion

In this study, we have demonstrated that the newly developed mTOR kinase inhibitor, PP242, can augment TRAIL-induced apoptosis in NSCLC cells (Fig. 1). Although rapamycin was previously shown to sensitize gliolastoma cells to TRAIL-induced apoptosis (29), we and others failed to demonstrate that rapalogs or mTOR knockdown could sensitize cancer cells, including gliolastoma cells, to TRAIL (30, 31). In the current study, knockdown of rictor rather than raptor sensitized cancer cells to TRAIL-induced apoptosis (Figs. 5 and S5). Thus it is very likely that inhibition of mTORC2 signaling results in sensitization of TRAIL-induced apoptosis. To the best of our knowledge, this is the first report of the cooperative induction of apoptosis between an mTOR kinase inhibitor (e.g., PP242) or mTORC2 inhibition and TRAIL. Given that TRAIL is being tested as a cancer therapeutic agent in clinical trials (1), further study of the potential application of the PP242 and TRAIL combination in cancer therapy (e.g., NSCLC) is warranted.

DR4, DR5 and c-FLIP are key components in the regulation of TRAIL-induced apoptosis. DR4 and DR5 are receptors for TRAIL that initiate apoptosis upon binding with TRAIL and c-FLIP is the major inhibitor that suppresses TRAIL/death receptor-induced apoptosis (2). Modulation of the levels of these proteins (e.g., upregulation of DR4 and/or DR5 and/or downregulation of c-FLIP) generally results in sensitization of cancer cells to TRAIL-induced apoptosis (44). Survivin is a major inhibitor of the intrinsic apoptotic pathway and has also been suggested to regulate TRAIL-induced apoptosis (45). In this study, PP242 did not apparently increase DR5 expression, but reduced the levels of FLIP\textsubscript{S} and survivin in two tested NSCLC cell lines. However, enforced expression of ectopic FLIP\textsubscript{S}, but not survivin, conferred resistance of NSCLC cells to the combination of PP242 and TRAIL as evaluated with both the annexin V assay and caspase cleavage (Figs. 3). These results suggest that downregulation of FLIP\textsubscript{S}, but not survivin, plays a critical role in mediating synergistic induction of apoptosis by PP242 and TRAIL. We also noted that DR4 expression was increased in H157 cells but not in H226 cells upon PP242 treatment, suggesting a cell line-dependent modulation. Whether DR4 upregulation also contributes to cooperative induction of apoptosis by the PP242 and TRAIL combination in some cell lines (e.g., H157) needs further investigation.

As an mTOR kinase inhibitor, PP242 efficiently inhibited both mTORC1 and mTORC2 signaling in our cell systems, evidenced by effective suppression of the phosphorylation of both S6 and Akt (Fig. 4), two well-known downstream markers of the mTORC1 and mTORC2. A previous study using gliolastoma cells suggested that mTORC1 signaling (i.e., mTOR/S6K signaling) positively regulates FLIP\textsubscript{S} translation and that inhibition of this pathway downregulates FLIP\textsubscript{S} through suppressing its translation and enhances TRAIL-induced apoptosis (29). In our study, mTOR knockdown reduced the levels of both FLIP\textsubscript{S} and survivin. However, we detected reduced levels of survivin, but not c-FLIP, in our cell systems exposed to rapamycin (Fig. 4E). Moreover, disruption of the mTORC2 (by rictor knockdown), but not the mTORC1 (by raptor knockdown), reduced FLIP\textsubscript{S} levels (Figs. 4 and S3) although knockdown of raptor decreased survivin levels (Fig. S3). Consistently, two other mTOR kinase inhibitors, INK128 and BEZ237, also reduced FLIP\textsubscript{S} levels effectively (Fig. S4). Similar to our observations using PP242, knockdown of rictor, but not raptor,
augmented TRAIL-induced apoptosis (Figs. 5 and S5). These findings together clearly indicate that it is inhibition of the mTORC2 that positively regulates FLIP<sub>S</sub> levels, leading to enhancement of TRAIL-induced apoptosis. Our data also suggest that the mTORC1 positively regulates survivin expression, supporting the previous notion that mTORC1 signaling positively regulates survivin translation (38).

Our current results are consistent with our previous findings that inhibition of cap-dependent translation (e.g., with eIF4E siRNA or rapamycin) failed to decrease c-FLIP levels and augment TRAIL-induced apoptosis in other NSCLC cells (31). In this study, we found that knockdown of rictor did not decrease survivin levels although mTOR or raptor knockdown did reduce its expression (Figs. 4 and S3). Thus, it is likely that the mTORC2-mediated regulation of c-FLIP may involve a novel mechanism independent of cap-dependent translation. We explored what this mechanism might be. It is known that c-FLIP including FLIP<sub>L</sub> and FLIP<sub>S</sub> are rapidly turned over proteins subjected to regulation through ubiquitin/proteasome-mediated protein degradation (6–8). Some small molecules negatively regulate c-FLIP levels through this mechanism as we demonstrated previously (33, 46). In this study, we found that PP242 failed to decrease FLIP<sub>S</sub> levels in the presence of a proteasome inhibitor, increased c-FLIP ubiquitination and reduced the stability of FLIP<sub>S</sub> protein (Fig. 6). All of these results indicate that PP242 reduces FLIP<sub>S</sub> levels by facilitating its degradation through the ubiquitin/proteasome-dependent pathway. In agreement, knockdown of rictor substantially decreased FLIP<sub>S</sub> stability and increased FLIP<sub>S</sub> ubiquitination, whereas expression of ectopic rictor elevates FLIP<sub>S</sub> levels by enhancing its stability (Fig. 6). These data clearly indicate that modulation of the mTORC2 activity through genetic means also alters FLIP<sub>S</sub> levels by regulating its stability. Collectively, we suggest that mTORC2 signaling positively regulates FLIP<sub>S</sub> levels through negatively controlling its degradation. To the best of our knowledge, this is the first study suggesting mTORC2 regulation of c-FLIP degradation.

Currently, mechanisms underlying c-FLIP degradation have not been fully elucidated. Cbl is a pro-oncogene with E3 ubiquitin ligase activity and has been suggested to be involved in the development of TRAIL resistance in cancer cells through enhancing death receptor degradation and activating Akt (47–49). In our study, we found that inhibition of Cbl by knocking down its expression not only elevated basal levels of FLIP<sub>S</sub>, but also prevented FLIP<sub>S</sub> from reduction induced by either PP242 or rictor knockdown (Fig. 7). Thus, Cbl is clearly important for mTORC2 inhibition-induced FLIP<sub>S</sub> degradation. In other words, mTORC2 negatively controls or inhibits Cbl-dependent FLIP<sub>S</sub> degradation. Our ongoing studies seek to understand how the mTORC2 exerts this effect.

Akt is known to be a major substrate of mTORC2. Some studies have suggested that Akt is involved in positive regulation of c-FLIP expression, likely at transcriptional levels (41–43). However, Akt has also been shown to physically interact with and phosphorylate FLIP<sub>L</sub>, leading to its degradation (50). We recently have shown that the Akt inhibitor API-1 induces c-FLIP degradation independent of Akt inhibition (51). Moreover, celecoxib can induce c-FLIP degradation while increasing Akt phosphorylation (46). Nonetheless, the role of Akt in positive regulation of c-FLIP, particularly FLIP<sub>S</sub> degradation has not been clearly established. In our experimental conditions, PP242 activated Akt signaling in H157 cells while suppressing the Akt signaling in H226 cells evidenced by the findings that PP242 increased the levels of p-Akt (308), p-GSK3 and p-FOXO3a in H157 cells, but decreased them in H226 cells (Fig. S2B). Regardless, PP242 decreased FLIP<sub>S</sub> levels in both cell lines (Fig. 2). Moreover, enforced activation of Akt (e.g., expression of myr-Akt) did not impair the ability of PP242 to decrease FLIP<sub>S</sub> levels (Fig. S7). Hence we suggest that PP242- or mTORC2 inhibition-induced FLIP<sub>S</sub> degradation is unlikely to be the consequence of Akt inhibition or activation.
In summary, the current work has revealed a novel biological function of the mTORC2, i.e., regulation of Cbl-dependent FLIPs degradation and TRAIL-induced apoptosis, hence providing the first evidence connecting mTORC2 signaling to the regulation of death receptor-mediated apoptosis. Our findings also highlight a novel strategy to use mTOR kinase inhibitors in combination with TRAIL for cancer therapy. Thus, further investigation in this direction is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Fig. 1. PP242 and TRAIL combination synergistically decreases cell survival (A), enhances cleavage of caspases (B) and induces apoptosis (C)

A. The given cell lines were plated on 96-well cell culture plates and treated the next day with the given doses of PP242 alone, 50 ng/ml TRAIL alone or their combination. After 24 h, cell numbers were estimated using the SRB assay. Columns, means of four replicate determinations; Bars, ± SDs. B and C. The given cell lines were treated with 20 ng/ml TRAIL alone, 1 μM PP242 alone, and their combination. After 16 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis (B) and for annexin V staining of apoptotic cells (C). CF, cleaved form.
Fig. 2. PP242 reduces c-FLIPs and survivin levels
The indicated cell lines were treated with different concentrations of PP242 as indicated for 16 h (A and B) or with 1μM PP242 for the given times (C). The cells were then harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis.
Fig. 3. Enforced expression of ectopic FLIP<sub>S</sub> (A and B), but not surviving (C and D), abrogates synergistic induction of apoptosis (A and D) and cleavage of caspases (B and C) by the combination of PP242 and TRAIL.

The indicated H157-derived cell lines were treated with 1 μM PP242 alone, 25 ng/ml TRAIL alone and their combination. After 16 h, the cells were harvested for annexin V staining of apoptotic cells (A and D) and for preparation of whole-cell protein lysates and subsequent Western blot analysis (B and C). CF, cleaved form. Columns, means of duplicate determinations; Bars, ± SDs.
Fig. 4. Effects of PP242 on suppression of mTORC signaling (A), impact of disruption of mTORCs on FLIP$_S$ abundance (B–D) and modulation of c-FLIP and survivin levels by rapamycin (E).

A. The indicated cell lines were treated with different concentrations of PP242 as indicated for 16 h. B–D. The indicated cell lines were transfected with control, mTOR (B), raptor (C) or rictor (D) siRNAs for 48 h. E. The indicated cell lines were treated with different concentrations of rapamycin as indicated for 24 h. After the aforementioned treatments or transfections, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Ctrl, control.
Fig. 5. Knockdown of rictor, but not raptor, enhances the ability of TRAIL to decrease cell survival (A) and to induce apoptosis (B) and caspase activation (C).

A, The indicated shRNA-transfected stable cell lines were plated on 96-well cell culture plates and treated the next day with the given doses of TRAIL. After 24 h, cell numbers were estimated using the SRB assay. Data, means of four replicated determinations; Bars, ± SDs. B and C, The given cell lines were treated with 20 ng/ml TRAIL. After 16 h, the cells were harvested for annexin V staining of apoptotic cells (B) and for preparation of whole-cell protein lysates and subsequent Western blot analysis (C). CF, cleaved form.
Fig. 6. PP242 (A–C) or rictor knockdown (D–F) destabilizes FLIP$_S$ protein, whereas enforced expression of rictor stabilizes FLIP$_S$ (G and H).

A, PP242 reduces FLIP$_S$ stability. H157 cells were treated with DMSO or 1 μM PP242 for 16 h. The cells were then washed with PBS 3 times and refed with fresh medium containing 10 μg/ml cycloheximide (CHX). At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Protein levels were quantified with NIH Image J software (Bethesda, MA) and were normalized to actin. The results were plotted as the relative FLIP$_S$ levels compared to those at the time 0 of CHX treatment (bottom panel).

B, The proteasome inhibitor MG132 inhibits FLIP$_S$ reduction by PP242. H157 and H1299 cells were pre-treated with 20 μM MG132 for 30 minutes prior to the addition of 1 μM PP242. After co-treatment for an additional 4 h, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis.

C, PP242 increases FLIP$_S$ ubiquitination. H1299 cells were co-transfected with HA-ubiquitin and Flag-FLIP$_S$ plasmids using Lipofectamine 2000 reagent for 24 h. The cells were then pre-treated with 20 μM MG132 for 30 minutes and then co-treated with 1 μM PP242 for another 4 h. Whole-cell protein lysates were then prepared for IP using anti-Flag antibody followed by Western blotting (WB) using anti-HA antibody for detection of ubiquitinated FLIP$_S$ (Ub-FLIP$_S$) and anti-Flag antibody for detection of ectopic FLIP$_S$.

D and E, Rictor knockdown decreases FLIP$_S$ stability. H226 cells were transfected with
control (Ctrl) or rictor siRNA for 48 h. Similar to the assay in panel A, the cells were treated with CHX and harvested at the given time post CHX treatment for preparation of whole-cell protein lysates and subsequent Western blotting to detect FLIP<sub>S</sub>. Rictor knockdown efficiency was demonstrated with Western blotting in D. F, Rictor knockdown increases FLIP<sub>S</sub> ubiquitination. H157-scramble and H157-shRictor cells were co-transfected with HA-ubiquitin and Flag-FLIP<sub>S</sub> plasmids using Lipofectamine 2000 reagent for 48 h followed with exposure to 20 μM MG132 for additional 4 h. Ubiquitinated FLIP<sub>S</sub> (Ub-FLIP<sub>S</sub>) was then detected as described in C. G and H, HEK293 cells were co-transfected with FLIP<sub>S</sub> and rictor expression plasmids for 48 h. The cells were harvested for Western blotting to detect the indicated proteins (G) or exposed to CHX as we did in panel A for FLIP<sub>S</sub> stability assay (H).
Fig. 7. Knockdown of Cbl rescues FLIP$_S$ reduction induced by PP2A (A and B) or rictor siRNA (C).

A. The indicated cell lines were transfected with control (Ctrl) or Cbl siRNA for 48 h and then exposed to 1 μM PP242 for an additional 16 h. B. The indicated stable transfectants derived from H1299 cells were treated with 1 μM PP242 for 16 h. C. The indicated cell lines were co-transfected with control (Ctrl) plus rictor siRNAs or Cbl plus rictor siRNA for 48 h. After these treatments or transfections, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis.