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Research Article

TRAIL induces proliferation of human glioma cells by c-FLIP$_L$-mediated activation of ERK1/2

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Abstract. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in TRAIL-sensitive human malignant glioma cells. We show for the first time that TRAIL stimulates cell growth in TRAIL-resistant glioma cells. TRAIL-induced cell growth in resistant cells occurred through increased cell cycle progression as determined by flow cytometry and Western blot analysis of retinoblastoma protein phosphorylation. Western blot analysis of TRAIL-treated resistant cells revealed phosphorylation of ERK1/2 proteins and *in vitro* kinase analysis confirmed the activation of the ERK1/2 kinases. Inhibition of MEK1 eliminated both TRAIL-induced ERK1/2 activation and cell proliferation. In addition, siRNA inhibition of c-FLIP expression eliminates TRAIL-induced ERK1/2 activation and proliferation. Furthermore, overexpression of c-FLIP$_L$ potentiates TRAIL-induced ERK1/2 activation and proliferation of resistant glioma cells. Our results have shown for the first time that TRAIL-induced ERK1/2 activation and proliferation of TRAIL-resistant human glioma cells is dependent upon the expression of the long form of the caspase-8 inhibitor c-FLIP$_L$.

Keywords. TRAIL, apoptosis, proliferation, c-FLIP, ERK1/2, glioma.

Introduction

The tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamily of proteins is made up of a group of ligands and receptors that are directly coupled to signaling pathways for cell apoptosis, differentiation, survival, and proliferation [1]. TNF-related apoptosis-inducing ligand (TRAIL) [2, 3] has recently emerged as a novel anticancer agent due to its ability to trigger apoptosis in tumor cells while sparing normal cells *in vitro* and *in vivo* [4, 5]. TRAIL induced apoptosis is mediated by two death receptors, DR4/TRAIL-R1 [6–9] and DR5/TRAIL-R2 [9–14], which mediate apoptosis by activation of caspase-8 in a Fas-associated death domain (FADD)-dependent manner [10, 15–18]. Activated caspase-8 cleaves downstream effector caspase-3 [19], which in turn cleaves its substrates such as DNA fragmentation factor 45 (DFF45) [20] in the execution of programmed cell death. Due to its potential as an anti-cancer agent, much research has focused on elucidating the mechanisms of TRAIL-induced apoptosis and its modulation.

The ability to induce apoptosis is the best known characteristic of the death ligand and receptor families, but the capacity of the TNFR and Fas systems to function in aspects of cell proliferation, survival, and differentiation of both normal and neoplastic tissues is well documented [21]. Unlike the TNFR and Fas

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systems, however, little is known about the non-apoptotic functions of TRAIL and its receptors in normal cells and even less in neoplastic cells. This is despite data from the earliest phases of TRAIL research indicating that activation of TRAIL receptors can activate nuclear factor-κB (NF-κB) pathway for cell survival [9, 10]. Reports have been emerging that indicate that TRAIL has non-apoptotic functions such as the regulation of erythroid cell maturation and hematopoiesis [22–24], endothelial cell function and proliferation [25, 26], proliferation of synovial fibroblasts isolated from patients with rheumatoid arthritis [27], as well as proliferation of lymphoma and leukemic cells [28]. Additionally, it has recently been shown that TRAIL can promote tumor metastasis and invasion of TRAIL-resistant cholangiocarcinoma and pancreatic ductal adenocarcinoma cells *in vivo* [29, 30]. We now present the first report of TRAIL-induced proliferation in a solid tumor.

TRAIL may activate several pathways for cell survival and/or proliferation. TRAIL has been reported to activate protein kinase Akt pathway for endothelial cell proliferation [25]. TRAIL has long been known to activate the NF-κB pathway for cell survival [9, 10, 31, 32], metastasis and invasion [29, 30], and cytokine release [33, 34]. TRAIL has also been shown to activate extracellular signal-regulated kinase 1/2 (ERK1/2)/mitogen-activated protein kinase (MAPK) pathway for erythroid maturation [23], endothelial cell proliferation [25] and melanoma cell survival [35]. In this study we show that TRAIL stimulates cell proliferation through cell cycle progression dependent on activation of the ERK1/2 pathway in TRAIL-resistant human glioma cells. Furthermore, we show that in glioma cells resistant to TRAIL-induced apoptosis, TRAIL-induced proliferation and activation of the ERK1/2 pathway is mediated by the long form of the caspase-8 inhibitor cellular Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (c-FLIP$_L$). This is the first report that c-FLIP not only functions as an inhibitor of DR4/DR5-mediated apoptosis, but that it has an additional function in mediating death receptor-induced proliferation.

Methods and materials

Materials and antibodies. Recombinant non-tagged soluble human TRAIL (amino acids 114–281) was from PeproTech, Inc. (Rocky Hill, NJ). Mouse monoclonal antibodies used were anti-human caspase-8 from MBL (Nagoya, Japan), c-FLIP NF6 from Alexis Biochemicals (Lausanne, Switzerland), and anti-human DFF45 from StressGen Biotechnologies Inc. (Victoria, Canada). Rabbit polyclonal antibodies to human caspase-3 and ERK1/2 were obtained from StressGen, anti-human phospho-p44/42 MAPK (Thr202/Tyr204), retinoblastoma protein (pRb) and phospho-pRb (Ser780) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson IP Laboratories (West Grove, PA). Bradford protein assay quantification reagents were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA) and enhanced chemiluminescence reagents for Western blot analysis were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The MEK1/2 inhibitor, PD98059, was obtained from Cell Signaling Technology Inc. The caspase-8 inhibitor carbobenzoxy-isoleucyl-glutamyl-[O-methyl]threonyl-aspartyl-[O-methyl]-fluoromethyl-ketone (z-IETD-fmk), was purchased from R & D Systems Inc. (Minneapolis, MN). All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Human glioma cell lines. The human glioma cell lines LN-71, LN-443, LN-405, LN-992, T98G, U343MG, U118MG, and D247MG a gliosarcoma cell line were previously described (American Type Culture Collection, Bethesda, MD) [36]. The cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution, all purchased from Invitrogen (Carlsbad, CA).

Flow cytometric analysis. Cell surface expression of DR4 and DR5 was measured by flow cytometry. Briefly, 0.1 μg/ml phycoerythrin-conjugated anti-human DR4 and DR5 (mouse IgG1) or mouse IgG1 (negative control) were added to 1×10^6 cells in 200 μl immunofluorescence buffer (2% FBS and 0.02% sodium azide in PBS). Cells were incubated for 1 h in the dark at 4°C, washed with immunofluorescence buffer then suspended in 500 μl PBS. For all samples 1×10^4 cells were analyzed using a Becton Dickinson FACScan (Mountain View, CA), and the data were processed using CellQuest software (Becton Dickinson).

Cell cycle analysis. Cell cycle phase analysis was carried out by propidium iodide staining for DNA quantitation [37]. Briefly, cells grown in a subconfluent culture were either left untreated or treated as described in results. Cells were collected by trypsinization, washed with PBS, and fixed by incubation in 70% ethanol overnight at 4°C. Cells were washed in PBS again, and resuspended in PBS with 0.1% Triton

X-100, 20 μg/ml propidium iodide, and 200 μg/ml DNase-free RNase A and incubated for 30 min at 20°C in the dark. For all samples 2×10^4 cells were analyzed using a Becton Dickinson FACScan using CellQuest software, and the cell cycle data were processed using ModFit LT 2.0 (Verity Software House Inc.).

Western blot analysis. Cells grown in a subconfluent culture were lysed in lysis buffer [30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma)] on ice for 30 min, centrifuged at 18 000 *g* for 15 min at 4°C, and the supernatants were collected as the cell lysates. Equal amounts of protein from each sample was separated by SDS-PAGE on 10–15% gels and transferred to nitrocellulose membranes purchased from Bio-Rad.

Cell death, caspase cleavage and MAP kinase. For quantification of cell viability, cells were seeded into 96-well plates at 2×10^4 cells/well, incubated for 24 h at 37°C then treated with the indicated doses of TRAIL, PD98059, z-IETD-fmk alone, or in combination for the times indicated in results. Relative cell number was determined by crystal violet assay as previously described [38]. For caspase and DFF45 cleavage, subconfluent cells were treated with the indicated doses of TRAIL at 37°C for the indicated times, and the cells were lysed in lysis buffer and subjected to Western blotting. *In vitro* MAPK/ERK1/2 activity was analyzed using p44/p42 MAPK Assay Kit (Cell Signaling Technology Inc.) according to the manufacturer's protocols.

siRNA transient transfection. siRNA duplex targeting nucleotides 535–555 of the prodomain of both the long and short forms of c-FLIP (c-$FLIP_L$ and c-$FLIP_S$) were synthesized by Qiagen Inc. (Alameda, CA). The sense strand used is 5′-UCGGGGACUUGGCUGAACUUU-3′, and the antisense strand is 5′-AGUUCAGCCAAGUCCCCGACA-3′. Cells were seeded in 6-well plates at 2.5×10^5 cells per well. siRNAs were transfected into the cells using TransMessenger Transfection Reagent from Qiagen according to the manufacturer's protocol. Cells were allowed to grow for 36 h following transfection then treated as outlined in results. Subsequently, cell death, Western blot and cell cycle analyses were performed as outlined above.

Generation of stable cell lines. The mammalian expression vector pEFrsFLAG containing c-$FLIP_L$ was kindly provided by Dr. Peter H. Krammer [39]. Cells were seeded in 6-well plates at 4×10^5 cells per well. Plasmids were transfected into the cells using Lipofectamine 2000 from Invitrogen (Burlington, ON, Canada). Cells were allowed to grow 36 h, selected using 0.8 mg/ml G418 for 7 weeks, treated as outlined in the results, and analyses were performed as outlined above.

Results

TRAIL triggers proliferation in glioma cell lines resistant to TRAIL-induced apoptosis. The responses of eight human malignant glioma cell lines to TRAIL were examined by crystal violet assay (Fig. 1A). LN-71 and U343MG cell lines were sensitive to apoptosis induced by 100 ng/ml TRAIL treatment, while the T98G cell line was found to be slightly sensitive. Western blot analyses of the LN-71 and U343MG cell lines after TRAIL treatment was in accord with TRAIL killing by apoptotic mechanisms. This was evidenced by detection of the proteolytically active caspase-8 (p18), caspase-3 (p20 and p17), and DFF45 cleavage products (p25, p17, and p11) (Fig. 1C) with kinetics similar to those previously observed in cell lines sensitive to TRAIL-induced apoptosis [18]. In contrast, after 24 h of TRAIL treatment there was about a 20% increase in crystal violet staining of U118MG, D247MG, LN-443, LN-405, and LN-992 cell lines when compared to untreated controls (Fig. 1B). TRAIL does not induce apoptosis in U118MG, D247MG, or LN-992 cells as Western blots revealed no cleavage of caspase-8, caspase-3, or DFF45 (Fig. 1D). Flow cytometry revealed expression of DR4 and DR5 in all cell lines, with the exception of LN-71, which only expressed DR5 (see Table 1).

Table 1. Expression of TRAIL receptors on glioma cells.

Cell line	DR4/TRAIL-R1	DR5/TRAIL-R2
LN-71	–	++
T98G	+	++
U118MG	+	++
U343MG	+	++
D247MG	++	++
LN-443	++	++
LN-405	+	++
LN-992	+	+

–, Not detectable; +, weakly expressed; ++, strongly expressed.

To confirm TRAIL-stimulated cell proliferation in the resistant cell lines, the effect of TRAIL on the cell cycle of U118MG, D247MG, and LN-992 cell lines was examined in two ways. First, an analysis of cell cycle

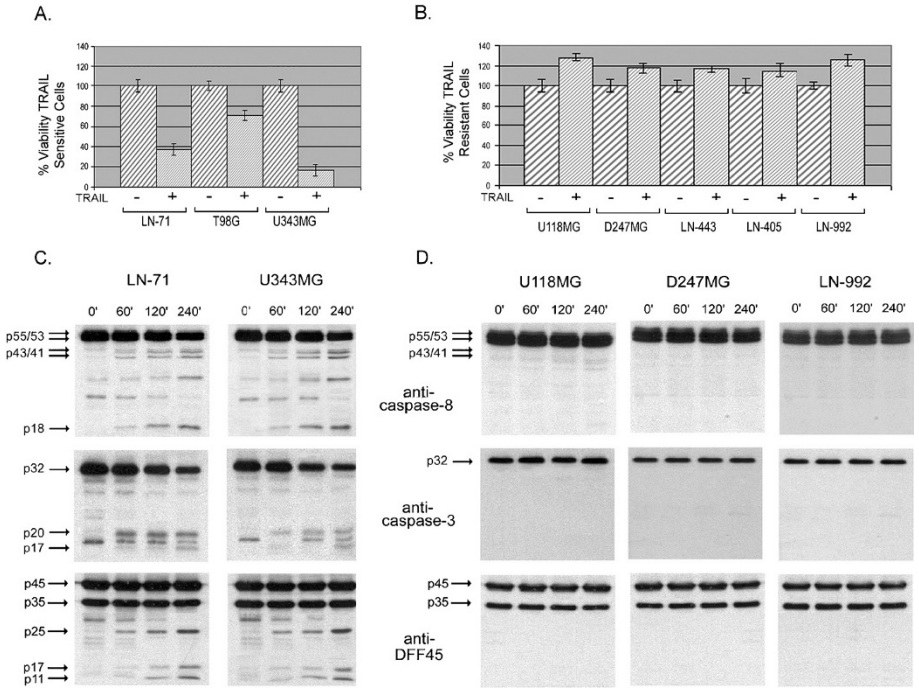

Figure 1. TRAIL-induced cell death and growth in human glioma cell lines. TRAIL-sensitive (*A*) and -resistant (*B*) glioma cell lines were seeded in 96-well plates at 2×10^4 cells per well, untreated or treated with TRAIL (+) for 24 h and subjected to crystal violet assay for cell viability. Each value represents the mean ± SD of eight samples. Untreated controls are represented as 100%. The sensitive (*C*) and resistant cell lines (*D*) were treated with TRAIL for the times as indicated on top of the panels and subjected to Western blot analysis for the cleavage of caspase-8, caspase-3 and DFF45.

phase distributions before and after 24 h of TRAIL treatment was performed by flow cytometry of propidium iodide-stained cells. The cell cycle analysis revealed an approximately 10% reduction in the number of cells in the G_1 phase of the cell cycle, and an increase in the number of cells in S phase and G_2 phases of the cell cycle of all three cell lines after 24-h treatment with 33 ng/ml TRAIL (Fig. 2A). This phenomenon was only observed in high-density cultures of 90–95% confluency (data not shown). Second, we examined phosphorylation of pRb at Ser780 in the three cell lines. pRb is ultimately responsible for inhibition of cell cycle progression from G_1 to S phase, inhibition that is relieved by its phosphorylation by cyclin/cdk complexes [40]. Western blots detected a dramatic increase in Ser780 phosphorylation after 24 h of TRAIL treatment in all three cell lines analyzed (Fig. 2B). These results suggest two functional outcomes of TRAIL treatment in human glioma cell lines. First, TRAIL induces caspase cascade for apoptosis in sensitive glioma cell lines. Second, TRAIL induces proliferation in TRAIL-resistant glioma cell lines. Cell cycle phase and phospho-pRb analyses suggest that TRAIL induces proliferation by accelerating the transition from G_1 to S phase of the cell cycle.

TRAIL activates the ERK1/2 pathway in resistant glioma cell lines. To explore the molecular mechanisms in TRAIL-induced cell growth, we examined the resistant glioma cell lines to determine if TRAIL-induced cell cycle progression occurs through activation of the ERK1/2 pathway or the Akt pathway. ERK1/2 kinases are activated through dual phosphorylation by MAP kinase/ERK kinase (MEK) [41]. First, we treated U118MG, D247MG, and LN-992 cell lines with 33 ng/ml TRAIL and Western blots revealed robust phosphorylation of ERK1/2 p44/42 proteins in all three cell lines starting at 5 min and peaking at 10–30 min after TRAIL treatment (Fig. 3A). The phosphorylation continued as long as the cells were treated with TRAIL, suggesting that TRAIL triggers a sustained activation of the ERK1/2 pathway. Next, we treated the resistant cell lines with PD98059, a cell permeable pharmacological inhibitor of the MEK1 [42], to determine if the MEK1 inhibitor prevents ERK1/2 phosphorylation. Indeed, treatment of the resistant cell lines with 20 μM PD98059 effectively inhibited TRAIL-induced phosphorylation of ERK1/2 p44/42 proteins in the cells (Fig. 3B). Furthermore, we examined the ERK1/2 kinase activity to assess whether TRAIL-induced phosphorylation of the ERK1/2 protein results in the functional activation of the kinases. Extracts from TRAIL-treated human glioma cell lines were capable of phosphorylating Elk-1, an immediate downstream substrate of the ERK1/2 kinases [43], whereas the treatment of the cells with PD98059 inhibited the phosphorylation of Elk-1 (Fig. 3C). We also examined activation of the Akt pathway by TRAIL in TRAIL-resistant glioma cell lines. Akt was found to be constitutively activated in U118MG, D247MG, and LN-992 cells, and activation of Akt by TRAIL treatment beyond baseline was not observed, and this

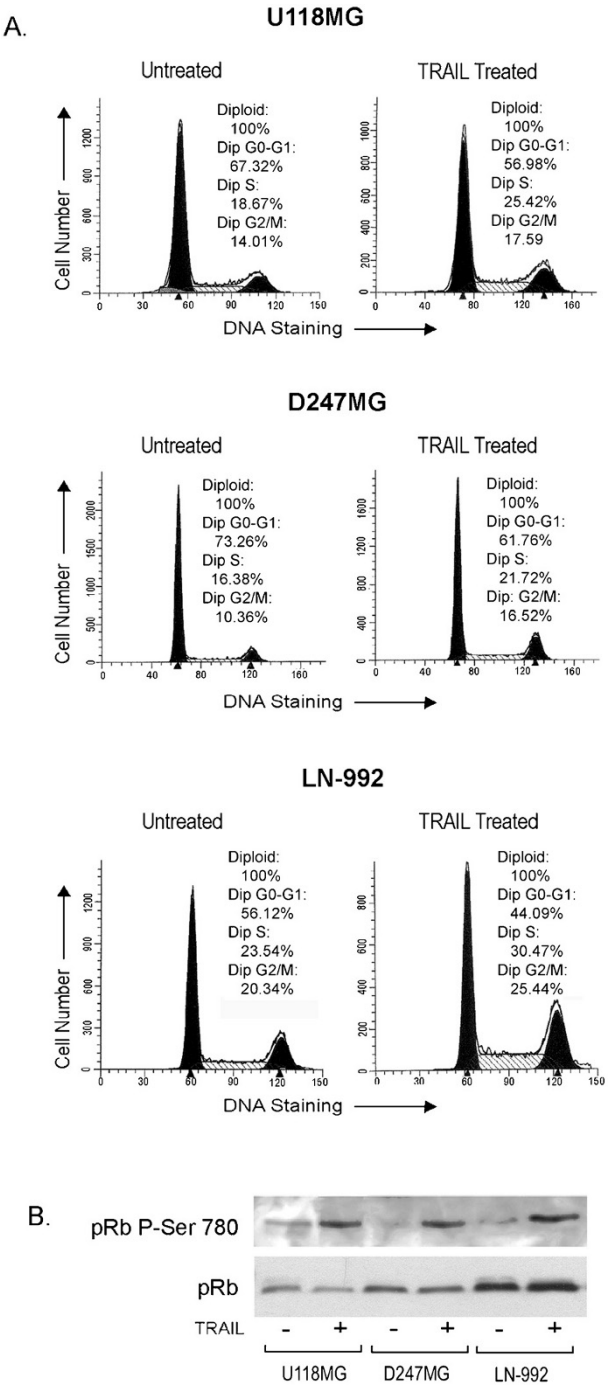

Figure 2. TRAIL-induced cell proliferation through cell cycle progression. (*A*) Cell cycle analysis. TRAIL-resistant U118MG, D247MG, and LN-992 glioma cell lines were treated with 33 ng/ml TRAIL for 24 h, fixed with 70% ethanol, followed by propidium iodide staining of DNA. DNA content was determined by flow cytometry, and cell cycle phase analysis was performed using ModFit LT software. Shown are tracings of one of five experiments for each cell line. Insets are the percentages of cells in G0/G1, S, and G2/M phases of the cell cycle. (*B*) Western blot analysis of pRb phosphorylation. Cell lines were treated with TRAIL as described in (*A*) above and subjected to Western blots using antibodies to phosphorylated pRb (pRb P-Ser 780) and unphosphorylated pRb.

avenue was not explored further (data not shown). The results indicate that TRAIL stimulation leads to activation of the ERK1/2 kinases and that inhibition of MEK1 prevents TRAIL-induced ERK1/2 kinase activation.

TRAIL-induced proliferation depends on activation of the ERK1/2 pathway. The role of the TRAIL-activated ERK1/2 pathway in TRAIL-induced proliferation of human glioma cell lines was further examined by experiments performed with PD98059 to inhibit MEK1-mediated ERK1/2 activation upon TRAIL treatment. Treatment of TRAIL-resistant cell lines with 33 ng/ml TRAIL in the presence of PD98059 for 24 h eliminated the observed increase in TRAIL-induced cell growth in U118MG, D247MG, and LN-992 cell lines (Fig. 4A). The ERK1/2 activation has been suggested to inhibit TRAIL-induced apoptosis in melanoma cells [35] and activated T cell [44]. However, inhibition of MEK1 with PD98059 did not sensitize the cells to TRAIL killing, as shown in crystal violet assay (Fig. 4A). To further confirm this, we examined caspase-8, caspase-3, and DFF45 cleavage in the cells treated with PD98059 and TRAIL and showed that TRAIL did not induce apoptosis under these conditions as there was no cleavage of caspase-8, caspase-3, or DFF45 (data not shown).

To ensure that TRAIL-induced proliferation in resistant glioma cell lines was indeed due to the ERK1/2 activation, we inhibited ERK1/2 pathway and then analyzed the cell cycle phase distributions of U118MG, D247MG, and LN-992 cells after exposure to TRAIL. The results showed no significant difference in the cell cycle phase distributions of the cell lines treated with PD98059 alone and the combination of PD98059 and TRAIL (Fig. 4B). These results suggest that TRAIL-induced proliferation of human glioma cell lines is dependent on activation of the ERK1/2 pathway. However, inhibition of MEK1-mediated ERK1/2 pathway does not potentiate TRAIL-induced apoptosis in these human glioma cell lines.

c-FLIP mediates TRAIL-induced ERK1/2 activation in resistant glioma cell lines. The mechanism through which TRAIL activates the ERK1/2 cascade is unknown. We have shown that TRAIL induces apoptosis in TRAIL-sensitive glioma cell lines through caspase-8-initiated caspase cascade (Fig. 1). In contrast, TRAIL activates the ERK1/2 pathway in TRAIL-resistant cells for cell growth (Fig. 4). It has been shown that the caspase-8 inhibitor c-FLIP is expressed and recruited to the TRAIL-DISC in TRAIL-resistant but not in -sensitive glioma cell lines [18]. To examine if c-FLIP mediates ERK1/2

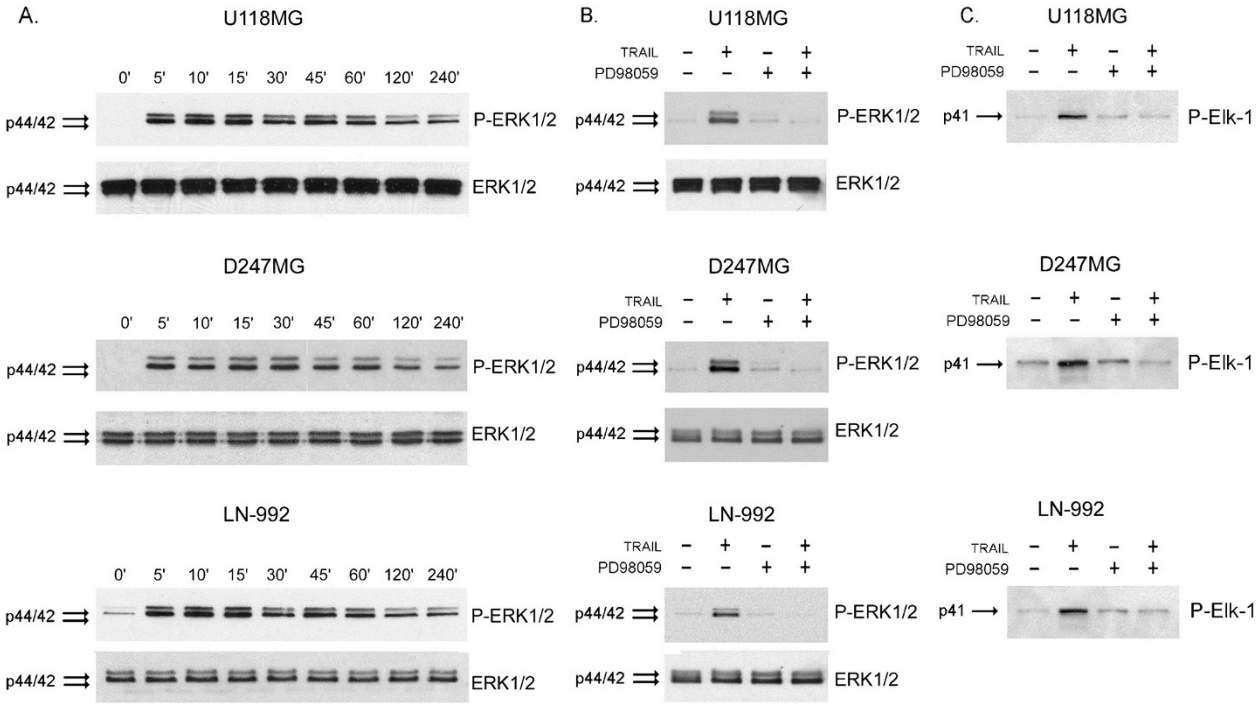

Figure 3. TRAIL triggers activation of the ERK1/2 pathway in TRAIL-resistant glioma cells. (*A*) Kinetic analysis of phosphorylation of ERK1/2 proteins. U118MG, D247MG, and LN-992 cell lines were treated with 33 ng/ml TRAIL for the times indicated. Phosphorylated ERK1/2 proteins as well as total ERK1/2 proteins were examined on Western blots. (*B*) Effects of MEK1 inhibitor PD98059 on ERK1/2 activation. Cell lines were either left untreated, treated with 33 ng/ml TRAIL for 15 min, 50 μM PD98059 for 75 min, or 50 μM PD98059 for 60 min followed by 33 ng/ml TRAIL for 15 min. Cell lysates were examined by Western blot for phosphorylated and total ERK1/2 proteins. (*C*) ERK1/2 *in vitro* kinase assay. Cell extracts obtained from glioma cell lines that were treated as described in (*B*) above, and 200 μg cell extract from each cell line was analyzed for its ability to phosphorylate a recombinant GST-Elk-1 fusion protein. Phosphorylation of the Elk-1 fusion protein was determined using an anti-phospho-Ser383 Elk-1 antibody on Western blots.

activation by TRAIL in glioma cell lines, we designed a synthetic siRNA duplex targeting the prodomain of c-FLIP$_L$ and c-FLIP$_S$ and showed that transfection of this siRNA in U118MG cells suppresses c-FLIP$_L$ and c-FLIP$_S$ expression (Fig. 5A). The siRNA treatment restored partial sensitivity to apoptosis induced by 100 ng/ml TRAIL treatment as shown by Western blotting for cleavage of caspase-8, caspase-3, and DFF45 (Fig. 5A) and cell viability assay for cell death (Fig. 5B). Inhibition of c-FLIP$_L$ and c-FLIP$_S$ with siRNA eliminates ERK1/2 activation in the resistant cells (Fig. 5C). These results indicate that c-FLIP mediates ERK1/2 activation in TRAIL-resistant glioma cells and may be responsible for TRAIL-induced glioma cell line proliferation.

To determine that c-FLIP-mediated activation of the ERK1/2 pathway is responsible for TRAIL-induced proliferation of U118MG cells, we simultaneously inhibited c-FLIP expression with c-FLIP siRNA and inhibited the caspase-8-initiated caspase cascade with the specific caspase-8 inhibitor z-IETD-fmk. The simultaneous inhibition of c-FLIP and caspase-8 eliminated both TRAIL-induced apoptosis and growth in U118MG cells after exposure to 33 ng/ml TRAIL for 24 h, as determined by crystal violet staining (Fig. 6A). Furthermore, Western blot analysis determined that activation of ERK1/2 and phosphorylation of pRb at Ser780 induced by 33 ng/ml TRAIL treatment for 15 min and 24 h, respectively, is completely eliminated by inhibition of c-FLIP expression (Fig. 6B). The role of c-FLIP in TRAIL-induced glioma cell line proliferation was finally confirmed by cell cycle analysis using propidium iodide staining. With caspase-8 inhibited, U118MG cells showed increased cell cycle phase progression after 33 ng/ml TRAIL treatment for 24 h as evidenced by a decrease in the G1 phase population of cells and increase in the S phase population (Fig. 6C). This increase in cell cycle phase progression did not occur after c-FLIP expression was inhibited by siRNA treatment (Fig. 6C).

To further explore the involvement of c-FLIP isoforms in TRAIL-induced ERK1/2 activation and glioma cell line proliferation, we created U118MG cells stably expressing c-FLIP$_L$ (Fig. 7). Overexpression of c-FLIP$_L$ in U118MG cell potentiated proliferation induced by 33 ng/ml TRAIL treatment from 125% to 140% over the course of 24 h (Fig. 7A).

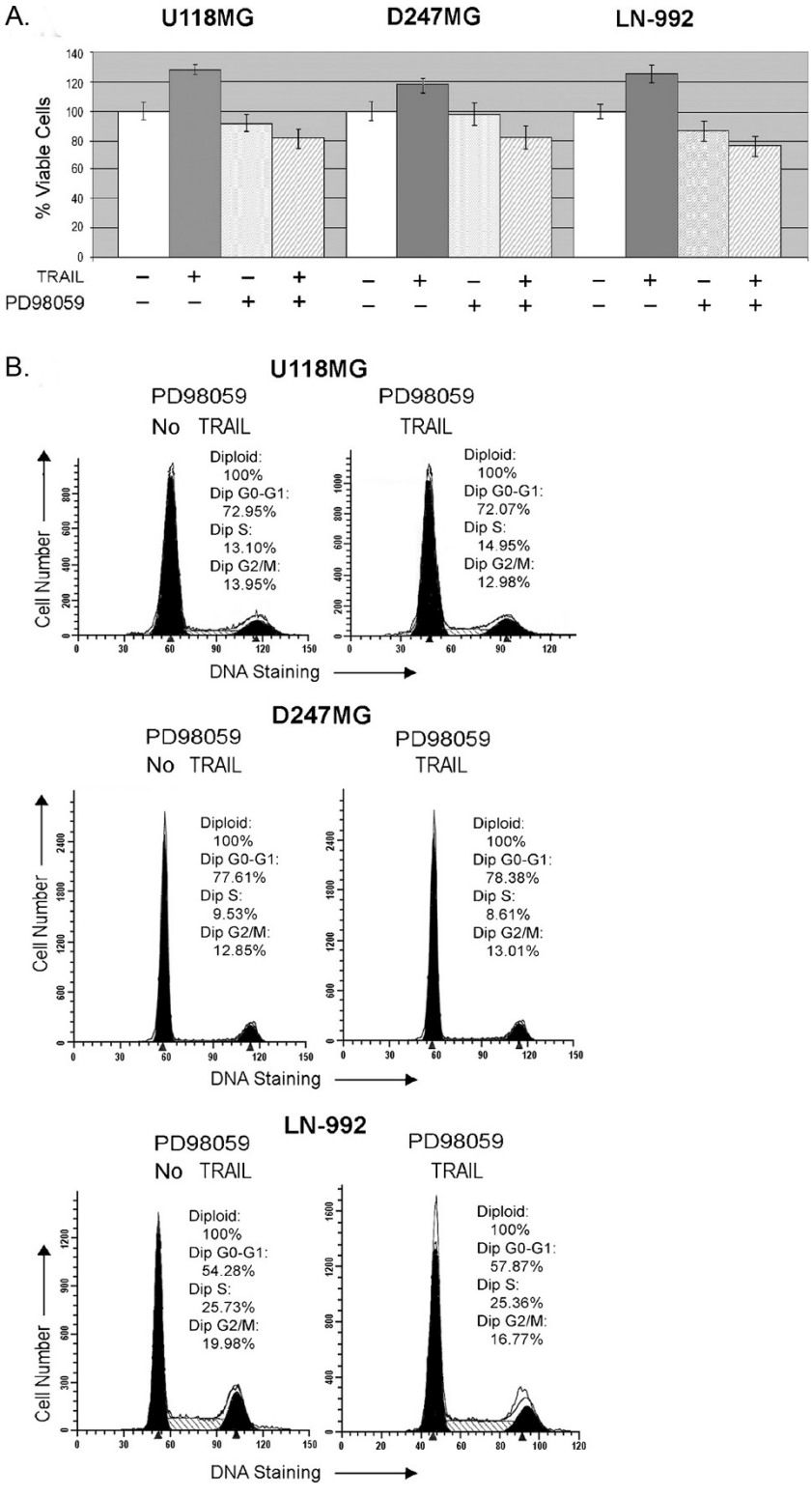

Figure 4. Inhibition of the ERK1/2 pathway eliminates TRAIL-induced proliferation while having no influence on apoptosis. (*A*) The effect of MEK1 inhibitor PD98059 on the viability of glioma cells. U118MG, D247MG, and LN-992 were left untreated (–) or treated (+) with 33 ng/ml TRAIL or 50 μM PD98059 alone or in combination for 24 h and subjected to crystal violet assay (*A*). Each value represents the mean ± SD of eight samples and experiments were repeated three times. (*B*) Effect of MEK1 inhibitor PD98059 on TRAIL-induced cell cycle progression. U118MG, D247MG, and LN-992 glioma cell lines were treated as in (*A*) and subjected to propidium iodide staining of DNA. DNA content was determined by flow cytometry, and cell cycle phase analysis was performed using ModFit LT software. Shown are tracings of one of five experiments for each cell line. Insets are the percentages of cells in G0/G1, S, and G2/M phases of the cell cycle.

Increased proliferation after 33 ng/ml TRAIL treatment was concomitant with an increase in ERK1/2 activation after 15 min of TRAIL treatment, and an increase in phosphorylation of pRb at Ser780 and in cell cycle progression after 24-h treatment (Fig. 7B, C). These results suggest that TRAIL induces proliferation of human glioma cell lines by c-FLIP$_L$ mediated activation of the ERK1/2 pathway in human glioma cell lines.

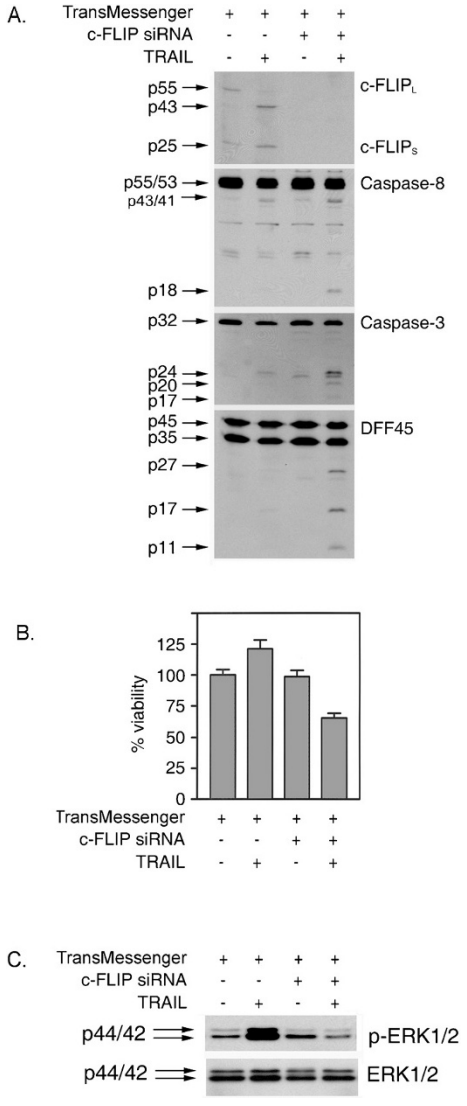

Figure 5. Inhibition of c-FLIP expression restores partial sensitivity to TRAIL-induced apoptosis and eliminates ERK1/2 activation. (*A*) U118MG cells were treated as indicated in the figure with: TransMessenger transfection reagents alone for 36 h; TransMessenger transfection reagents for 36 h and 100 ng/ml TRAIL for 6 h; TransMessenger transfection reagents for 36 h and actual transfection of c-FLIP-siRNA for 36 h; or TransMessenger transfection reagents with transfection of c-FLIP-siRNA for 36 h and 100 ng/ml TRAIL for 6 h; and were then subjected to Western blot analysis for the expression of c-FLIP$_L$ and c-FLIP$_S$, as well as for the cleavage of caspase-8, caspase-3, and DFF45. (*B*) U118MG cells were treated as in (*A*) except they were treated with 100 ng/ml TRAIL for 24 hours and subjected to crystal violet assay for cell viability. Non-TRAIL treated cells are represented as 100% viable in both the TransMessenger transfection control and c-FLIP-siRNA-transfected groups. (*C*) U118G cells were treated as above, except that they were treated with TRAIL for 30 min and subjected to Western blot analysis for phosphorylated and total ERK1/2 proteins.

Discussion

TNFR and Fas have been shown to mediate cell death and proliferation pathways [21]. The first observation of induction of tumor cell proliferation by a death receptor was by TNF-α stimulation of the growth of a human glioma cell line [45]. This observation has been extended and TNFR and Fas have been shown to promote proliferation in several other human glioma cell lines [46–48]. It has been shown that TRAIL activates a caspase-8-initiated caspase cascade for cell death in TRAIL-sensitive human glioma cells [18, 38]. Here, we show for the first time that TRAIL can stimulate proliferation of TRAIL-resistant human glioma cell lines through cell cycle progression. TRAIL-induced cell proliferation occurs by activation of ERK1/2 pathway in resistant glioma cell lines. The MEK/ERK1/2 pathway has been shown to be required for cell proliferation by promoting G_1 to S phase cell cycle progression [49, 50]. Constitutive activation of the ERK1/2 pathway has been shown to be able to transform mammalian cells to a neoplastic phenotype *in vitro* [51, 52]. Furthermore, activation of the ERK1/2 pathway has been suggested to contribute to the cancerous phenotype of human gliomas [53, 54] and the proliferation of rat C6 glioma cell lines [48]. The MEK/ERK1/2 pathway is a central signal transduction pathway that transmits signals from multiple cell surface receptors and indeed platelet-derived growth factor has been shown to induce proliferation in glioma cells through an ERK1/2-dependent mechanism [55]. In this study, we show that ERK1/2 pathway is involved in death receptor-mediated cell proliferation in glioma cells.

MEK1 inhibition and dominant negative mutants have been shown to increase the sensitivity of HeLa and melanoma cells to TRAIL-induced apoptosis [35, 56]. Adenovirus-mediated expression of active MEK1 in HeLa cells inhibits TRAIL-induced cleavage of caspase-8, suggesting that the MEK/ERK1/2 pathway protection may occur upstream of caspase-8 [56]. In contrast, others have reported that ERK1/2 protection seems downstream of caspase-8 [35]. To further complicate the issue, there is a report that epidermal growth factor-induced ERK1/2 activation does not protect breast cancer cells from TRAIL-induced apoptosis [57]. The HeLa, melanoma and breast cell lines examined in the above studies are partially sensitive to TRAIL-induced apoptosis [35, 56, 57]. In contrast inhibition of the ERK1/2 pathway does not fully sensitize TRAIL-resistant colon adenocarcinoma cells to TRAIL treatment [58]. These have prompted us to examine the ERK1/2 pathway in TRAIL-resistant glioma cell lines. The results indicate that TRAIL activates the ERK1/2 pathway but not the caspase-8-initiated cascade; however, inhibition of the TRAIL-induced ERK1/2 pathway fails to potentiate TRAIL-induced activation of caspase-8-initiated caspase cascade for cell death, suggesting that ERK1/2

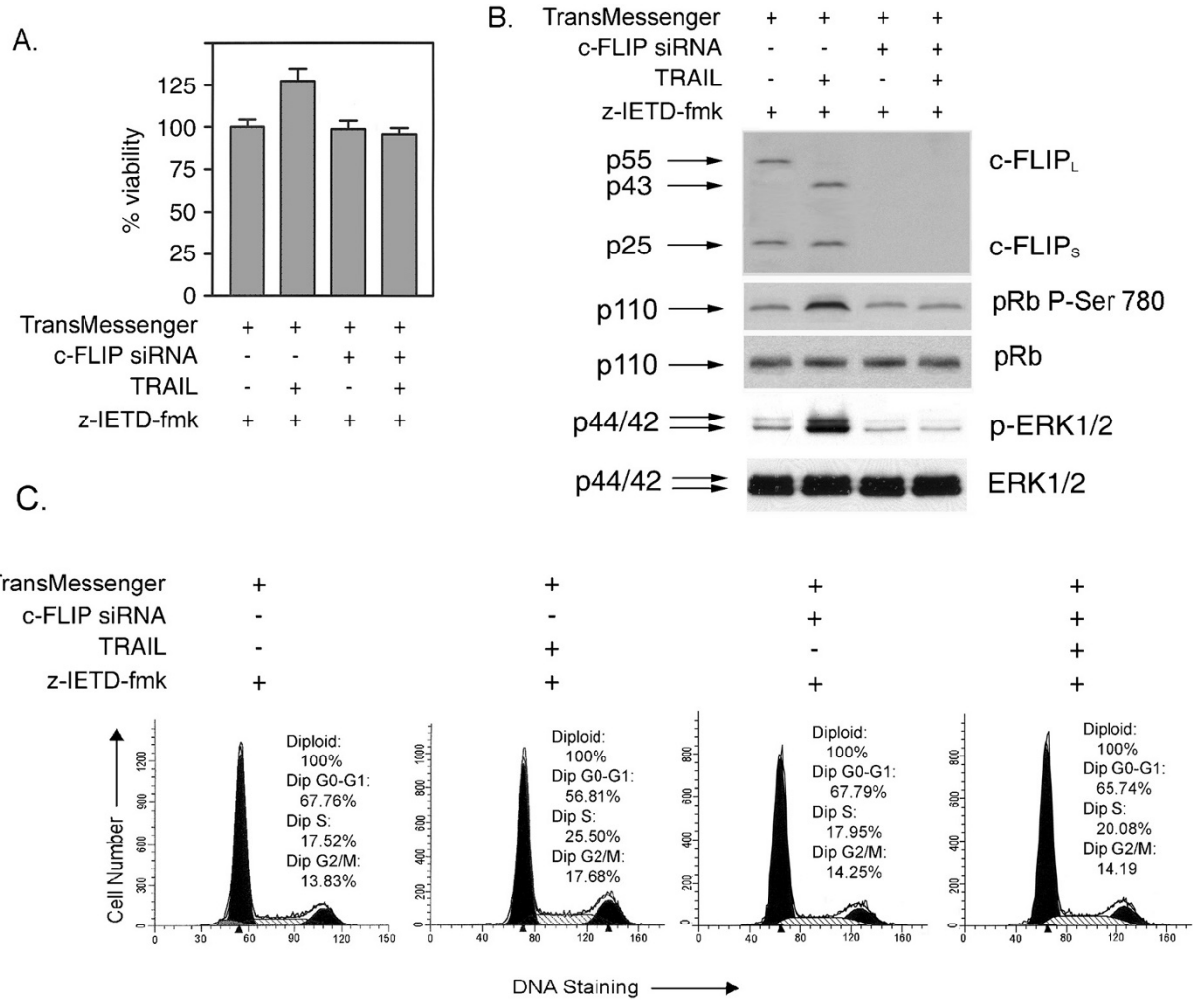

Figure 6. Inhibition of c-FLIP expression eliminates TRAIL-induced proliferation of TRAIL-resistant glioma cells. (*A*) U118MG cells were treated with 33 ng/ml TRAIL in the presence of z-IETD-fmk for 24 h after treatment with TransMessenger transfection reagents alone for 36 h or after actual transfection of c-FLIP-siRNA for 36 h and subjected to crystal violet assay for cell viability. Non-TRAIL-treated cells are represented as 100 % viable in both the TransMessenger transfection control and c-FLIP-siRNA-transfected groups. (*B*) U118MG cells were treated as in (*A*) with the exception that they were treated with TRAIL for 24 h, and subjected to Western blot analysis for c-FLIP, total pRb and pRb phosphorylated on Ser780, or treated with 33 ng/ml TRAIL for 30 min and subjected to Western blot analysis for total and phosphorylated ERK1/2 proteins. (*C*) U118MG cells were treated as in (*A*) and subjected to propidium iodide staining of DNA. DNA content was determined by flow cytometry, and cell cycle phase analysis was performed using ModFit LT software. Shown are tracings of one experiment of three for each treatment. Insets are the percentages of cells in G0/G1, S, and G2/M phases of the cell cycle.

pathway activation does not contribute to inhibition of TRAIL-induced apoptosis in cell lines fully resistant to TRAIL.

TRAIL-induced apoptosis in sensitive glioma cells occurs through recruitment of FADD and caspase-8 to the TRAIL receptors, leading to the assembly of the death-induced signaling complex (DISC) where caspase-8 completes two step cleavage [18]. It has been shown that c-FLIP is not recruited to the TRAIL DISC in cell lines sensitive to TRAIL-induced apoptosis [18, 38]. This has been shown for both the LN-71 and the U343MG TRAIL-sensitive cell lines used in this study. Furthermore, inhibition of the TRAIL-induced caspase cascade, and TRAIL-induced apoptosis, by either the caspase-8 inhibitor z-IETD-fmk or by the pan-caspase inhibitor z-VAD-fmk does not reveal any TRAIL-dependent increases in proliferation in TRAIL-sensitive glioma cell lines [18, 38]. However, in resistant glioma cells c-FLIP is recruited to the DISC to inhibit caspase-8 cleavage [18].

The *c-FLIP* gene is expressed as four mRNA splice variants, but only two forms of protein are expressed, c-FLIP$_S$ and c-FLIP$_L$ [39, 59, 60]. Both c-FLIP$_S$ and c-FLIP$_L$ are recruited to the DISC, where they inhibit the second step cleavage of caspase-8 in resistant glioma cells [18]. c-FLIP proteins have been reported to enhance FasL-activated ERK1/2 pathway in CD3-activated human T lymphocytes [61]. In addition, the

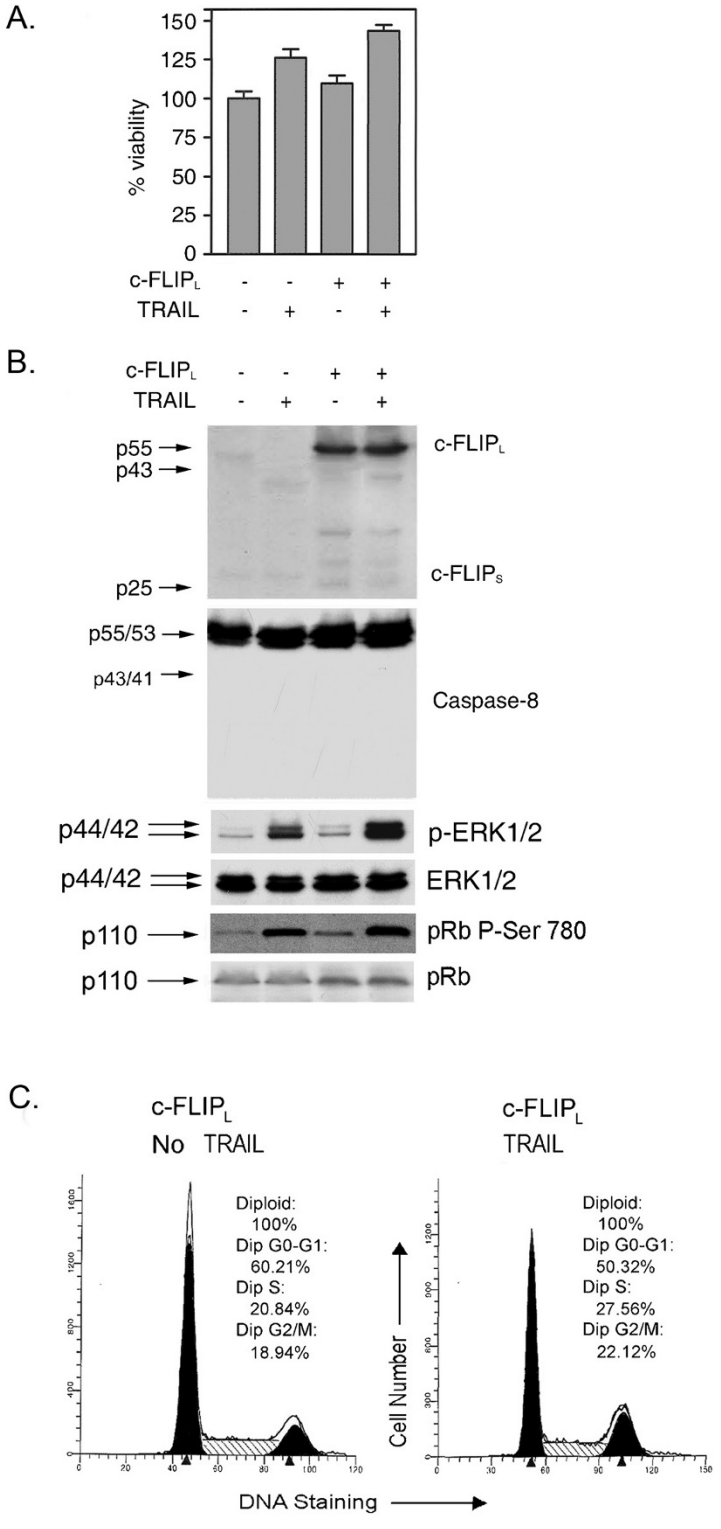

Figure 7. Overexpression of c-FLIP$_L$ potentiates TRAIL-induced proliferation of TRAIL-resistant glioma cells. (*A*) U118MG vector transfected or U118MG-c-FLIP$_L$ cells were either left untreated or treated with 33 ng/ml TRAIL for 24 h and then subjected to crystal violet assay for cell viability. Untreated cells are represented as 100% viable in both the TransMessenger transfection control and c-FLIP-siRNA-transfected groups. (*B*) The same cells were treated as in (*A*) with the exception that they were treated with TRAIL for 24 h, and subjected to Western blot analysis for c-FLIP, caspase-8, total pRb and pRb phosphorylated on Ser780, or treated with 33 ng/ml TRAIL for 30 min and subjected to Western blot analysis for total and phosphorylated ERK1/2 proteins. (*C*) Same cells were treated as in (*A*) and subjected to propidium iodide staining of DNA. For comparison purposes, represented are U118MG vector controls and U118MG-c-FLIP$_L$ cells treated with TRAIL. DNA content was determined by flow cytometry, and cell cycle phase analysis was performed using ModFit LT software. Shown are tracings of one experiment of three for each treatment. Insets are the percentages of cells in G0/G1, S, and G2/M phases of the cell cycle.

FADD/caspase-8/c-FLIP complex has been shown to be involved in ERK1/2 activation in the TNFR system [62]. Transfection of c-FLIP$_L$ in pancreatic β cells protects the cells from FasL-induced apoptosis and in the meantime promotes the cell growth [63]. In this study, we have shown for the first time that expression of c-FLIP not only protects human glioma cells from TRAIL-induced apoptosis but also that, specifically, c-FLIP$_L$ mediates TRAIL-induced ERK1/2 activation, which promotes TRAIL-induced cell growth in

glioma cells. These results suggest that TRAIL can trigger both proliferative and apoptotic pathways in cancer cells, but the TRAIL-activated proliferative pathway can proceed only if the apoptotic pathway is inhibited by the caspase-8 inhibitor c-FLIP.

Mechanistically, a number of studies have implicated c-FLIP$_L$ in the activation of the ERK1/2 signaling cascade. As early as 2000, Kataoka et al. [61] showed that c-FLIP is recruited to the Fas DISC upon receptor engagement by FasL. Their research shows that Raf-1, the upstream activating kinase of the ERK1/2 pathway, is recruited into the DISC in a c-FLIP$_L$-dependent manner, and that the activation of the ERK1/2 cascade, as measured by MEK1/2 and ERK1/2 phosphorylation, is enhanced in cells overexpressing c-FLIP$_L$ [61]. TNF-α-mediated proliferation of a gastric cancer cell line, AGS, is dependent on the expression of c-FLIP, and Raf-1. In AGS cells, c-FLIP was found to recruit Raf-1 to the TNFR complex [64]. These data point to a direct link between death receptor-mediated c-FLIP$_L$ recruitment and the activation of the ERK1/2 signaling cascade *via* Raf-1, and lend credence to our hypothesis that TRAIL-induced proliferation of glioma cell lines is by c-FLIP-mediated activation of the ERK1/2 pathway.

TRAIL and its agonists are currently under development as anti-cancer agents. One of the major obstacles in the use of TRAIL in the treatment of cancers is that many cancer cells, particularly in primary cultures prepared from patient samples, are resistant to TRAIL-induced apoptosis [38, 65]. To make things worse, this study shows for the first time that TRAIL activates the ERK1/2 pathway and induces the growth of resistant human glioma cells by increasing cell cycle progression. However, TRAIL can simultaneously activate proliferative and apoptotic pathways and inhibition of caspase-8 by c-FLIP switches the cells from cell death to cell proliferation. The data presented in this study further suggest that combination therapies with TRAIL and other reagents targeting anti-apoptotic genes such as c-FLIP need to be developed to provide effective strategies for TRAIL-induced apoptosis in resistant glioma cells.

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