Biochemical and Biophysical Research Communications 414 (2011) 129–134

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of eEF-2 kinase sensitizes human glioma cells to TRAIL and down-regulates Bcl-xL expression

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ARTICLE INFO

Article history:
Received 7 September 2011
Available online 16 September 2011

Keywords:
eEF-2 kinase
TRAIL
Bcl-xl
Apoptosis
Glioblastoma

ABSTRACT

Elongation factor-2 kinase (eEF-2 kinase, also known as calmodulin-dependent protein kinase III), is a unique calcium/calmodulin-dependent enzyme that inhibits protein synthesis by phosphorylating and inactivating elongation factor-2 (eEF-2). We previously reported that expression/activity of eEF-2 kinase was up-regulated in several types of malignancies including Gliomas, and was associated with response of tumor cells to certain therapeutic stress. In the current study, we sought to determine whether eEF-2 kinase expression affected sensitivity of glioma cells to treatment with tumor the necrosis factor-related apoptosis-inducing ligand (TRAIL), a targeted therapy able to induce apoptosis in cancer cells but causes no toxicity in most normal cells. We found that inhibition of eEF-2 kinase by RNA interference (RNAi) or by a pharmacological inhibitor (NH125) enhanced TRAIL-induced apoptosis in the human glioma cells, as evidenced by an increase in apoptosis in the tumor cells treated with eEF-2 kinase siRNA or the eEF-2 kinase inhibitor. We further demonstrated that sensitization of tumor cells to TRAIL was accompanied by a down-regulation of the anti-apoptotic protein, Bcl-xL, and that overexpression of Bcl-xL could abrogate the sensitizing effect of inhibiting eEF-2 kinase on TRAIL. The results of this study may help devise a new therapeutic strategy for enhancing the efficacy of TRAIL against malignant glioma by targeting eEF-2 kinase.

1. Introduction

Glioblastoma multiforme (GBM), one of the common childhood malignancies and a cancer of increasing significance in adults, is a fatal disease characterized by survival of glioma cells following initial treatment, invasion through the brain, and ultimately resistance to treatments [1]. To improve the treatment outcome of this devastating disease, new therapies are urgently needed. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered a promising candidate as an anticancer agent based on its ability to trigger rapid apoptosis and its specific cytotoxicity in malignant cells [2]. TRAIL belongs to the TNF family, and is able to induce caspase-8-dependent apoptosis through its binding to the death receptors DR4 (TRAIL-RI) and DR5 (TRAIL-RII) to form the death-inducing signaling complex (DISC) and to trigger the recruitment of Fas-Associated protein with Death Domain (FADD) [3]. Nevertheless, despite expression of TRAIL receptor in tumor cells, cellular insensitivity to TRAIL-induced apoptosis is still often encountered [4]. How tumor cells become insensitive to TRAIL and how to sensitize tumor cells to this therapy remain poorly understood.

Elongation factor-2 (eEF-2) kinase is an essential enzyme that participates in regulation of protein synthesis under stressful conditions. This kinase phosphorylates eEF-2, a 100 kDa protein that promotes ribosomal translocation from the A to the P-site, which is the reaction that induces movement of mRNA along the ribosome during translation [5]. Phosphorylation of eEF-2 at Thr56 by eEF-2 kinase terminates peptide elongation by decreasing the affinity of eEF-2 for the ribosome. Previous studies found that the activity and expression of eEF-2 kinase is up-regulated in several types of cancers including glioma and breast cancer [6,7], and that inhibiting this kinase results in a decreased viability of tumor cells [8]. We have recently reported that inactivation of eEF-2 kinase suppresses autophagy but promotes apoptosis under various stresses such as nutrient deprivation, growth factor inhibition [9], Akt inhibition [10] and energy stress caused by the glycolytic inhibitor, 2-deoxy-D-glucose [11]. These studies suggest that over-expression of eEF-2 kinase in tumor cells may contribute to apoptosis resistance, which is often associated with failure of cancer therapy.

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.09.038

Therefore, in this study we intended to determine the effects of eEF-2 kinase activity on sensitivity of glioma cells to TRAIL. We found that inhibiting eEF-2 kinase by genetic or pharmacologic approaches can sensitize glioma cells to TRAIL treatment, and this sensitizing effect is associated with the down-regulation of the anti-apoptotic protein Bcl-xL.

2. Materials and methods

2.1. Cell lines and culture

The human glioma cell lines U251 and T98G were purchased from American Type Culture Collection (Manassas, VA, USA); the normal human astrocyte cell line, SVGp12, was a kind gift from Dr. James Connor (Penn State College of Medicine). T98G cells were cultured in Ham's F-10: DMEM (10:1) medium; U251 and SVGp12 cells were cultured in DMEM medium. All of the cell culture media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. All cultures were monitored routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 3 months and new lines propagated from frozen stocks.

2.2. Reagents and antibodies

Recombinant human TRAIL was purchased from PeproTech (Rocky Hill, NJ); 1-Hexadecyl-2-methyl-3-(phenyl methyl)-1*H*-imidazolium iodide (NH125) and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA); antibodies to PARP, Caspase 8, phospho-eEF-2 (Thr56), eEF-2 kinase were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies to Mcl-1, XIAP, and Bcl-xL were purchased from BD Biosciences (San Diego, CA, USA). α-Tubulin antibody was obtained from Santa Cruz (Santa Cruz, CA, USA); Antibody Microarray Assay Kit was obtained from Full Moon Biosystems (Sunnyvale, CA, USA). All of the cell culture media and other reagents were from Invitrogen (Carlsbad, CA, USA).

2.3. siRNA transfection

siRNA duplexes targeting eEF-2 kinase and control siRNA were prepared by Dharmacon RNAi Technologies (Lafayette, CO, USA). Transfection of siRNA was performed according to the manufacturer's protocol. Briefly, cells in exponential phase of growth were plated in six-well cell culture plates at 1×10^5 cells per well, grown for 24 h, and then transfected with siRNA using Oligofectamine and OPTI MEMI-reduced serum medium (Invitrogen, Carlsbad, CA). The concentrations of siRNA were chosen based on dose–response studies.

2.4. Bcl-xL plasmid and transfection

U251 and T98G cells subjected to inhibition of eEF-2 kinase were transiently transfected with an empty vector or a Bcl-xL-expressing plasmid (a gift from Dr. Wang HG, Penn State College of Medicine) using FuGENE six transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, the transfected tumor cells were treated with TRAIL and used for further experiments. The overexpression of Bcl-xL was confirmed by Western blotting.

2.5. Western blot

Cells were lysed in M-PER mammalian protein extraction reagent (Pierce Biotechnology, Inc., Rockford, IL) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA), followed by centrifugation at 14,000*g* for 10 min. At the end of

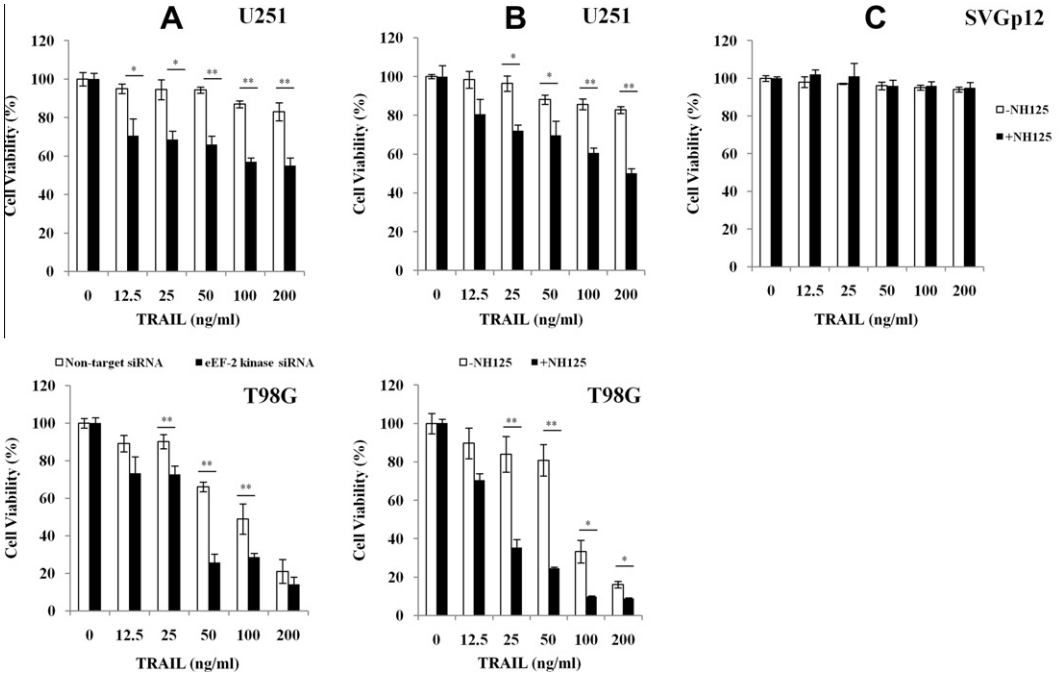

Fig. 1. Inhibition of eEF-2 kinase enhances sensitivity of glioma cells to TRAIL. (A) Human glioma cell lines U251 and T98G with or without silencing of eEF-2 kinase expression were treated with the indicated concentrations of TRAIL for 48 h. At the end of treatment, cell viability was measured by MTT assay. (B) U251 and T98G cells were treated with the indicated concentrations of TRAIL for 48 h in the presence or absence of 0.5 μM of NH125. At the end of treatment, cell viability was measured by MTT assay. (C) Normal human astrocytes were treated with the indicated concentrations of TRAIL for 48 h in the presence or absence of NH125 (0.5 μM). At the end of treatment, cell viability was measured by MTT assay. Each point represents mean ± SD of triplicate determinations; results shown are the representative of three identical experiments. $^*P < 0.05$; $^{**}p < 0.01$, *t*-test.

centrifugation, cell lysates were collected and protein concentrations of cell lysates were measured. Protein (10–20 μg) were resolved by SDS–PAGE, and then transferred to PVDF membrane (Bio-Rad Hercules, CA, USA). The blots were incubated with primary antibodies in 3% BSA/TBST at 4 °C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. The protein signals were detected by ECL method.

2.6. Apoptosis assays

Apoptosis was assayed by: (1) flow cytometric analysis of Annexin V and 7-AAD staining. Briefly, 100 μL of Guava Nexin reagent (Millipore, Bedford, MA, USA) was added to 1×10^5 cells (in 100 μL) and the cells were incubated with the reagent for 20 min at room temperature in the dark. At the end of incubation, the cells were analyzed by a Guava 16 EasyCyte™ Plus FlowCytometry System (Millipore); (2) Western blot analysis of the cleaved PARP and caspase-8.

2.7. Cell viability assay

Cell viability was measured by MTT assay. Briefly, cells were plated at a density of 5×10^3 cells per well on 96-well plates and subjected to different treatment. Following 48 h incubation at 37 °C in a humidified atmosphere containing 5% CO_2/95% air, the cells were incubated for another 4 h with MTT reagent. The formazan product was dissolved in DMSO and read at 570 nm on a Victor3 Multi Label plate reader (PerkinElmer, Boston, MA, USA).

2.8. Statistical analysis

All data are expressed as means ± standard deviation (SD), and the data were compared using the Student's *t*-test. The results considered significant at $P < 0.05$ (*) or $P < 0.01$ (**).

3. Results

To determine whether or not the increased expression and activity of eEF-2 kinase in tumor cells affected efficacy of TRAIL, we first compared the cellular viability of glioma cells with or without silencing of eEF-2 kinase following treatment with a series of concentrations of TRAIL. Fig. 1A shows that silencing of eEF-2 kinase expression by siRNA significantly enhanced the cytotoxicity of TRAIL in the human glioma cell lines, U251 and T98G. Combinatorial treatment with TRAIL and the small molecule inhibitor of eEF-2 kinase, NH125, also sensitized tumor cells to TRAIL, as compared to the treatment with TRAIL alone (Fig. 1B). Neither NH125 and TRAIL alone, nor the combination of NH125 and TRAIL showed cytotoxicity in normal human astrocytes, SVGp12 (Fig. 1C).

We next examined the effects of inhibiting eEF-2 kinase on TRAIL-induced apoptosis in those glioma cells. We found that both eEF-2 kinase-targeted siRNA and the inhibitor of the kinase,

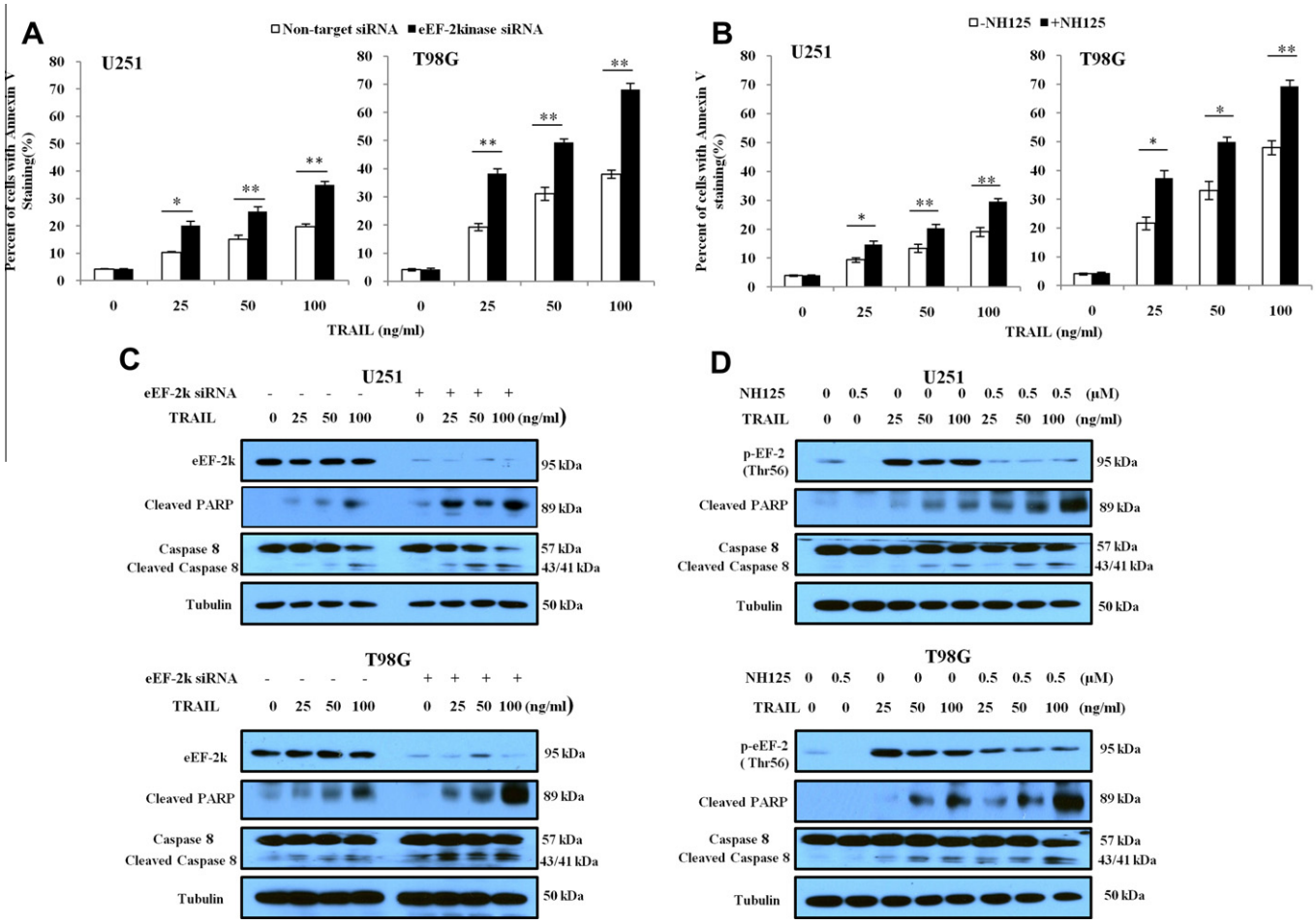

Fig. 2. Inhibition of eEF-2 kinase augments TRAIL-induced apoptosis in glioma cells. (A) U251 and T98G cells with or without silencing of eEF-2 kinase expression were treated with the indicated concentrations of TRAIL for 24 h. (B) U251 and T98G cells were treated with the indicated concentrations of TRAIL for 24 h in the presence or absence of 0.5 μM of NH125. At the end of treatment, apoptosis was determined by flow cytometric analysis of Annexin V staining (A and B) and Western blot analysis of PARP and cleaved caspase-8 (C and D). α-Tubulin was used as a loading control. Each bar represents mean ± SD of triplicate determinations; results shown are the representative of three identical experiments. *$P < 0.05$; **$P < 0.01$, *t*-test.

NH125, could significantly augment the apoptosis induced by TRAIL, as indicated by the increases in Annexin V staining (Fig. 2A and B). The augmenting effect of eEF-2 kinase inhibition on TRAIL-induced apoptosis was also demonstrated by increases in the levels of cleaved caspase-8 and cleaved PARP (Fig. 2C and D), two biochemical indicators of apoptosis. To further explore pathways possibly involved in the augmentation of TRAIL-induced apoptosis by inactivating eEF-2 kinase, based on our comparisons of the apoptosis-related proteins in glioma cells with or without silencing of eEF-2 kinase expression, which showed differential levels of the proteins known to be involved in resistance to TRAIL, including XIAP, survivin, Bcl-xL, Mcl-1 (Supplemental data), we examined the expression of these anti-apoptotic proteins in the treated cells. Fig. 3 shows that as compared to TRAIL treatment alone, the combination of TRAIL with either eEF-2 kinase-targeted siRNA (Fig. 3A) or NH125 (Fig. 3B) led to a reduction of Bcl-xL protein in U251 and T98G cells, as determined by Western blot. The decrease of Bcl-xL was most remarkable in the glioma cells treated with 100 ng/mL of TRAIL. The expression of survivin, XIAP and Mcl-1 did not appear to be altered in the cells subjected to both TRAIL and inhibition of eEF-2 kinase, as compared to TRAIL treatment alone (Fig. 3).

To verify the role of Bcl-xL down-regulation in conferring sensitivity of glioma cells to TRAIL, we transfected U251 and T98G cells with a Bcl-xL-expressing plasmid, and then analyzed apoptosis in the cells co-treated with the inhibitors of eEF-2 kinase and TRAIL or vehicle. Fig. 4 demonstrates that forced expression of Bcl-xL partially blocked the augmentation of TRAIL-induced apoptosis by eEF-2 kinase inhibition (Fig. 4A and B: siRNA; Fig. 4C and D: NH125), as indicated by the decreases in Annexin V staining and in the amounts of cleaved caspase-8 and PARP. These results suggest that the down-regulation of Bcl-xL is likely to be responsible for the sensitizing effect of inhibiting eEF-2 kinase on TRAIL efficacy.

4. Discussion

Although TRAIL can cause variable cytotoxicity in human cancer cells both *in vitro* and *in vivo* [12], approximately one-third of human malignancies are resistant to TRAIL treatment, and an additional one-third only have a moderate response [13]. TRAIL resistance can result from a variety of mechanisms, which can occur at various points in the apoptotic pathway or in other cellular signaling pathways [14,15]. Here, we report that eEF-2 kinase, a critical regulator of protein synthesis, plays an important role in determining sensitivity of glioma cells to TRAIL, and that inhibiting eEF-2 kinase cooperates with TRAIL in killing glioma cells.

To explore the pathways underlying the sensitizing effect of eEF-2 kinase inhibition on TRAIL-induced apoptosis, we compared the expression of XIAP, survivin, Bcl-xL, and Mcl-1 (Fig. 3), as the balance between the levels of these apoptosis-regulatory proteins are known to be associated with sensitivity of tumor cells to TRAIL [16]. Among those apoptosis-related proteins examined, we found a significant reduction only in the anti-apoptotic protein Bcl-xL, in the cells co-treated with TRAIL and eEF-2 kinase inhibitors (Fig. 3), suggesting that the effect of eEF-2 kinase on TRAIL-induced apoptosis might be mediated through modulating Bcl-xL expression. The role of Bcl-xL in altering the sensitivity of tumor cells to TRAIL-induced apoptosis in tumor cells subjected to eEF-2 kinase inhibition was further verified by the experiments showing that forced expression of Bcl-xL blocked the sensitizing effect of NH125 or eEF-2 kinase - targeted siRNA on TRAIL-induced apoptosis (Fig. 4). Nevertheless, the precise mechanism by which eEF-2 kinase regulates Bcl-xL expression remains unclear, and would need further studies. Bcl-xL resides within the mitochondrial membrane where it acts by inhibiting adaptor molecules needed for activation of the effector caspases [17], and is known to suppress apoptosis induced by TRAIL [18–20] and some other therapeutic insults [21]. Our results provide additional evidence for the critical role of Bcl-xL in inhibiting TRAIL-induced apoptosis.

In order to improve and reinforce the efficacy of TRAIL in cancer therapy, many strategies and approaches to modulating TRAIL sensitivity have been reported. For instance, it has been shown that TRAIL in combination with irinotecan (CPT-11) increased the expression of the pro-apoptotic protein, Bax, but decreased Bcl-xL expression in prostate cancer cells [22]. PS-341, a proteasome inhib-

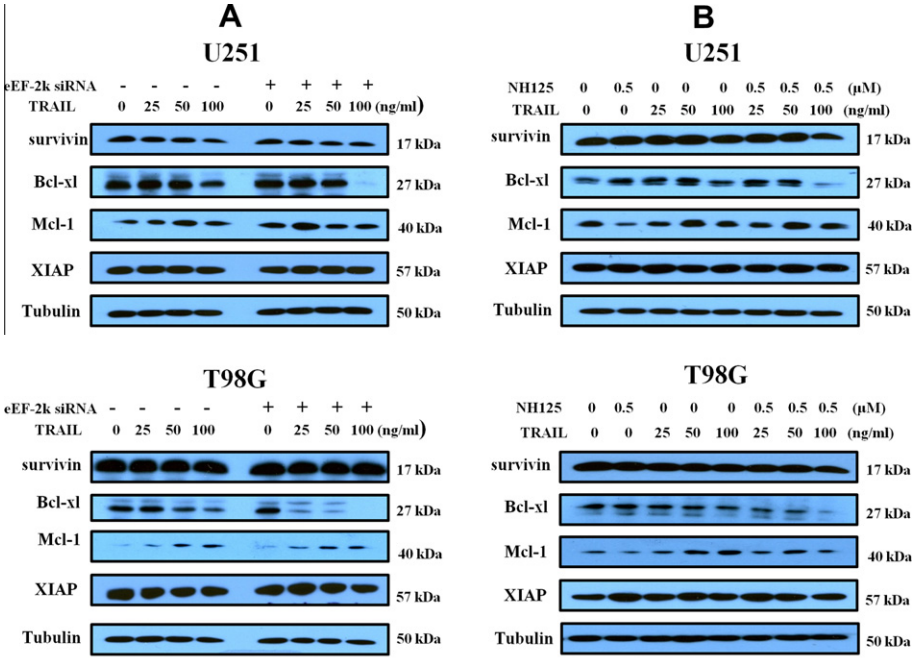

Fig. 3. Inhibition of eEF-2 kinase cooperates with TRAIL modulated Bcl-xL expression in glioma cells. (A) U251 and T98G cells with or without silencing of eEF-2 kinase expression were treated with the indicated concentrations of TRAIL for 24 h. (B) U251 and T98G cells were treated with the indicated concentrations of TRAIL for 24 h in the presence or absence of 0.5 μM of NH125. At the end of treatment, survivin, XIAP, Bcl-xL and Mcl-1 were examined by Western blot. Tubulin was used as a loading control.

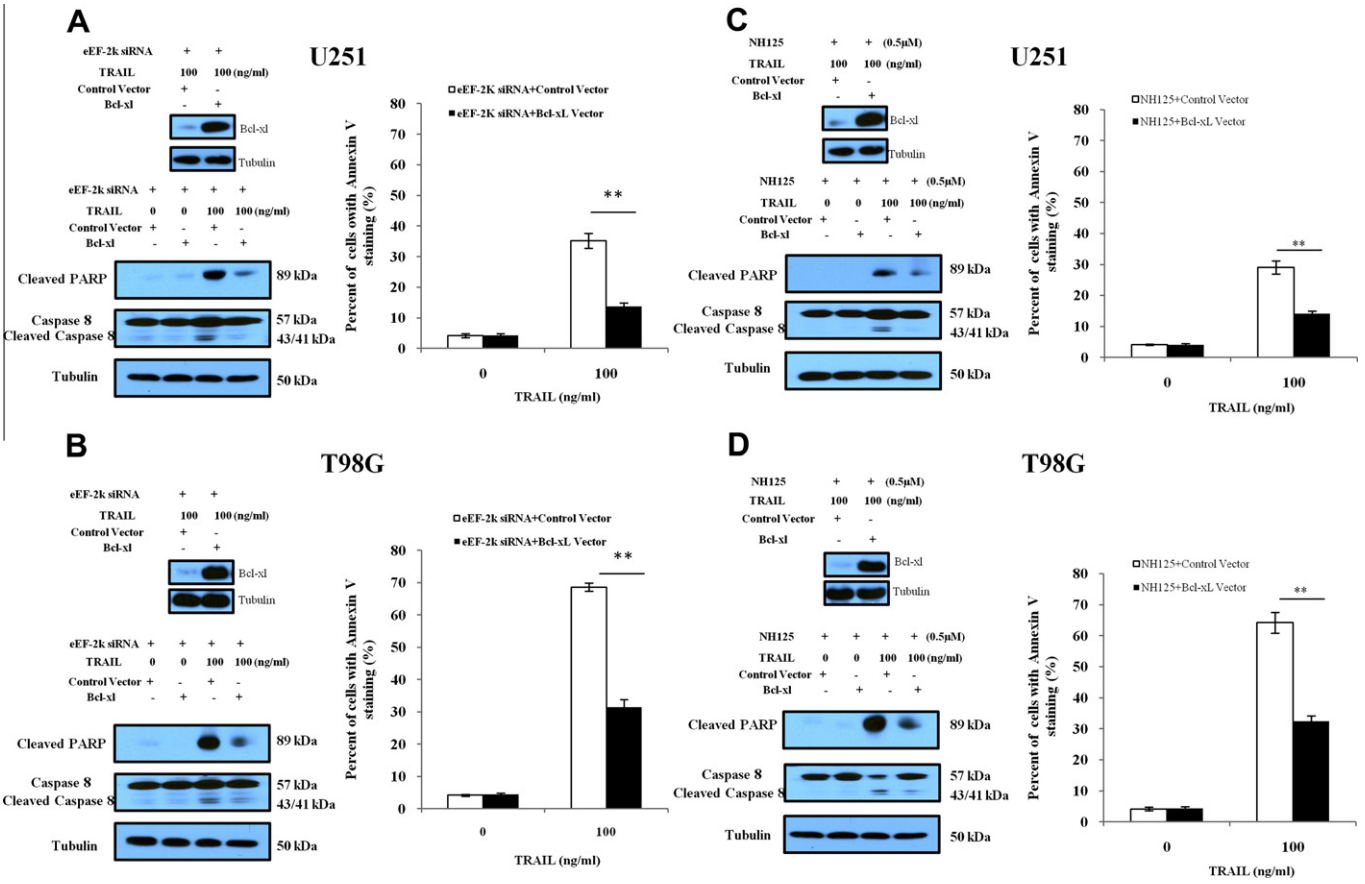

Fig. 4. Forced expression of Bcl-xL diminishes the sensitizing effect of eEF-2 kinase inhibition on TRAIL-induced apoptosis in glioma cells. (A) U251 and (B) T98G cells with silencing of eEF-2 kinase by siRNA were transfected with an empty control vector or Bcl-xL-expressing plasmids for 24 h, followed by treatment with the indicated concentrations of TRAIL for another 24 h. At the end of treatment, cell lysates were prepared and analyzed for protein levels of Bcl-xL, PARP, and cleaved caspase-8 by Western blot analysis. Apoptosis was determined by flow cytometric analysis of Annexin V staining. (C) U251 and (D) T98G cells were transfected with an empty control vector or a Bcl-xL-expressing plasmids for 24 h, followed by treatment with the indicated concentrations of TRAIL and 0.5 μM of NH125 for another 24 h. At the end of treatment, cell lysates were prepared and analyzed for protein levels of Bcl-xL, PARP, and cleaved caspase-8 by Western blot analysis. Apoptosis was determined by flow cytometric analysis of Annexin V staining. Each point represents mean ± SD of triplicate determinations; results shown are the representative of three identical experiments. $^{**}P < 0.01$, *t*-test.

itor, was shown to enhance the TRAIL-induced cytotoxicity through decreasing Bcl-2 and Bcl-xL in malignant glioma cells [23]. We show here that inhibiting eEF-2 kinase can significantly enhance glioma cells sensitivity to TRAIL-induced apoptosis, likely via down-regulating the expression of the anti-apoptotic protein, Bcl-xL. Taken together, the results of the current study reveal eEF-2 kinase as a potential new target that can be exploited to reinforce the efficacy of TRAIL in killing tumor cells, and may thus provide a rationale for combined use of TRAIL and an eEF-2 kinase inhibitor as a new therapeutic strategy for malignant glioma or other types of cancers.

Acknowledgments

This study was supported by a grant (R01CA135038) from the US Public Health Service and by the National Natural Sciences Foundation of China (81072146).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.038.

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