The Tumor Necrosis Factor-like Weak Inducer of Apoptosis (TWEAK)-Fibroblast Growth Factor-inducible 14 (Fn14) Signaling System Regulates Glioma Cell Survival via NFκB Pathway Activation and BCL-X\textsubscript{L}/BCL-W Expression*

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Nhan L. Tran‡, Wendy S. McDonough‡, Benjamin A. Savitch‡, Thomas F. Sawyer‡, Jeffrey A. Winkles§, and Michael E. Berens¶

The Fn14 gene encodes a type Ia transmembrane protein that belongs to the tumor necrosis factor receptor superfamily. We recently showed that fibroblast growth factor-inducible 14 (Fn14) is overexpressed in migrating glioma cells in vitro and in glioblastoma multiforme clinical specimens in vivo. To determine the biological role of Fn14 in brain cancer progression, we examined the activity of Fn14 as a potential mediator of cell survival. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-stimulated glioma cells had increased cellular resistance to cytotoxic therapy-induced apoptosis. Either TWEAK treatment or Fn14 overexpression in glioma cells resulted in the activation of NFκB and subsequently the translocation of NFκB from the cytoplasm to the nucleus. In addition, Fn14 activation induced BCL-X\textsubscript{L} and BCL-W mRNA and protein levels, and this effect was dependent upon NFκB transcriptional activity. Substitution of a putative NFκB binding site identified in the BCL-X\textsubscript{L} promoter significantly decreased Fn14-induced transactivation. Furthermore Fn14-induced transactivation of the BCL-X\textsubscript{L} promoter was also diminished by the super-repressor IκB\textalpha~ mutant, which specifically inhibits NFκB activity, and by mutations in the NFκB binding motif of the BCL-X\textsubscript{L} promoter. Additionally small interfering RNA-mediated depletion of either BCL-X\textsubscript{L} or BCL-W antagonized the TWEAK protective effect on glioma cells. Our results suggest that NFκB-mediated up-regulation of BCL-X\textsubscript{L} and BCL-W expression in glioma cells increases cellular resistance to cytotoxic therapy-induced apoptosis. We propose that the Fn14 protein functions, in part, through the NFκB signaling pathway to up-regulate BCL-X\textsubscript{L} and BCL-W expression to foster malignant glioblastoma cell survival. Targeted therapy against Fn14 as an adjuvant to surgery may improve management of invasive glioma cells and advance the outcome of this devastating cancer.

Members of the tumor necrosis factor (TNF) family are type II transmembrane proteins that are involved in the regulation of various cellular responses, including proliferation, differentiation, and apoptosis (1, 2). These proteins can exist as both membrane-associated and soluble forms and generally function as homotrimers (1). TNF-like weak inducer of apoptosis (TWEAK) is a type II membrane protein of the TNF ligand superfamily (3) that induces various cellular responses such as proliferation, survival, apoptosis, and migration (4). In both endothelial cells and astrocytes, TWEAK promotes cell proliferation and not death (5–7). TWEAK can also stimulate angiogenesis in vivo (8).

The receptor for TWEAK, fibroblast growth factor-inducible 14 (Fn14), is a member of the TNF superfamily of receptors and is characterized as a type I transmembrane receptor lacking a cytoplasmic death domain (9–11). Fn14 is an immediate-early response gene whose expression is directly activated following exposure to growth factors, fetal calf serum, and phorbol ester in fibroblasts (9, 10). Human Fn14 contains 129 amino acids, making it the smallest member of the TNF receptor superfamily identified to date (9–11). The expression of Fn14 is high in a variety of tissues including heart, placenta, kidney, lung, and pancreas and is relatively low in brain and liver (10). In cancerous tissues, Fn14 expression is elevated in hepatocellular carcinomas (10), glioblastoma multiforme (12), and pancreatic cancer (13). In addition, TWEAK binding to Fn14 or overexpression of Fn14 protein promotes nuclear factor-κB (NFκB) pathway activation that may drive the expression of several NFκB-regulated genes (14). In fact, the cytoplasmic domain of the Fn14 receptor contains a single TNF receptor-associated factor binding site flanked by two conserved threonine residues (11, 14). TNF receptor-associated factors link transmembrane receptors to the NFκB pathway and several serine/threonine protein kinase cascades, including c-Jun NH\textsubscript{2}-terminal kinase, p38, and extracellular signal-regulated kinase, that generally function to promote cellular survival and proliferation (15).

Dysregulated NFκB proteins play a role in malignant transformation by either providing continued positive growth stimuli such as that mediated by cytokines or by inhibiting apoptotic pathways (16). NFκB functions as a dimer composed of

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†To whom correspondence should be addressed: The Translational Genomics Research Institute, Neurogenomics Div., 445 North Fifth St., Phoenix, AZ 85004. Tel.: 602-343-8400; Fax: 602-343-8440; E-mail: mberens@tgern.org.

‡ The abbreviations used are: TNF, tumor necrosis factor; Fn14, fibroblast growth factor-inducible 14; TWEAK, TNF-like weak inducer of apoptosis; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase; siRNA, small interfering RNA; NFκB, nuclear factor-κB; PIPES, 1,4-piperazinedithanesulfonic acid; DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride; wt, wild type; E1, envelope protein 1.
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the RelA (p65) and NFκB1 (p50) or NFκB2 (p52) subunits. In normal resting cells, NFκB is sequestered in the cytoplasm by virtue of binding to IκB (17, 18). Cytokines such as TNF, interleukin-1, and epidermal growth factor trigger a cascade of signaling events after binding to their transmembrane receptors ultimately leading to the activation of IκB kinase, which phosphorylates IκB at two serine residues, Ser-32/36 (19, 20). Phosphorylated IκB is rapidly ubiquitinated and degraded through the 26 S proteasome pathway, releasing NFκB. Free NFκB translocates to the nucleus and binds to the promoter regions of target genes and activates their transcription (17, 21).

Both BCL-2 and BCL-X₇ are NFκB-inducible genes. Members of the BCL-2 gene family include the proapoptotic proteins BAD, BIK, and BID and the antiapoptotic proteins BCL-2, BCL-X₇, and BCL-W (22). High expression levels of antiapoptotic BCL-2-related proteins have been found in many tumors, and up-regulation of BCL-2 and BCL-X₇ has been shown to be a key element in malignancy (23) and drug resistance (24, 25). BCL-2 overexpression has been observed in several glioma cell lines and in glioma biopsies of various histological grade (26, 27) and may confer resistance to radiotherapy and chemotherapy (28–30). Malignant glioblastoma multiforme displays highly infiltrative behavior and resistance to chemo- and radiotherapy constituting major obstacles for successful therapy and patient outcome (31, 32).

To elaborate the role of Fn14 in glioma pathobiology, we examined Fn14 activation as a potential mechanism by which cell survival is fostered. We showed that TWEAK-stimulated glioma cells had increased cellular resistance to cytotoxic therapy-induced apoptosis. In addition, we demonstrated that activation of NFκB by the TWEAK-Fn14 ligand-receptor system underlies the molecular basis for resistance to apoptosis induction in glioma cells. Moreover our data indicated that NFκB protected glioma cells from cytotoxic therapy-induced apoptosis, in part, by up-regulating expression of the BCL-X₇ and BCL-W proteins.

MATERIALS AND METHODS

Cell Culture Conditions—Human astrocystoma cell lines T98G (American Type Culture Collection (ATCC), Manassas, VA) and SF767 were maintained in minimum essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) in a 37 °C, 5% CO₂ atmosphere at constant humidity. After subconfluent infection, the xL-expressing cells were plated into a 6-well plate and cultured for 24 h prior to infection.

Antibodies, Reagents, Western Blot Analysis, and Immunofluorescence—Polyclonal antibodies to IκBα and BCL-2 and monoclonal antibody to BAX were obtained from Cell Signaling Technology Inc. (Beverly, MA). Antibody to the p65 subunit of NFκB was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Poly(ADP-ribose) polymerase (PARP) antibodies and the ε-tubulin monoclonal antibody were obtained from Upstate Biotechnology (Lake Placid, NY). BCL-W polyclonal antibodies were obtained from Stressgen Biotechnologies (San Diego, CA), and monoclonal antibodies specific to BCL-X₇ were purchased from Zymed Laboratories Inc.. Monoclonal antibodies recognizing both BCL-X₇ and BCL-X₈ were obtained from Chemicon International (Temecula, CA). Monoclonal antibody to proliferating cell nuclear antigen was obtained from BD Transduction Laboratories. Human recombiant TWEAK was purchased from PeproTech (Rock Hill, NJ), and human recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was purchased from BIOSOURCE. Laminin from normal resting cells, NFκB1 (p50), or NFκB2 (p52) subunits. In normal resting cells, NFκB is sequestered in the cytoplasm by virtue of binding to IκB (17, 18). Cytokines such as TNF, interleukin-1, and epidermal growth factor trigger a cascade of signaling events after binding to their transmembrane receptors ultimately leading to the activation of IκB kinase, which phosphorylates IκB at two serine residues, Ser-32/36 (19, 20). Phosphorylated IκB is rapidly ubiquitinated and degraded through the 26 S proteasome pathway, releasing NFκB. Free NFκB translocates to the nucleus and binds to the promoter regions of target genes and activates their transcription (17, 21).

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TCT ATA; w-2, region 1733–1754, 5'-GTG GCC AGT AGT GCT GAT CTA; w-3, region 3259–3210, 5'-CTC GCC CCT GCG ATT ATT AAT), and GL2 luciferase (region 153–173, 5'-AAC GTA CGG GGA ATA CTT CGA TAG). Si oligos were described previously (39). Small interfering RNA duplex formation was performed according to the manufacturer’s instructions.

Transient transfection of siRNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were plated in a 6-well plate at 2.0 x 10^5 cells/well in 1.5 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% serum and without antibiotics. Transfections were carried out according to the manufacturer’s protocol after cells were fully spread (6 h postplating). BCL-XL and BCL-W small interfering RNAs were transfected at 20 nmol/liter. No cell toxicity was observed at 20 nmol/liter siRNA. Using quantitative PCR, we verified that the siRNA oligos to BCL-XL and BCL-W specifically inhibited the expression of BCL-XL and BCL-W, respectively, but not other members of the BCL-2 family (i.e., BCL-2 and BAX). Maximum inhibition was achieved by day 2–3 after transfection, and cells were assayed at day 3 or 4 post-transfection.

Apoptosis Assays—Apoptotic cells were evaluated by nuclear morphology of DAPI-stained cells as described previously (40). Briefly cells with condensed, fragmented chromatin were manually scored as apoptotic cells. At least five fields (total of 1000 cells) were evaluated, and data are reported as apoptotic cells/total cells x 100. Verification of apoptotic cells was conducted by co-immunofluorescence staining using a monoclonal antibody against activated cleaved caspase 3 (Promega). At least 1000 cells per treatment were evaluated for condensed chromatin and activated caspase 3. In addition, nuclear PARP proteolytic cleavage was assessed by immunoblotting analysis of cellular lysates as described above using antibodies recognizing both the intact (116-kDa) and proteolytic (85-kDa) forms of PARP. In certain experiments, TWEAK (200 ng/ml) was preincubated with Fas14-Fc decoy receptor (2.5 μg/ml) or control mouse IgG as described previously (7, 12).

Transfection and Dual Luciferase Reporter Assays—Dual luciferase reporter assays were performed according to the manufacturer’s protocols (Promega). Cells were plated in a 24-well tissue culture dish and then incubated in normal growth medium for 24–48 h until 70% confluence was reached. Cells were transiently transfected with 0.5 μg of the Renilla luciferase reporter gene (Clontech), BCL-X promoter/luciferase reporter gene (pGL2(2484)) or mutant pGL2-BM (41), or pGL basic promoter/enhancerless luciferase reporter gene (Promega) using Effectene reagent (Qiagen). In some cases, cells were co-transfected with pCMV-βGalM or control pcDNA3.1 (1 μg). As an internal standard, all plasmids were co-transfected with 50 ng of pRL-TK (Promega), which contains the Renilla luciferase gene. At 6 h post-transfection, the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 0.1% fetal bovine serum. In certain experiments cells were either treated with 100 ng/ml TWEAK or infected with adenoviruses expressing Fn14wt, Fn14ct, or LacZ. All cells were harvested 48 h after transfection, washed twice with phosphate-buffered saline, and lysed in passive lysis buffer. All treatments were done in triplicate for each experiment. Luciferase activity was measured using a TD-20/20 luminometer (Turner Designs, Sunnydale, CA) and normalized against the activity of the Renilla luciferase gene for differences in transfection efficiency. The results were expressed as relative light units of luciferase activity to Renilla activity.

RESULTS
TWEAK Protects Glioma Cells from Cytotoxicity-Induced Apoptosis through Activation of the Fas14 Receptor—TRAIL, a member of the TNF family, exhibits selective cytotoxicity for glioma cells versus non-neoplastic astrocytes in vitro (42). Unlike TRAIL, TWEAK induces cell proliferation and not apoptosis when added to astrocytes (5, 6, 43). Here we investigated the effect of TWEAK on glioma cell survival. TRAIL treatment of T98G glioma cells induced apoptosis (Fig. 1A) indicated by cells showing condensed, fragmented chromatin revealed using a DAPI nuclear morphology staining technique (40). Apoptotic cells were further validated by co-immunofluorescent detection of activated caspase 3 (data not shown). Pretreatment of cells with TWEAK for 2 h prior to TRAIL addition reduced TRAIL-induced apoptosis by 50% (Fig. 1A). Progressive reduction of the percentage of cells undergoing TRAIL-induced apoptosis was observed using longer preincubations with TWEAK. No changes in base-line apoptosis was observed in cells treated with TWEAK alone (Fig. 1A). Immuno-
results suggest that TWEAK induces glioma cell survival via activation of the Fn14 receptor. We also observed that TWEAK treatment of the TRAIL-resistant glioma cell line SF767 inhibited camptothecin-induced apoptosis (Fig. 1C).

To confirm that the biological effect of TWEAK on glioma cell survival was a result of signaling initiated by interaction with the Fn14 receptor, we infected both T98G and SF767 glioma cells with recombinant replication-deficient adenoviruses expressing either Fn14wt or a truncated Fn14 protein missing amino acids 112–129 (Fn14tCT), a region containing the TNF receptor-associated factor binding sequence motif as described by Brown et al. (14). It has been reported previously that Fn14tCT cannot activate downstream pathways such as the NFκB pathway (14). We found that overexpression of Fn14tCT inhibited TWEAK suppression of cytotoxic therapy-induced apoptosis as compared with overexpression of Fn14wt (Fig. 2, A, lanes c and f, and B, lanes o and p). Whereas expression of Fn14wt had no cytotoxic effect on glioma cells (Fig. 2, A, lane g, and B, lane q), apoptosis was detected in cells expressing Fn14tCT (Fig. 2, A, lane j, and B, lane t). To determine whether Fn14 overexpression could promote cell survival, glioma cells were infected with Fn14wt adenoviruses prior to addition of TWEAK and liberation of NFκB from the cytoplasm to the nucleus after 2–4 h (Fig. 3A, b and c, arrows) similar to treatment with the positive control NFκB activator phorbol 12-myristate 13-acetate (Fig. 3A, d). Blocking TWEAK binding to endogenous Fn14 by preincubation with Fn14-Fc decay receptor prevented TWEAK-induced NFκB translocation to the nucleus (Fig. 3A, e). However, preincubation of TWEAK with control mouse IgG did not prevent NFκB translocation to the nucleus (Fig. 3A, f). To further validate TWEAK induction of NFκB nuclear translocation, we isolated the nuclear fraction of cells stimulated with TWEAK and immunoblotted for the NFκB p65 subunit. Densitometric analysis revealed an increased level of p65 protein in the nuclear lysates of cells exposed to TWEAK with a 2-fold increase after 30 min compared with untreated cells (Fig. 3B), consistent with the immunofluorescence findings.

NFκB translocation and transcriptional activity are inhibited by its association with IκBα proteins (48). For NFκB nuclear translocation and transcriptional activation to occur, phosphorylation of IκBα has to occur at serine residues 32 and 36, which results in proteosome-mediated IκBα degradation and liberation of NFκB (17). Immunoblot analysis of whole cellular lysates of T98G cells after TWEAK treatment using an anti-IκBα antibody showed an induction of IκBα phosphorylation over the indicated time (Fig. 3C). Phosphorylation of IκBα was detected after 30 min and persisted to the 4-h time point. Concomitantly the level of endogenous IκBα protein decreased, as expected, upon TWEAK treatment, corresponding to IκBα phosphorylation. In addition, translocation of NFκB to the nucleus in glioma cells promoted high transcriptional activity as shown in Fig. 3D. T98G cells were transfected with the NFκB enhancer/luciferase reporter plasmid and the Renilla luciferase gene to correct for transfection efficiency. Luciferase assays revealed that upon TWEAK treatment there was a 45-fold induction of NFκB transcriptional activity over the activation level detected in the vector-transfected cells. However, TWEAK-stimulated NFκB transcriptional activity was sup-

![Diagram](http://www.jbc.org/content/3486/3/3486/F2.large.jpg)
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FIG. 3. Effect of TWEAK treatment on NFκB cellular localization, IκBα phosphorylation, and NFκB activation in glioma cells. A, T98G cells were cultured under reduced serum and then treated with solvent control phosphate-buffered saline (a) TWEAK (100 ng/ml) for 2 (b) or 4 h (c) or with TPA (100 nM) for 2 h (d). In certain experiments, TWEAK was preincubated with soluble Fn14-Fc decoy receptor (e) or control mouse IgG (f) prior to addition. Cells were fixed and stained for the p65 subunit of NFκB or proliferating cell nuclear antigen (PCNA). Arrows indicate NFκB p65 immunostaining in the cell nucleus (N), and asterisks indicate NFκB p65 in the cytoplasm. Bar, 20 μm. B, T98G cells were treated with 100 ng/ml TWEAK for various time periods as indicated above. Following treatment the cells were lysed, and nuclear extracts were prepared and immunoblotted for either the p65 subunit of NFκB or proliferating cell nuclear antigen (PCNA). C, whole cell lysates of TWEAK-treated cells were immunoblotted for phospho-IκBα, total IκBα, or α-tubulin. D, cells were co-transfected with the NFκB enhancer/luciferase reporter plasmid and the pRL-TK plasmid in combination with either the pCDNA3.1 vector (V) or the pCMV-IκBαM (IκBαM) plasmid. Cells were then cultured for 16 h under reduced serum conditions prior to TWEAK addition. After 6 h, cell lysates were prepared and assayed for luciferase and Renilla activity using the dual luciferase reporter assay system. The promoter activity was normalized to the activity of the Renilla and further normalized to untreated cells. The results are expressed as relative light units of luciferase activity to Renilla activity. Values represent mean ± S.D. of observations from three independent experiments, each carried out in duplicate. WB, Western blot.

FIG. 4. TWEAK induces BCL-XL and BCL-W mRNA and protein expression. A, total RNA was extracted from cells treated with TWEAK for various time periods and then analyzed by quantitative PCR for BCL-XL and BCL-W mRNA and normalized to histone H3.3 expression. Values for BCL-XL and BCL-W were then normalized to untreated control. Data represent the mean and S.D. of three independent experiments. B, immunoblot analyses of BCL-XL, BCL-W, BCL-2, BAX, and α-tubulin protein levels upon TWEAK treatment. Cellular lysates were separated by 12% SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with antibodies to BCL-XL, BCL-W, BCL-2, and BAX.

pressed when cells were co-transfected with the super-repressor form of IκBα mutant (IκBαM), which specifically suppresses NFκB activity (Fig. 3D).

Regulation of BCL-XL and BCL-W Expression by the TWEAK-Fn14 Ligand-Receptor System Is Dependent upon NFκB Activity—NFκB has been shown to regulate the expression of genes that actively participate in controlling cell survival (BCL-2, BCL-XL, survivin, and the inhibitor of apoptosis protein family) (17, 21). To understand the mechanisms of TWEAK-induced inhibition of apoptosis in glioma cells, we examined the mRNA levels of several BCL-2-related genes, including both antiapoptotic genes (BCL-2, BCL-W, and BCL-XL) and proapoptotic genes (BCL-XS and BAX) using real time quantitative PCR analysis. We reasoned that increased resistance to apoptosis might be attributed to increased expression of the antiapoptotic genes. Cells treated with TWEAK for various lengths of time showed no changes in BCL-2 and BAX mRNA levels, and BCL-XS mRNA expression could not be detected in unstimulated or TWEAK-stimulated cells (data not shown). However, BCL-XL and BCL-W transcripts were induced in a time-dependent manner upon TWEAK treatment. Increased BCL-XL and BCL-W mRNA expression was detected at 4 h, and maximal induction was noted at the last time point examined, 24 h (Fig. 4A). The effect of TWEAK on BCL-XL and BCL-W mRNA expression was inhibited when cells were infected with an adenovirus expressing the Fn14ct protein (Fig. 5A, lane d).

To determine whether the regulation of BCL-XL and BCL-W
mRNA expression by TWEAK was dependent upon NFκB activity, we inhibited NFκB activation using a cell-permeable pharmacological peptide inhibitor, SN50. SN50 contains a nuclear localization sequence of the p50 subunit of NFκB and functions to inhibit the translocation of the NFκB active complex into the nucleus (49). TWEAK-stimulated BCL-X<sub>L</sub> and BCL-W mRNA expression was repressed in the presence of SN50 (Fig. 5A, lane e), while the cell-permeable inactive control peptide, SN50M, had no effect on TWEAK-stimulated BCL-X<sub>L</sub> and BCL-W mRNA expression (Fig. 5A, lane f). Similarly cells infected with IκBαM also had reduced TWEAK-stimulated BCL-X<sub>L</sub> and BCL-W mRNA expression (Fig. 5A, lane g).

Changes in BCL-X<sub>L</sub> and BCL-W protein levels following TWEAK stimulation were analyzed by Western blot analysis. As shown in Fig. 4B, BCL-X<sub>L</sub> protein levels increased in a time-dependent manner upon TWEAK addition. Densitometric analysis of the BCL-X<sub>L</sub> signal intensity revealed a 4-fold induction after 24 h of TWEAK treatment. Interestingly an increase in BCL-X<sub>L</sub> protein levels was observed as early as 30 min. In comparison, little change in BCL-W protein expression was detected over 8 h of TWEAK exposure, but a maximal 2-fold increase was detected at the 16- and 24-h time points (Fig. 4B).

In contrast, TWEAK did not change BAX or BCL-2 protein levels (Fig. 4B); in addition, BCL-X<sub>S</sub> protein expression was not detected in unstimulated or TWEAK-stimulated cells (data not shown). Moreover both inhibition of Fn14 signaling by Fn14<sup>tCT</sup> and NFκB inactivation by IκBαM suppressed TWEAK-elevated BCL-X<sub>L</sub> and BCL-W protein expression after 24 h (Fig. 5B), corroborating the changes in mRNA expression.

**TWEAK Addition or Fn14 Overexpression Transactivates the BCL-X Promoter via the NFκB Pathway**—We assessed whether TWEAK could enhance BCL-X promoter activity via NFκB activation. Studies by Tsukahara et al. (41) identified the NFκB binding motif at positions −848 to −840 of the BCL-X promoter. This putative NFκB element is reported to be a binding site for the p65 and p50 subunits of NFκB (41). T98G glioma cells were co-transfected with a luciferase reporter proceeded by a 5′ deleted portion of the BCL-X promoter containing the NFκB binding site (pGL848) along with the Renilla luciferase plasmid. As shown in Fig. 6, the luciferase activity in cells stimulated with TWEAK was consistent with 8-fold higher than that in non-treated cells (compare lanes a and b). However, the luciferase activity in TWEAK-stimulated cells infected with Fn14<sup>tCT</sup> (Fig. 6, lane d) was almost equivalent to that in control cells. Similarly forced overexpression of Fn14 receptor independent of TWEAK resulted in a 6-fold induction of luciferase activity as compared with LacZ control (Fig. 6, compare lanes a and c). Furthermore a mutated NFκB-luc plasmid, pGL2xBM, which has the same length as pGL2(848) but possesses CC-to-GG mutations at positions −841 and −840 within the NFκB motif,
To investigate the role of NFκB in TWEAK-induced glioma cell survival, we antagonized NFκB activity using the super-repressor IκBαM mutant. T98G glioma cells were infected with IκBαM or LacZ adenoviruses for 24 h. Cells were cultured in reduced serum for 16 h prior to treatment with or without TWEAK and TRAIL. Cells were then fixed 24 h later and immunostained for DAPI and active caspase 3. The number of total and apoptotic cells was determined as described under “Materials and Methods.” Values represent the mean and S.D. of five replicate measurements.

Inhibition of NFκB Activity Antagonizes TWEAK-induced Cell Survival—To investigate the role of NFκB in TWEAK-induced glioma cell survival, we antagonized NFκB function using the super-repressor IκBαM mutant. T98G glioma cells were infected with IκBαM or control LacZ adenoviruses. Cells were then pretreated with TWEAK for 6 h prior to addition of TRAIL. As described earlier, TWEAK conferred resistance to TRAIL-induced apoptosis (Fig. 7, lanes c and d). However, inhibition of NFκB function by IκBαM suppressed TWEAK-induced cell survival (Fig. 7, lane e). Cell survival induced by forced overexpression of Fn14 was also inhibited by IκBαM (Fig. 7, lanes f and g). Similar data were observed for SF767 cells (data not shown). Taken together, our results show that TWEAK-Fn14 ligand-receptor signaling to the NFκB transcription factor fosters glioma cell resistance toward cytotoxic therapy-induced apoptosis.

Small Interfering RNA-mediated Depletion of BCL-X or BCL-W Inhibits TWEAK-induced Cell Survival—To further examine whether BCL-X and BCL-W are critical for TWEAK-mediated glioma cell survival, we inhibited the expression of BCL-X and BCL-W by transient transfection of small interfering RNA oligonucleotide duplexes. Three independent siRNA oligos were designed against BCL-X and BCL-W. Likewise, BCL-W siRNA oligos did not affect the expression levels of BCL-2 and BCL-W; similarly BCL-W siRNA oligos did not affect the expression levels of BCL-2 and BCL-W (Fig. 8A). Infection of protein expression was also verified by Western blot analysis using antibodies specific to either BCL-X or BCL-W (Fig. 8B) or BCL-W (Fig. 8C). Small interfering RNA-mediated depletion of BCL-X resulted in a 2-fold induction of apoptosis in T98G (Fig. 8D, lanes k and o) and SF767 (Fig. 8E, lanes k and o) cells in the presence of cytotoxic drugs as compared with cytotoxic drug-treated untransfected (Fig. 8, D and E, lane c) or control siRNA luciferase-transfected cells (Fig. 8, D and E, lane g). Likewise depletion of BCL-W antagonized TWEAK-induced cellular survival in both T98G and SF767 cells (Fig. 8, D and E, lane p). Interestingly, in T98G BCL-X-depleted cells, TWEAK conferred a semiprotective effect on TRAIL-induced apoptosis, suggesting that BCL-W may compensate for the TWEAK survival effect (Fig. 8D, lane l). However, in SF767 cells, shutdown of BCL-X suppressed camptothecin-induced apoptosis (Fig. 8E, lane l).

We next determined whether suppression of both BCL-X and BCL-W would result in the enhancement of cytotoxic therapy-induced apoptosis. Shutdown of both BCL-X and BCL-W expression in untreated or control luciferase siRNA-treated cells resulted in ~20–25% cellular apoptosis (Fig. 8, D and E, lane q), highlighting the constitutive survival function of these mediators. In the presence of cytotoxic drugs, ~60–80% apoptotic cells were observed, which was 20% higher compared with shutdown of either BCL-X or BCL-W alone (Fig. 8, D and E, lane s). In addition, these cells were refractory to TWEAK-induced cell survival. Thus, these results suggest that both BCL-X and BCL-W are critical for the TWEAK survival response.

DISCUSSION

In the present study we described a role for the TWEAK-Fn14 ligand-receptor system in promoting survival of glioma cells through the transcriptional regulation of two antiapoptotic BCL-2 family members, BCL-X and BCL-W. We demonstrated that the NFκB pathway is an important downstream target of TWEAK-Fn14 signaling using both a pharmacological peptide inhibitor (SN50) and a super-repressor IκBα mutant. Activation of the Fas receptor resulted in elevated expression of BCL-X and BCL-W. Inhibition of NFκB function diminished BCL-X and BCL-W expression, suggesting that TWEAK-Fn14 signaling promotes glioma cell survival by small interfering RNA oligos antagonized TWEAK regulation of cell survival, subsequently making glioma cells susceptible to cytotoxic therapy-induced apoptosis. Moreover, we showed that activation of the BCL-X promoter by Fn14 was regulated through NFκB, supporting the notion that the TWEAK-Fn14 ligand-receptor system plays a role in apoptosis prevention in glioma cells.

Elevated NFκB activity has been observed in various carcinoma cells and glioblastoma multiforme (50–52). NFκB activation in malignant cells can result in resistance to certain chemotherapeutic agents, irradiation, and cytokines, an important characteristic of malignant glioma (53–56). This activation may contribute to cellular resistance to cytotoxic interventions by preventing apoptosis. We speculate that inhibition of NFκB may confer sensitivity of glioma cells to these agents. TWEAK has been shown to induce NFκB activation via the Fas receptor, which results in a rapid (14) and long lasting NFκB activation via IκBα and p100 regulation (45). Our results are consistent with this latter report; indeed both IκBα phosphorylation and NFκB nuclear translocation were observed for as long as 4 h post-TWEAK stimulation and then diminished over time. Previously we demonstrated that Fn14 mRNA is highly expressed in glioma cells in vivo and enhanced in migrating cells in vitro (12). It is possible that the pathophysiological roles of TWEAK-Fn14 signaling may contribute to constitutive NFκB activity in invasive glioma cells and hence lead to resistance to chemo- and irradiation therapies.

Both BCL-X and BCL-W belong to the subfamily of antiapoptotic BCL-2 family members that share several antiapoptotic features with BCL-2. These proteins are able to differentially block chemo- and irradiation therapy-induced cell death (24). The balance between antiapoptotic and proapoptotic BCL-2 family members has been described as a pri-
mary event in determining the susceptibility to apoptosis through maintaining the integrity of the mitochondria and inhibiting activation of the caspase cascade (22). High expression levels of antiapoptotic BCL-2-related proteins have been found in many tumors, and up-regulation of these proteins has been shown to be a key element in tumor malignancy and drug resistance (22, 24). BCL-2 and BCL-XL overexpression has been observed in several glioma cell lines and in glioma biopsies regardless of histological grade (26, 27, 57). Down-regulation of BCL-2 and/or BCL-XL expression using antisense oligonucleotides abolishes tumorigenicity and enhances chemosensitivity in human malignant glioma cells (57–59). In addition, overexpression of BCL-2 inhibits TRAIL-induced apoptosis in various carcinoma cell lines and also in gliomas (59). However, in this study, we observed changes in BCL-XL and BCL-W expression consequent to TWEAK-Fn14 signaling but no alterations in BCL-2 or BCL-XS expression. In addition, depletion of BCL-XL and BCL-W levels antagonized the TWEAK survival response, suggesting that up-regulation of BCL-XL and BCL-W may be critical for TWEAK protection against cytotoxic therapy-induced apoptosis.

The BCL-X promoter is distinct from the BCL-2 promoter and is regulated by different transcriptional activators (60, 61). The BCL-X gene encodes a full-length pre-mRNA transcript that is capable via alternative RNA splicing to produce several protein products with either antiapoptotic (BCL-X long) or proapoptotic (BCL-X short) activity (60). Different promoter regions have been described in the regulation of the expression of these splice variants (60). Differences in the use of the promoter region resulting in the increased expression of BCL-XL or BCL-XS are attributed to cell type and differentiation status of the cell (62). In certain cell types, transcription of the BCL-X gene is controlled by NFkB (41, 63). Binding sites for the active NFkB subunits p56/RelA and c-Rel have been demonstrated using functional analysis of the BCL-X promoter (41, 63, 64). Our results demonstrate that TWEAK-Fn14 signaling is able to increase the promoter activity of the BCL-X gene and that this response is dependent upon NFkB activation. TWEAK-mediated BCL-X promoter activation was profoundly inhibited by a super-repressor mutant of IκBα (Fig. 6) or by introduction of mutations in the NFκB-like element in the mouse BCL-X promoter constructs. Thus, these findings further support the role of NFkB in TWEAK-induced BCL-X expression.

The highest level of BCL-XL mRNA expression was observed at 24 h post-TWEAK stimulation. Although the protein level of BCL-XL corresponded to the mRNA expression at 24 h, rapid increase of this protein level was observed 30 min after
TWEAK addition (Fig. 5A). These data argue for the presence of additional signal(s) from the TWEAK-Fn14 ligand-receptor system that potentially influence the protein stability of BCL-XL. One mechanism by which the cellular level of BCL-XL can be regulated is through the activity of the AKT kinase. Activated AKT increases BCL-XL protein stability through the phosphorylation of the proapoptotic protein BAD on Ser-136 (22). In the absence of activated AKT, BAD forms heterodimers with BCL-XL and prevents the release of cytochrome c from the mitochondria (65, 66). This complex formation abrogates the antiapoptotic function of BCL-XL (67, 68), thus facilitating apoptosis via a cytochrome c-dependent pathway. Conversely when AKT is activated, BAD becomes phosphorylated and is sequestered in the cytoplasm by interacting with 14-3-3 scaffolding proteins; this in turn suppresses apoptosis (69). Preliminarily we observed phosphorylation of AKT on Ser-473 upon TWEAK stimulation and subsequently BAD phosphorylation on Ser-136. Current investigations are exploring the signaling pathway(s) from the Fn14 receptor that may impact the stability and function of BCL-XL at the protein level.

There is presently little information available on the regulation of BCL-W and the mechanism by which it suppresses cell death. Although the promoter of BCL-W is not characterized, our data suggests that BCL-W expression is regulated through the NFκB pathway since inhibition of NFκB activity suppresses TWEAK induction of BCL-W expression. It has been proposed that BCL-W is localized to the mitochondria and nuclear envelopes, the same sites where BCL-XL and BCL-2 reside (70, 71). Like BCL-2, increased levels of BCL-W can suppress cell death by blocking stress-activated protein kinase/c-Jun NH2-terminal kinase activation (72). In addition, BCL-W is expressed in various tissues including the brain, testis, heart, and intestines (70, 71) and plays an important antiapoptotic role in regulating the survival of neurons (73). Furthermore BCL-W expression is elevated in certain tumor cell lines of epithelial origin such as colonic, cervical, and breast cancer cells (71). In fact, our gene expression profiling of glioma cells from patient biopsy specimens identified BCL-W as a candidate gene up-regulated in invasive glioma cells (74). Immunohistochemical analysis of BCL-W in glioma biopsy specimens confirmed that BCL-W was expressed in the invading cancer cells but not in the neighboring normal brain cells, implying that BCL-W expression may be important for invasive glioma cell survival. This result is similar to those reported in infiltrative morphotypes of gastric cancer by Lee and colleagues (72).

Our study further indicates that Fn14 overexpression independent of TWEAK may drive the promoter activity of BCL-XL, and this activity is not observed if there are mutations in the NFκB binding motif. In fact, we found that glioma cells overexpressing Fn14 were able to suppress cytotoxic therapy-induced apoptosis, and cell survival was diminished when NFκB activity was suppressed. These data are consistent with a previous study demonstrating that Fn14 overexpression in NIH 3T3 cells resulted in increased NFκB transcriptional activity (14). In our earlier report, we demonstrated that Fn14 expression is induced in migration-activated glioma cells in vitro and significantly increases according to tumor grade with the highest levels in glioblastoma tissue specimens (12). In comparison, TWEAK mRNA levels are low in glioblastoma samples relative to normal brain tissue (12). It is possible that overexpression of Fn14 in glioblastoma multiforme may result in the aberrant activation of NFκB resulting in increased transcriptional activity of survival factors such as BCL-XL and BCL-W. This may possibly explain how invasive glioma cells affect resistance toward chemotherapeutic and cytotoxic agents. In the treatment of glioma, sensitivity or resistance of tumor cells to cytotoxic therapy has substantial clinical consequences. However, the molecular mechanisms and/or intrinsic factors controlling cellular resistance are not well understood. In the present study, regulation of two key NFκB genes, BCL-XL and BCL-W, by the TWEAK-Fn14 ligand-receptor system enhanced glioma cell resistance to both TRAIL- and camptothecin-induced apoptosis. Our results offer a potential mechanism by which the TWEAK-Fn14 signaling system can contribute to the regulation of glioma cell survival potentially by up-regulation of BCL-XL and BCL-W expression. Thus, understanding the function of Fn14 may lead to the development of effective therapies against invasive gliomas.

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Nhan L. Tran, Wendy S. McDonough, Benjamin A. Savitch, Thomas F. Sawyer, Jeffrey A. Winkles and Michael E. Berens

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