Tumor Necrosis Factor–Like Weak Inducer of Apoptosis Stimulation of Glioma Cell Survival Is Dependent on Akt2 Function

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Abstract

Malignant gliomas are the most common primary brain tumors. Despite intensive clinical investigation and significant technical advances in surgical and radiation treatment, the impact on clinical outcome for patients with malignant gliomas is disappointing. We have previously shown that tumor necrosis factor–like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor superfamily, can stimulate glioma cell survival via binding to the Fn14 receptor, activation of the NF-κB pathway, and upregulation of *BCL-X$_L$* gene expression. Here, we show that TWEAK treatment of glioma cells leads to phosphorylation of Akt and BAD. TWEAK stimulation results in the phosphorylation of both Akt1 and Akt2. However, small interfering RNA (siRNA)–mediated depletion of either Akt1 or Akt2 showed that BAD serine 136 phosphorylation is dependent specifically on Akt2 function. Depletion of Akt2 expression by siRNA also abrogates TWEAK-stimulated glioma cell survival, whereas no effect on glioma cell survival was observed after siRNA-mediated depletion of Akt1 expression. Surprisingly, although siRNA-mediated depletion of BAD in glioma cells abrogates cytotoxic- and chemotherapy-induced apoptosis, TWEAK still displays a strong protective effect, suggesting that BAD serine 136 phosphorylation plays a minor role in TWEAK-Akt2–induced glioma cell survival. We also report here that *AKT2* gene expression levels increased with glioma grade and inversely correlate with patient survival. Additionally, immunohistochemical analysis showed that Akt2 expression positively correlates with Fn14 expression in glioblastoma multiforme specimens. We hypothesize that the TWEAK-Fn14 signaling axis functions, in part, to enhance glioblastoma cell survival by activation of the Akt2 serine/threonine protein kinase. (Mol Cancer Res 2009;7(11):1871–81)

Introduction

Glioblastoma multiforme (GBM) is the most malignant form of all primary adult brain tumors (1). Although significant technical advances in surgical and radiation treatment for brain tumors have emerged, their impact on clinical outcome for patients has been disappointing (2-4). Of the features that characterize GBM, arguably none is more clinically significant than the capacity of glioma cells to infiltrate into normal brain tissue (5). These invasive cells render tumor resection ineffective, and confer resistance to chemotherapy and radiation therapy. To date, little is known about the molecular mechanisms that contribute to the resistance phenotype.

The tumor necrosis factor (TNF) ligand superfamily and their cognate receptors are involved in the regulation of various cellular responses including proliferation, differentiation, and apoptosis (6). Of interest, tumor necrosis factor–like weak inducer of apoptosis (TWEAK) and its receptor Fn14 are members of the tumor necrosis factor and tumor necrosis factor receptor superfamilies, respectively, and TWEAK-Fn14 axis signaling has been implicated in cancer progression and survival (7). We reported that Fn14 mRNA expression is upregulated in migration-stimulated glioma cells *in vitro* and invading cells *in vivo* (8, 9). Across tumor grades, Fn14 is most significantly overexpressed in GBM tissue, whereas in normal brain tissue, the expression of Fn14 is minimal-to-absent (8, 9). TWEAK binding to the Fn14 receptor activates the NF-κB signaling pathway (10, 11). In our earlier report, we showed that two key NF-κB–inducible proteins, Bcl-x$_L$ and Bcl-w, contribute to TWEAK-enhanced glioma cell resistance to cytotoxic therapy–induced apoptosis (11).

One means by which Bcl-x$_L$ cellular levels are regulated is through activation of the serine/threonine kinase Akt/protein kinase B, a downstream effector of the phosphoinositide 3-kinase (PI3K) pathway (12). To date, three members of this family, Akt1, Akt2, and Akt3, have been identified and are independently activated by phosphorylation on conserved serine residues at aa473 (Akt1), aa474 (Akt2), or aa472 (Akt3), as well

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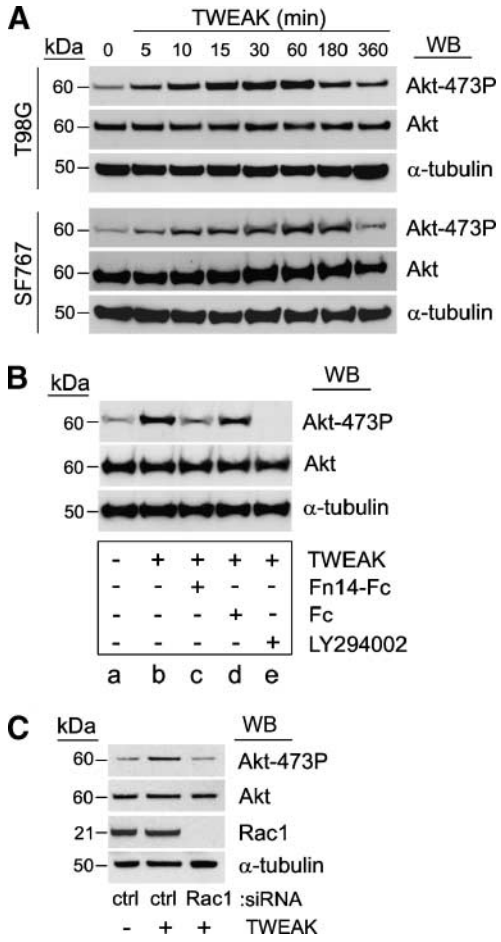

FIGURE 1. TWEAK-induced Akt Ser-473 phosphorylation is dependent on PI3K and Rac1. **A.** T98G and SF767 glioma cells were treated with TWEAK and then harvested at the indicated time. Protein lysates were analyzed by Western blotting using antibodies to Akt Ser-473, total Akt, and α-tubulin. **B.** T98G cells were treated with TWEAK, TWEAK preincubated with Fn14 decoy receptor (Fn14-Fc), TWEAK preincubated with Fc protein, or pretreated with LY294002 for 10 min before TWEAK addition. Total cell lysates were collected after 10 min of TWEAK treatment and analyzed for phosphorylated Akt-473, total Akt, and α-tubulin. **C.** T98G cells were transfected with siRNA targeting control luciferase or Rac1 for 24 h. Cells were treated with TWEAK for 10 min and protein lysates were analyzed for phosphorylated Akt-473, total Akt, Rac1, and α-tubulin. Each panel is a representation of three independent experiments.

as on threonine residues at aa308 (Akt1), aa309 (Akt2), or aa305 (Akt3; ref. 13). The isoforms share many common substrates through their preferential phosphorylation of a motif with the sequence R*X*R*XX*(S/T) (ref. 14). Several Akt-regulated gene products that have roles in the regulation of apoptosis, including the proapoptotic proteins BAD and caspase-9 (15, 16), have been identified. Activated Akt increases Bcl-x_L protein stability through the phosphorylation of BAD on Ser-136 (15). Phosphorylated BAD is sequestered in the cytoplasm by interacting with 14-3-3 scaffolding proteins, thus blocking BAD binding to Bcl-x_L (15).

Although the three Akt isoforms are structurally homologous and share similar mechanisms of activation, they also exhibit distinct biological features. For instance, Akt1 is more highly expressed in tissues such as the thymus and lung, whereas Akt2 overexpression has been found in cancer of the ovary, breast, and pancreas where it has been implicated in the processes of invasion and metastasis (17-23). In contrast, Akt3 has been reported to be more limited in tissue distribution, with high levels in brain, heart, and kidney (24), and seems to function in promoting melanoma cell survival (25).

We previously reported that TWEAK is a survival factor for glioma cells. This effect depends on the activation of the NF-κB pathway and subsequent upregulation of Bcl-x_L and Bcl-w protein expression (11). Here, we show that TWEAK stimulation results in Akt activation and phosphorylaton of the proapoptotic protein BAD. Activation of Fn14 by TWEAK results in phosphorylation of both Akt1 and Akt2. However, siRNA-mediated depletion of Akt1 or Akt2 showed that BAD serine 136 phosphorylation is dependent specifically on Akt2 function. Inhibition of Akt2 expression by siRNA also abrogates TWEAK-induced glioma cell survival, whereas no effect on glioma cell survival was observed after siRNA-mediated inhibition of Akt1 expression. Additionally, although inhibition of BAD expression rescues glioma cells from cytotoxic- and chemotherapy-induced apoptosis, TWEAK still displays a strong protective survival effect. Furthermore, Akt2 mRNA expression levels positively correlate with both brain tumor grade and malignancy, and with poor clinical outcome. Finally, Akt2 protein levels correlate with Fn14 protein levels in GBM specimens, supporting the notion that Fn14 signaling through Akt2 may contribute to the poor response observed when patients with brain tumors are treated with chemotherapy and radiation therapy.

Results

TWEAK Stimulates Akt-Ser473 Phosphorylation through Activation of the Fn14 Receptor

We previously showed that TWEAK-stimulated glioma cells increase expression of the antiapoptotic protein Bcl-x_L, resulting in an enhancement of glioma cell survival (11). The protein kinase Akt is a critical regulator of cell survival and it has been reported that Akt activation can increase Bcl-x_L protein stability (26, 27); therefore, we evaluated whether TWEAK stimulation of glioma cells could affect the activation state of Akt by assessing phosphorylation of Akt on serine-473. In these experiments, we used a serine-473 phospho-specific antibody that monitors the phosphorylation status of all Akt isoforms [at either serine 473 (Akt1), serine 474 (Akt2), or serine 472 (Akt3)]. Immunoblot analysis of whole cellular lysates of glioma cells after TWEAK treatment showed an induction of Akt-Ser473 phosphorylation (Fig. 1A). Rapid phosphorylation of Akt-Ser473 was detected after 5 minutes for both T98G and SF767 cells upon TWEAK treatment, which then diminished after 1 hour. To investigate whether TWEAK stimulation of Akt-Ser473 phosphorylation could be blocked with a TWEAK antagonist, a soluble Fn14-Fc decoy receptor was applied (11, 28). Pretreatment of TWEAK with the Fn14-Fc decoy receptor before TWEAK application to the cells did not result in an increase in Akt-Ser473 phosphorylation, whereas phosphorylation of Akt-Ser473 was detected after control Fc treatment (Fig. 1B). These results suggest that TWEAK induces Akt-Ser 473 phosphorylation via binding to the Fn14 receptor.

Two additional experiments were conducted to further investigate TWEAK activation of Akt phosphorylation. First, to determine whether TWEAK induction of Akt-Ser473 phosphorylation was dependent on PI3K activity, glioma cells were treated with the PI3K inhibitor, LY294002. Akt-Ser473 phosphorylation was blocked in the presence of LY294002, thus indicating that TWEAK activation of Akt is dependent on PI3K (Fig. 1B). Second, we have reported that TWEAK can induce Rac1 activation (9). Because the PI3K-Akt pathway can be affected by Rac1 activity (29-31), we determined whether TWEAK-induced Akt-Ser473 phosphorylation was dependent on Rac1 by inhibiting Rac1 expression using a siRNA approach. siRNA-mediated depletion of Rac1 expression resulted in a >90% shutdown of Rac1 protein expression and abrogated TWEAK-induced Akt-Ser473 phosphorylation (Fig. 1C).

Analysis of Akt Isoform Expression Profiling Using a Publicly Available Database

Specific signaling roles for individual Akt isoforms have begun to emerge (19, 32, 33). As an initial approach to investigate which Akt isoforms could contribute to aberrant signaling in glioma cells, we surveyed probes for the three Akt isoforms on global expression arrays from a panel of 24 non-neoplastic and 111 anaplastic astrocytoma and GBM specimens [National Center for Biotechnology Information (NCBI) Gene Expression Omnibus data set GSE4290]. We found that both Akt1 and Akt2 mRNA expression levels correlate with increased glial tumor grade with highest expression in GBM specimens ($P < 0.001$; Fig. 2A). In contrast, Akt3 mRNA expression is relatively high in non-neoplastic brain specimens and seems to decrease in glial tumors, with low expression in GBM specimens ($P < 0.001$; Fig. 2A).

We applied principle component analysis to query the relationship between the expression of each of the Akt isoforms and patient outcome (9). By principle component analysis, GBM patients were segregated into two separate clusters displaying distinct Kaplan-Meier survival curves. Cluster 1 has a median survival time of 952 days (long term), whereas cluster 2 has a median survival of 401 days (short term; ref. 9). Analysis of the expression value for each Akt isoform showed that GBM patients in the short-term survival cluster had higher Akt1 ($P < 0.01$) and Akt2 ($P < 0.001$) mRNA levels than GBM patients in the long-term survival cluster. In contrast, Akt3 mRNA expression is significantly higher in GBM patients in the long-term survival cluster compared with GBM patients in the short-term survival cluster. These findings suggest that high Akt1 and Akt2 expression levels correlate with brain tumor grade and poor patient outcome.

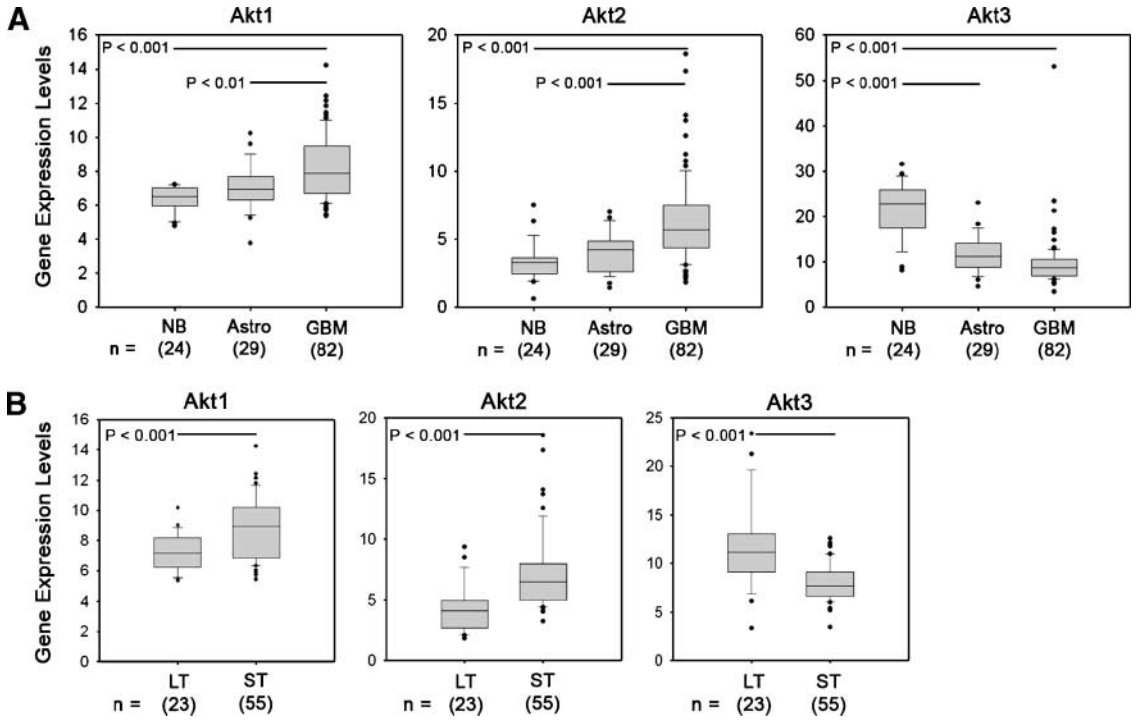

FIGURE 2. Gene expression profiling of Akt isoforms across non-neoplastic brain samples and brain tumor samples. **A.** mRNA expression levels of Akt1, Akt2, and Akt3 from NCBI Gene Expression Omnibus GDS1962 data set are presented as box-and-whisker plots. The box for each gene represents the interquartile range (25-75th percentile) and the line within this box is the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (•). Significance between the indicated classes of brain specimens was tested using a two-sample t test assuming unequal variances. NB, non-neoplastic brain; Astro, low-grade astrocytomas. **B.** Principal component analysis of brain tumors from NCBI Gene Expression Omnibus GDS1962 data set revealed two groups differing by their survival and were denoted as long-term (*LT*) survival and short-term (*ST*) survival. Box-and-whisker plots for Akt1, Akt2, and Akt3 expression in GBM specimens for each cluster are shown. Significance between the two populations was tested with a two-sample *t* test assuming unequal variances.

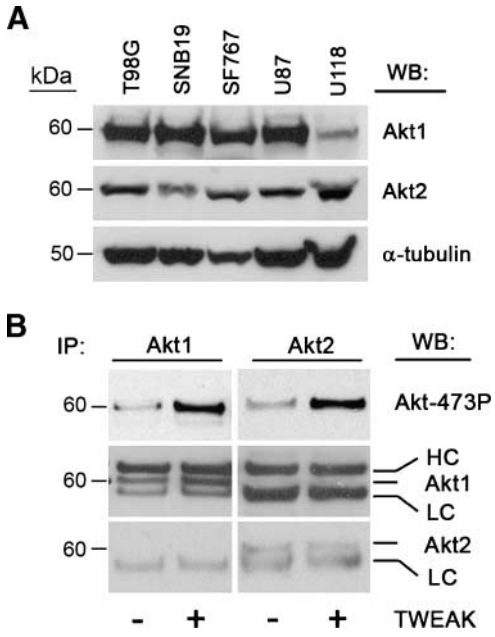

FIGURE 3. Akt1 and Akt2 are expressed in glioma cell lines and are phosphorylated in TWEAK-stimulated cells. **A.** T98G, SNB19, SF767, U87, and U118 cell lysates were analyzed for endogenous levels of Akt1 and Akt2 by Western blotting (*WB*). To monitor gel loading, α-tubulin protein levels were also analyzed. **B.** T98G cells were treated with TWEAK for 10 min. Cell lysates were subjected to immunoprecipitation with Akt1- or Akt2-specific antibodies and then immunoblotted for phospho Akt-473. HC, heavy chain IgG; LC, light chain IgG.

Akt1 and Akt2 Are Coexpressed in Glioma Cell Lines and Phosphorylated on TWEAK Stimulation

Because the results from the brain tumor expression profiling suggest survival roles for Akt1 and Akt2 in glioma cells, we examined the protein expression of Akt1 and Akt2 across five different glioma cell lines. Both Akt isoforms were present in all glioma cell lines (Fig. 3A).

To determine the effect of TWEAK stimulation on the specific phosphorylation status of serine residues Akt1-473 and Akt2-474, we immunoprecipitated the individual isoforms using isoform-specific antibodies, and then performed immunoblot analysis using a phospho-specific antibody to Akt-Ser473, which recognizes phospho-serine 473 (Akt1) and phospho-serine 474 (Akt2). We observed a 2-fold induction of phosphorylation of both Akt1and Akt2 in T98G cells following TWEAK treatment (Fig. 3B). Similar results were observed in SF767 cells (data not shown). These findings indicate that TWEAK stimulation can result in both Akt1 and Akt2 activation.

TWEAK-Induced Glioma Cell Survival Is Dependent on the Akt2 Isoform

We previously showed that TWEAK stimulation of glioma cells results in diminished cytotoxic therapy–induced apoptosis (11). To investigate the role of the Akt isoforms in TWEAK-induced cell survival, we inhibited the expression of Akt1 and Akt2 by transient transfection with siRNA duplexes, followed by treatment with the cytotoxic agents tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or camptothecin. Two independent siRNA oligonucleotides were used against Akt1 and Akt2. The level of protein inhibition for the siRNA-mediated depletion of Akt1 and Akt2 in the glioma cells is isoform specific and reached an 80% to 90% knockdown (Fig. 4A). Whereas depletion of Akt1 expression has a minimal effect on glioma cell survival (Fig. 4B and C, *e* and *i*), increased apoptosis was detected in cells when Akt2 expression was knocked down (Fig. 4B and C, *m* and *q*) compared with control siRNA transfection. Similar to our previous data, treatment of glioma cells transfected with control siRNA and treated with cytotoxic agents showed enhanced cellular apoptosis (Fig. 4B and C, *c*). Pretreatment of cells with TWEAK before the addition of the cytotoxic agents abrogated cytotoxic therapy–induced apoptosis (Fig. 4B and C, *d*). Depletion of Akt1 expression by siRNA did not abrogate the TWEAK protective effect (Fig. 4B and C, *h* and *k*). However, depletion of Akt2 expression resulted in a loss of TWEAK-induced cell survival (Fig. 4B and C, *p* and *t*) in the presence of the cytotoxic agents. These findings indicated that TWEAK-induced glioma cell survival is dependent on Akt2 function.

Because temozolomide (TMZ) is the standard of care for GBM patients, we investigated whether the TWEAK-Fn14-Akt2 pathway can suppress TMZ-induced glioma cell death. Cellular apoptosis was detected in glioma cells treated with TMZ (Fig. 4D and E, *c*). However, pretreatment of the cells with TWEAK before TMZ addition suppressed TMZ-induced cell death (Fig. 4D and E, *d*). Depletion of Akt2 expression negated the TWEAK survival effect (Fig. 4D and E, *p* and *t*), whereas no effect on TWEAK survival was detected upon Akt1 depletion (Fig. 4D and E, *h* and *l*). Taken together, our results show that TWEAK-Fn14 signaling through Akt2 can also suppress chemotherapy-induced cell death in glioma.

TWEAK Stimulation of BAD Serine-136 Phosphorylation Is Dependent on Akt2 Function

One mechanism by which Akt activation increases cell survival is through the phosphorylation of the proapoptotic protein BAD on Ser136 (12). We examined whether TWEAK-Fn14 signaling can induce BAD-Ser136 phosphorylation by monitoring the phosphorylation state of BAD on Ser136 using a cell-based ELISA kit. Upon TWEAK stimulation, the level of phosphorylated BAD-Ser136 increased ~2-fold (Fig. 5A*b* and B*j, gray bar*), whereas the levels of total Bad did not change significantly (Fig. 5A*b* and B*j, black bar*). To investigate whether a specific Akt isoform is critical for TWEAK-induced Bad-Ser136 phosphorylation, we inhibited the expression of Akt1 or Akt2 by transient transfection with siRNA oligonucleotide duplexes. Interestingly, depletion of Akt2 expression by siRNA oligonucleotides suppressed TWEAK-induced BAD-Ser136 phosphorylation [Fig. 5A (*g* and *h*) and B (*o* and *p*)], whereas no effect of BAD Ser-136 phosphorylation by TWEAK was observed in Akt1-depleted glioma cells [Fig. 5A (*e* and *f*) and B (*m* and *n*)].

siRNA-Mediated Depletion of BAD Inhibits Cytotoxic- and Chemotherapy-Induced Apoptosis in Glioma Cells

To further examine the role of BAD in glioma cell survival, we inhibited the expression of BAD by transient transfection of siRNA. Transfection of glioma cells with two independent

siRNA oligonucleotides showed an 80% to 95% reduction of BAD protein compared with no treatment control or control siRNA transfection (Fig. 6A). siRNA -mediated depletion of BAD antagonized cytotoxic therapy–induced cell death (Fig. 6B and C, compare *c, g*, and *k*) and chemotherapy-induced cell death (Fig. 6D and E, compare *c, g*, and *k*) in both T98G and SF767 cells. Interestingly, pretreatment of BAD-depleted glioma cells with TWEAK still provided a strong protection of glioma cells from apoptosis by cytotoxic (Fig. 6B and C, compare *g* and *h* and *k* and *l*) or chemotherapeutic (Fig. 6D and E, compare *g* and *h* and *k* and *l*) agents, similar to TWEAK stimulation in control siRNA–transfected glioma cells (Fig. 6B-E, *d*). This suggests that BAD is not the main mediator of the TWEAK protective effect in glioma cells.

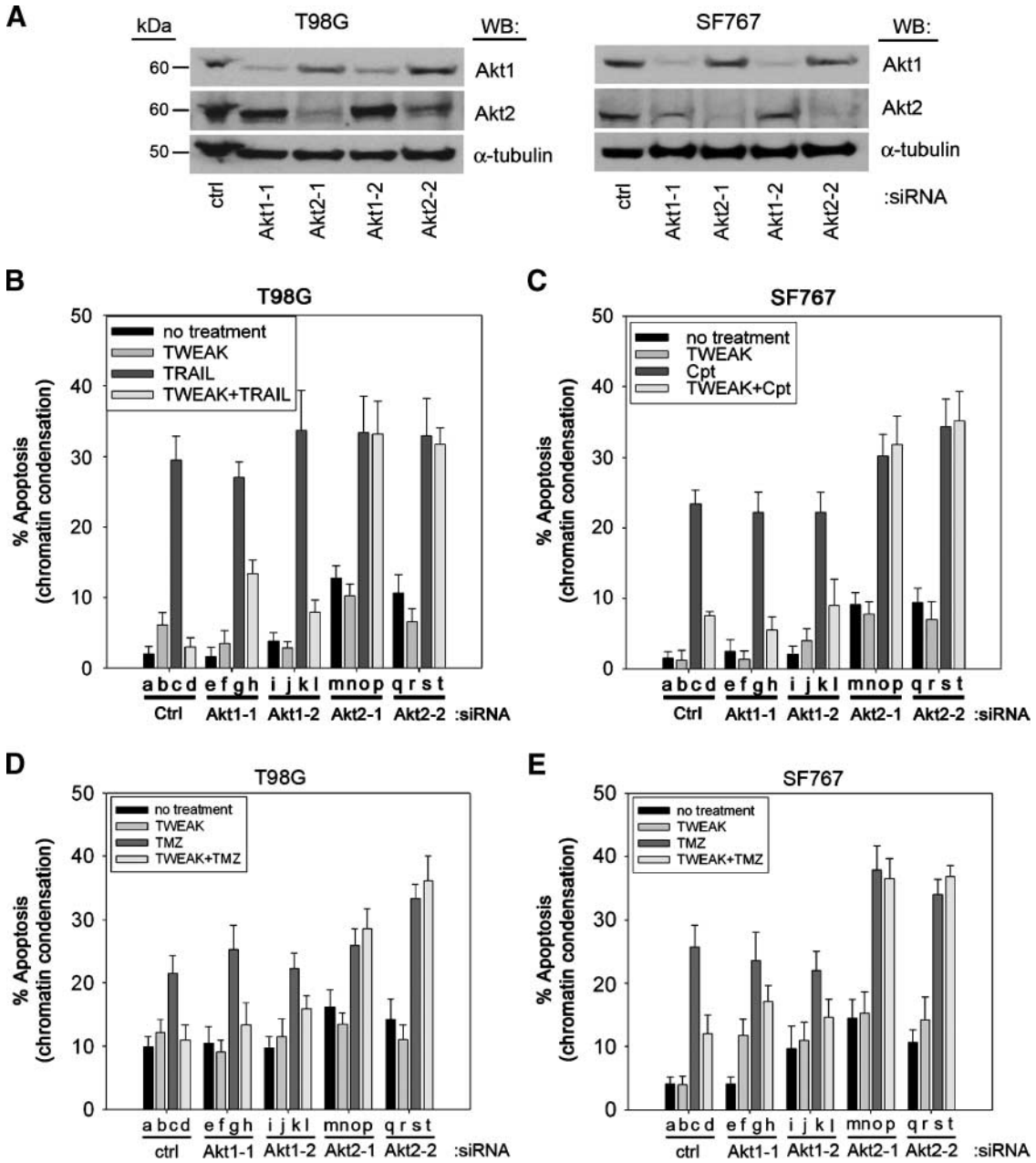

FIGURE 4. TWEAK stimulation of glioma cell survival depends on Akt2 function. **A.** Confirmation of Akt1 and Akt2 siRNA-triggered shutdown. T98G and SF767 glioma cells were transfected with siRNA oligonucleotides targeting control luciferase, Akt1, or Akt2; after 72 h, protein lysates were collected and analyzed by Western blotting (*WB*) for Akt1, Akt2, and α-tubulin. Each panel is a representation of three independent experiments. **B** to **E.** T98G and SF767 cells were transfected with siRNA targeting either control luciferase (*ctrl*), Akt1, or Akt2. Cells were then cultured in reduced serum for 16 h before treatment with TWEAK (100 ng/mL) alone or TWEAK pretreatment (100 ng/mL) for 2 h followed by the addition of TRAIL (100 ng/mL; **B**), Camptothecin (1 μmol/L; **C**), or TMZ (200 μmol/L; **D** and **E**). Cells were fixed 24 h later and stained for 4′,6-diamidino-2-phenylindole. Cells with condensed, fragmented chromatin were manually scored as apoptotic cells. At least 10 fields (total of 1,000 cells) were evaluated and data were reported as apoptotic cells/total cells × 100. Columns, mean of five replicate measurements; bars, SD.

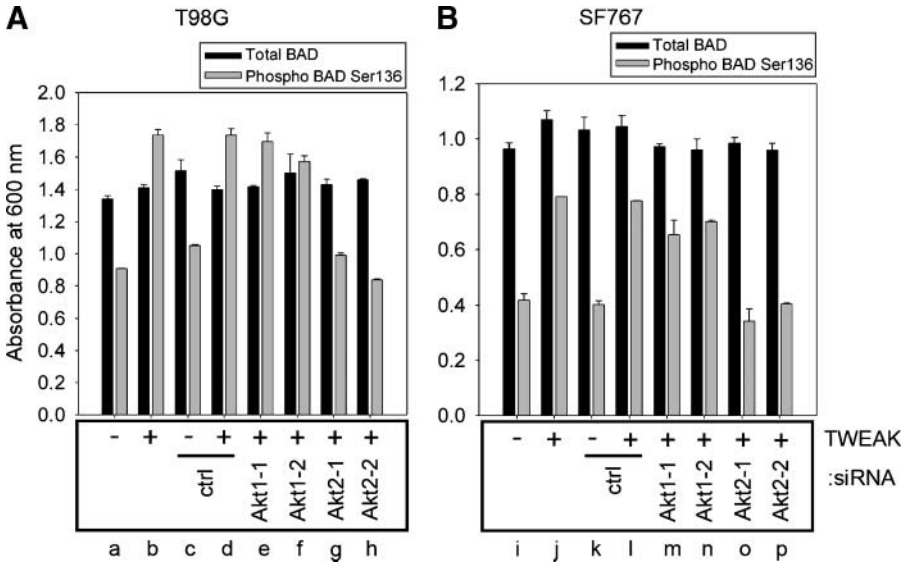

FIGURE 5. TWEAK-induced BAD phosphorylation on serine 136 is dependent on Akt2 function. T98G (**A**) and SF767 (**B**) glioma cells were transfected with siRNA oligonucleotides targeting control luciferase, Akt1, or Akt2. Twenty-four hours later, cells were cultured under reduced serum for 16 h before TWEAK addition for 10 min. Cellular lysates were collected and analyzed for phosphorylated BAD Ser-136 and total BAD by ELISA. Columns, mean from three independent experiments with each experiment conducted in triplicate; bars, SD.

Immunohistochemical Detection of Akt1, Akt2, and Fn14 on a Glioma Invasion Tissue Microarray

We have previously shown that the Fn14 receptor is highly expressed in glioma specimens by immunohistochemistry (IHC) analysis (8, 9). Here, we investigated the protein expression levels for Akt1 and Akt2 in relationship to Fn14 expression on an invasion glioma tissue microarray (TMA) consisting of 44 GBM specimen cases assembled to reflect dispersion of infiltrative gliomas. A semiquantitative scoring system of 0 to 4 for expression levels of each protein was used to reflect the staining intensity in the tumor cells. Examination of Akt1 and Akt2 levels in control non-neoplastic brain specimens from epileptogenic patients showed negative to weak staining, mainly in endothelial cells, neuronal cells, and reactive astrocytes (data not shown). In contrast, expression of both Akt1 (Fig. 7A and D) and Akt2 (Fig. 7B and E) was present in the GBM specimens and localized in the cytoplasm as well as the nucleus of tumor cells. Further analysis revealed no differences between the staining of Akt1 or Akt2 in the invasive cells compared with the core. However, the distribution of staining intensity between Akt1 and Akt2 among the GBM cases is significantly different ($P < 0.05$, χ^2 test; Table 1). The majority of the GBM cases were scored "moderately positive" for Akt1 expression in the core (60.0%) and invasive cells (69.2%) compared with "strong positivity" discerned in only 33.3% of the core and 23.1% in the rim. In comparison, the staining intensity distribution of Akt2 in the tumor core and invasive cells seems to be more strongly positive, 47.2% (core) and 55.6% (rim), whereas the moderately positive scores were identified in 50.0% (core) and 44.4% (rim). Similarly, moderate and strong staining for Fn14 in GBM cells was observed in the tumor core and invading cells in the rim, corroborating our previous results (Fig. 7C and F; ref. 9).

We assessed the correlation between the expression of Akt1, Akt2, and Fn14 in the 44 GBM cases (Table 2). No significant correlation was observed between Akt1 and Akt2. Also, no correlation was observed between Fn14 and Akt1. However, Akt2 protein expression positively correlated with Fn14 expression level ($r = 0.31$, $P < 0.02$) among the 44 GBM cases.

Discussion

Akt isoforms are known modulators of cellular processes including cell growth, metabolism, proliferation, and survival (34). Although these isoforms are activated through similar mechanisms involving PI3K, they seem to have different roles in cancer progression and survival (17, 19, 24, 25, 32, 34). In this study, we showed that Akt2 is an important kinase that functions downstream of the TWEAK-Fn14 axis to induce glioma cell survival. We showed that although Fn14 activation leads to both phosphorylation of Akt1 and Akt2, depletion of Akt2 expression but not Akt1 expression by siRNA targeting suppresses the survival signal of Fn14 in glioma cells *in vitro*.

In glioma cell line models *in vitro* and in clinical tissues samples *in situ*, heightened Akt activity has been detected, and has been correlated with loss of function of the phosphatase and tensin homologue (PTEN) tumor suppressor on chromosome 10q25 (35). In fact, a consequence of deregulated Akt activity that may underlie treatment failure derives from the role of Akt in directly regulating target signaling proteins through phosphorylation, including the proapoptotic machinery. Here, we showed that TWEAK can influence Akt2 function to induce glioma survival. Interestingly, of the various Akt protein survival substrates examined (mammalian target of rapamycin, Forkhead, and BAD), only BAD phosphorylation was observed consequent to TWEAK stimulation (data not shown). However, TWEAK still provided a strong protective

effect toward cytotoxic- and chemotherapy-induced apoptosis after siRNA-mediated depletion of BAD in glioma cells. This suggests that BAD function does not affect the ability of TWEAK to protect glioma cells against apoptosis, but it may potentially regulate its initial sensitivity. Our data are consistent with a previous report by Fan and colleagues (36), showing that in myeloid cells, BAD was not required for cell death following interleukin-3 withdrawal; thus, suggesting that BAD phosphorylation does not play a significant role in the apoptotic stimulus of interleukin-3–deprived myeloid cells. Although the mechanism by which TWEAK regulates glioma cell survival is unclear, it is possible that the TWEAK protective effect in

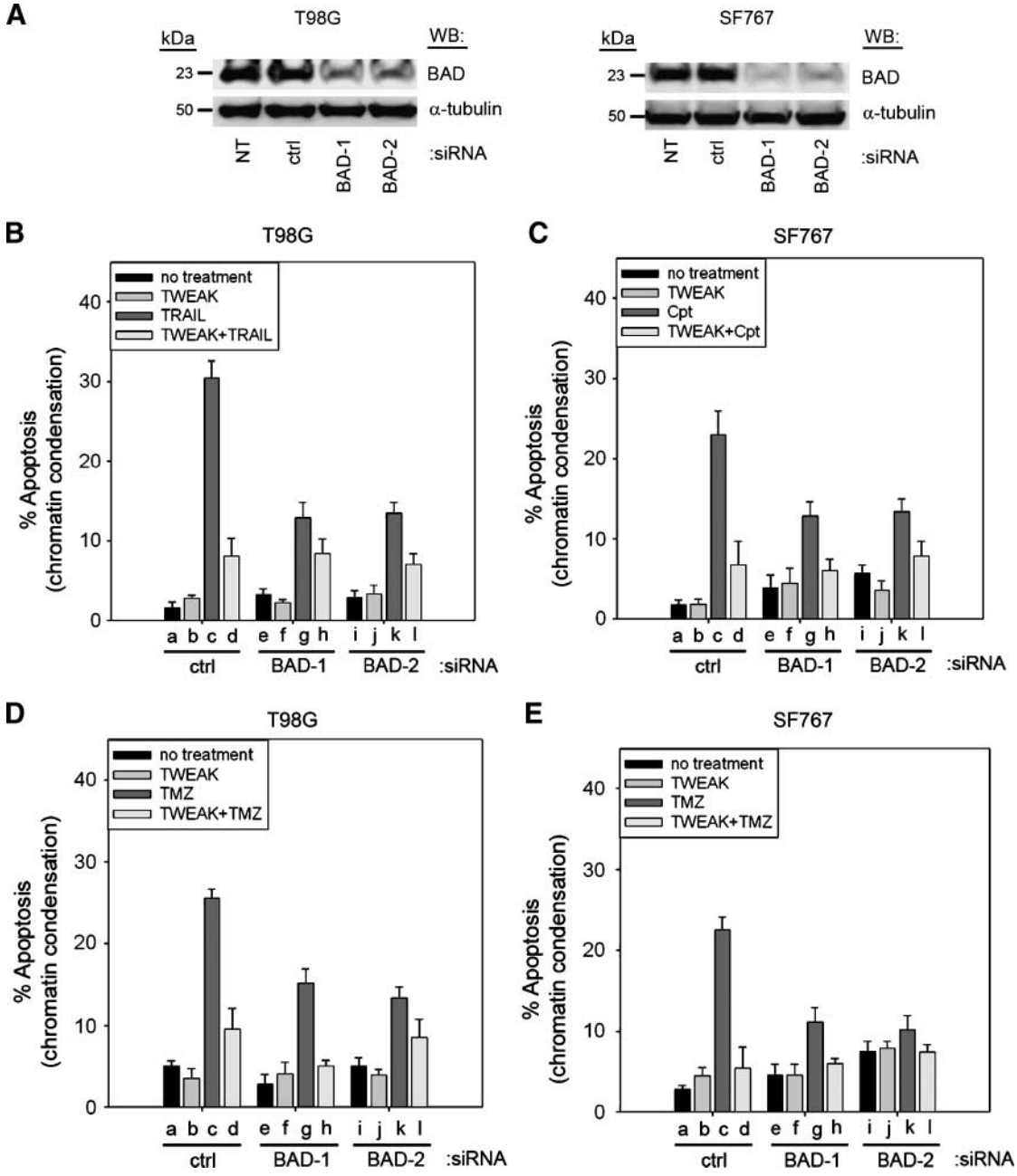

FIGURE 6. Depletion of BAD expression protects glioma cells from cytotoxic- and chemotherapy-induced apoptosis. **A.** T98G and SF767 glioma cells were transfected with siRNA oligonucleotides targeting control luciferase or BAD; after 72 h, protein lysates were collected and analyzed by Western blotting for BAD and α-tubulin. Each panel is a representation of three independent experiments. **B** to **E.** T98G and SF767 cells were transfected with siRNA targeting either control luciferase (*ctrl*) or BAD. Cells were then cultured in reduced serum for 16 h before treatment with TWEAK (100 ng/mL) alone or TWEAK pretreatment (100 ng/mL) for 2 h followed by the addition of TRAIL (100 ng/mL; **B**), Camptothecin (1 μmol/L; **C**), or TMZ (200 μmol/L; **D** and **E**). Cells were fixed 24 h later and stained for 4′,6-diamidino-2-phenylindole. Cells with condensed, fragmented chromatin were manually scored as apoptotic cells. At least 10 fields (total of 1,000 cells) were evaluated and data were reported as apoptotic cells/total cells × 100. Columns, mean of five replicate measurements; bars, SD.

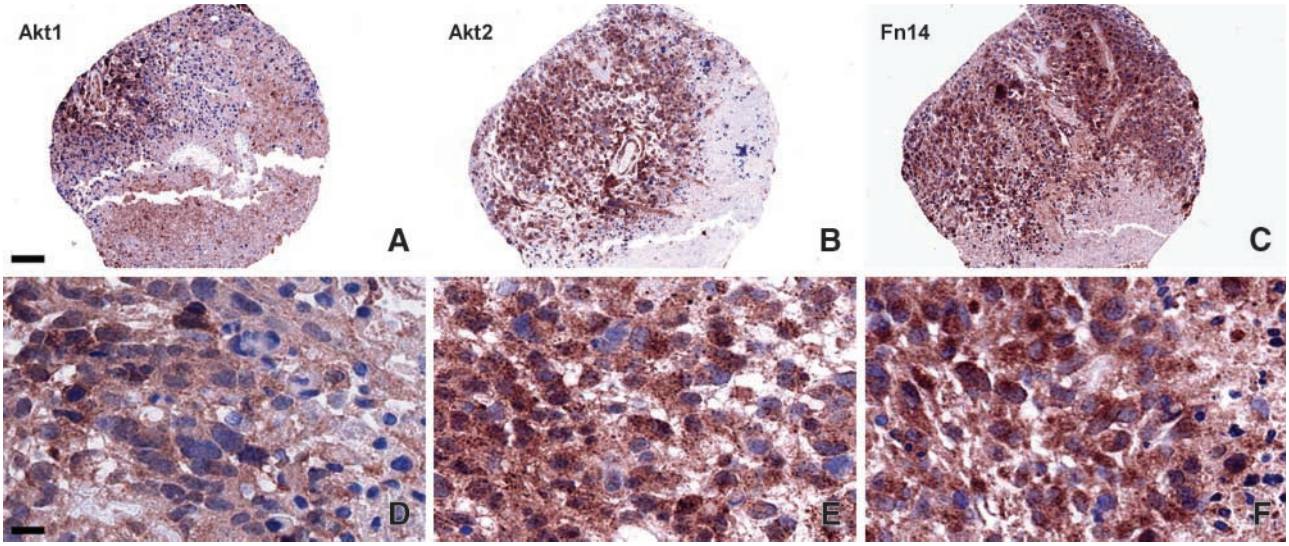

FIGURE 7. Immunohistochemical detection of Akt1, Akt2, and Fn14 during GBM invasion. Paraffin-embedded, formalin-fixed sections of the glioma invasion TMA were stained with antibodies against Akt1, Akt2, and Fn14. Panels are a representation of staining of Akt1, Akt2, and Fn14 from a single GBM case. Top (**A, B**, and **C**), ×10 magnification (*bar,* 100 μm). Bottom (**D, E**, and **F**), ×40 magnification (*bar,* 20 μm).

glioma cells may be dependent on other BH3-related proapoptotic proteins such as Bim and Puma.

TWEAK stimulation has also been shown to affect Akt phosphorylation in other cellular systems. For example, TWEAK inhibits insulin-induced Akt phosphorylation in hepatocytes (37). Also, TWEAK stimulation results in the transient induction of Akt phosphorylation within 15 minutes in C2C12 myoblasts (38), but prolonged TWEAK treatment (>2 hours) results in the inhibition of Akt activation (38, 39). In comparison, our result indicates that TWEAK stimulation of Akt phosphorylation in glioma cells is rapid and peaks at 1 hour. Diminished Akt phosphorylation is observed after 2 hours with basal phospho-Akt levels detected by 6 hours. Thus, these data suggest that the effect of TWEAK stimulation on intracellular signaling processes is cell-type specific; in the case of glioma cells, TWEAK activation of the Akt pathway promotes cell survival.

Accumulating evidence shows that dysregulated Akt2 function is more consistently detected in human malignancies than Akt1 and Akt3. Specifically, gene amplifications of Akt2 have been reported in cancers of the stomach, ovary, pancreas, and breast (21). Further, ectopic expression of Akt2 or depletion of Akt2 expression by shRNA, but not Akt1 and Akt3, leads to increased invasion and metastasis of human breast (19) and ovarian cancer cells (40). Moreover, ovarian cancer cells overexpressing constitutively active Akt2 become cisplatin resistant, whereas expression of dominant-negative Akt2 sensitized both cisplatin-sensitive and cistaplin-resistant ovarian cells to cisplatin-induced apoptosis (41). Likewise, we observed a significant decrease in TWEAK-induced glioma cell survival upon Akt2 depletion. This suggests that part of the response of Fn14-induced glioma cell survival to cytotoxic therapy–induced apoptosis involves an Akt2-dependent mechanism. It is also noteworthy that in glioma, antisense knockdown of Akt2 inhibits glioma cell growth *in vitro* and *in vivo* (42). Furthermore, siRNA treatment of Akt2 in breast epithelial cells revealed a role in the promotion of proliferation and cell survival downstream of insulin-like growth factor receptor (19).

Although there are a limited number of studies, it seems that all three Akt isoforms are widely expressed but the level of expression of each isoform may differ among tissues, thus modulating in part their biological activities. In brain specimens, Akt2 mRNA expression levels correlate directly with glial tumor grade and inversely with patient outcome, supportive of a functional role of Akt2 in glioma malignancy. Akt1 expression shows a similar trend; however, expression of Akt2 seems to be lower in non-neoplastic brain tissue than Akt1. Surprisingly, Akt3 expression is highest in non-neoplastic brain specimens but seems to decrease with glial tumor grade and is positively associated with patient survival, suggesting that Akt3 may have some tumor suppressor activity in gliomas. In fact, Akt3 has been reported to be highly expressed in the brain (43), suggesting that Akt3 may have important roles in normal brain cellular physiology.

Examination of Akt1 and Akt2 protein expression *in situ* portrays high Akt1 and Akt2 staining across clinical GBM specimens, thus corroborating with the gene expression profiling data. However, upon examination of Akt1 and Akt2 distribution among tumor cells residing at the core versus the adjacent rim region, Akt2 staining seems to be stronger in the tumor cells in the rim region compared with Akt1. A strong correlation between the protein expression levels of Akt2 and Fn14 was observed among the GBM cases, thus strengthening the clinical relevance of the TWEAK-Fn14-Akt2 pathway as an attractive axis for targeted therapy.

Overall, our studies uncovered a specific role for Akt2 as a unique driver of TWEAK-induced glioma cell survival. The study also argues for a better contextual understanding of Akt isoform expression, specifically Akt1 and Akt2, in glioma

progression in the likely scenario that one of the isoforms will prove to be a more appropriate potential drug target for glioma therapeutics. Importantly, target molecules along the same pathway may have a synergistic antitumor effect and help to prevent tumor drug resistance. We argue that targeted combination therapy against Fn14 and Akt2 as an adjuvant to surgery may offer a path to management of invasive glioma cells and consequently improve the prognosis of this devastating disease.

Materials and Methods

Cell Culture Conditions

Human astrocytoma cell lines T98G, U87, U118, SNB19 (American Type Culture Collection), and SF767 (University of California at San Francisco) were maintained in DMEM + 10% heat-inactivated fetal bovine serum in a 37°C, 5% CO_2 atmosphere. In all assays treated with TWEAK, cells were cultured in reduced serum (0.5% fetal bovine serum) for 16 h before stimulation with recombinant TWEAK at 100 ng/mL in DMEM + 0.1% bovine serum albumin for the indicated times.

Antibodies and Reagents

Bcl-2, BAX, Phospho-Akt (Ser 473), total Akt, Akt1, Akt3, Phospho-BAD (Ser 136), and total BAD antibodies were obtained from Cell Signaling Technology, Inc. Akt2 antibody was obtained from Santa Cruz Biotechnology, Inc. Monoclonal antibodies specific to Bcl-x_L were purchased from Zymed Laboratories, Inc. Monoclonal antibodies to Rac1 were purchased from BD Transduction Labs. Human recombinant TWEAK was purchased from PeproTech, and human recombinant TRAIL was purchased from Invitrogen. Laminin from human placenta and LY294002 were obtained from Sigma. Camptothecin was obtained from Calbiochem and TMZ was purchased from Sequoia Research Products.

Western Blot and Immunoprecipitation Assays

Immunoblotting and protein determination experiments were done as described previously (11). In some experiments, TWEAK (100 ng/mL) was preincubated with 2.5 μg/mL soluble Fn14-Fc decoy receptor or control Fc protein at 37°C for 15 min before it was added to cells (28). Also, in some cases, cells were pretreated with the PI3K inhibitor LY294002 (10 μmol/L) for 10 min before TWEAK addition. For immunoprecipitation, cells were lysed on ice for 10 min in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 0.5% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 2 mmol/L sodium vanadate, 10 μg/mL aprotinin, and 10 μg/mL leupeptin (Sigma). Equivalent amounts of protein (500 μg) were precleared and immunoprecipitated from the lysates, and then washed with lysis buffer followed by S1 buffer [10 mmol/L HEPES (pH 7.4), 0.15 mol/L NaCl, 2 mmol/L EDTA, 1.5% Triton X-100, 0.5% deoxycholate, and 0.2% SDS; ref. 37]. Samples were then resuspended in 2 × SDS sample buffer and boiled in the presence of 2-mercaptoethanol (Sigma), separated by SDS-PAGE, transferred to nitrocellulose for 1 h at 4°C, and proteins were detected as described previously (26).

siRNA Preparation and Transfection

siRNA oligonucleotides specific for Rac1 and GL2 Luciferase were previously described (44). Validated siRNA sequences for Akt1 (Akt1-1, Hs_AKT1_5HP; Akt1-2, Hs_AKT1_10_HP), Akt2 (Akt2-1, Hs_AKT2_5_HP; Akt2-2, Hs_AKT2_6_HP), and BAD (BAD-1, Hs_BAD_3_HP; BAD-2, Hs_BAD_4_HP) were purchased from Qiagen. Transient transfection of siRNA was carried out as previously described (44). All Akt-directed and Bad-directed siRNA were used at 25 nmol/L, and no cell toxicity was observed. Maximum inhibition of protein levels was achieved 72 h after transfection.

PhosphoBAD (Ser 136) Sandwich ELISA

Phosphorylation levels of BAD (Ser 136) were determined using a cell-based ELISA according to the manufacturer's instructions (Cell Signaling). Briefly, glioma cells were transfected with siRNA oligonucleotides against *AKT1, AKT2*, or nonmammalian GL2 luciferase control siRNA and incubated overnight. The cells were cultured overnight in DMEM with 0.5% fetal bovine serum before TWEAK or control PBS addition in DMEM with 0.1% bovine serum albumin for 15 min. Total cellular lysates were collected according to manufacturer's instructions using the PathScan Sandwich ELISA kit (Cell Signaling). Each sample (100 μg total protein) was diluted 1:1 in sample buffer for a final volume of 200 μL before addition to the designated microstrip wells previously coated by the manufacturer with the BAD rabbit monoclonal capture antibody. The microstrips were then sealed and incubated

Table 1. Analysis of Akt1, Akt2, and Fn14 Expression Levels by IHC on the Glioma Invasion TMA

	Percent of specimens			
	Negative	Weak	Moderate	Strong
Akt1 core	0.0	6.7	60.0	33.3
Akt1 edge	0.0	11.5	69.2	19.2
Akt1 rim	0.0	7.7	69.2	23.1
Akt2 core	0.0	2.8	50.0	47.2
Akt2 edge	0.0	0.0	60.6	39.4
Akt2 rim	0.0	0.0	44.4	55.6
Fn14 core	0.0	5.6	47.2	47.2
Fn14 edge	0.0	11.5	42.3	46.1
Fn14 rim	0.0	0.0	50.0	50.0

NOTE: The values shown are the percentage of cases at each staining intensity. A scoring system of 0 to 4 for expression levels of each protein was used to indicate the staining intensity in the tumor cells (0, negative; 1, weak; 2, moderate; 3-4, strong).

Table 2. Pearson's Correlation of Akt1, Akt2, and Fn14 Protein Expression in GBM Specimens

Antigen1	Antigen2	r	*P*
Akt1	Akt2	0.07	0.59
Akt1	Fn14	−0.02	0.92
Akt2	Fn14	0.31	**0.02**

NOTE: r, correlation coefficient; significant *P* value is bolded.

overnight at 4°C on a plate shaker. Following extensive washing (4× with wash buffer), the phosphoBAD (Ser 136) antibody (Cell Signaling #5282 1:1,000) and total BAD antibody (Cell Signaling #9292 1:1,000) were added to their designated wells and incubated for 1 h at 37°C. The wells were then washed 4× with wash buffer before addition of the horseradish peroxidase–linked secondary antibody. Samples were incubated at 37°C for 30 min, washed, and incubated with TMB substrate for 10 min. The kit's protocol was modified by omitting stop solution before reading absorbance at 600 nm (45).

Apoptotic Assay

Apoptotic cells were evaluated by nuclear morphology of 4′,6-diamidino-2-phenylindole–stained cells as described previously (11). Briefly, cells with condensed, fragmented chromatin were manually scored as apoptotic cells. At least five fields (total of 1,000 cells) were evaluated, and data reported as apoptotic cells/total cells × 100. Verification of apoptotic cells was conducted by coimmunofluorescence staining using a monoclonal antibody against activated cleaved caspase 3 (Promega). At least 1,000 cells per treatment were evaluated for condensed chromatin and activated caspase 3 (11).

Expression Profile Data Set of AKT *Isoforms in Human Gliomas and Non-Neoplastic Brain*

We mined an expression microarray database consisting of 135 clinically annotated brain tumor specimens publicly available at NCBI's Gene Expression Omnibus as data set GDS1962 for the different Akt isoforms. Snap-frozen specimens from epileptogenic foci ($n = 24$) and tumors (29 low-grade astrocytomas and 82 GBMs) with clinical information were collected at the Hermelin Brain Tumor Center, Henry Ford Hospital as previously described (9). Gene expression profiling as described previously (9) was conducted on all samples using Affymetrix U133 Plus 2 GeneChips according to the manufacturer's protocol at the Neuro-Oncology Branch at the National Cancer Institute. For our analysis, gene expression data were normalized in two ways: per chip normalization and per gene normalization across all samples in the collection. For per chip normalization, all expression data on a chip were normalized to the 50th percentile of all values on that chip. For per gene normalization, the data for a given gene were normalized to the median expression level of that gene across all samples. Gene expression differences were deemed statistically significant using parametric tests where variances were not assumed equal (Welch ANOVA). Expression values were then filtered for highly variable (differentially expressed) genes (coefficient of variation, >30%) across samples, producing a list of 7,322 genes. Principal component analysis was done to discern possible relationships between subgroups of samples as previously described (9), and Kaplan-Meier survival curves were developed for each principal component cluster. One cluster had a median survival time of 401 d (short-term survival) and the other cluster had a median survival time of 952 d (long-term survival). Box-and-whisker plots for *AKT1, AKT2*, and *AKT3* expression levels in each cluster derived from principal component analysis were graphed. Significance between the two populations was tested with a two-sample *t* test assuming unequal variances.

Glioblastoma Tissue Microarray and IHC

Sections were obtained from a glioma invasion TMA master block containing representative punches of tumor core, edge, and invasive rim from 44 clinically annotated cases of WHO grade IV GBM specimens (according to standardized criteria from 10 contributing institutes) as previously described (46). The GBM samples were obtained from patients who underwent primary therapeutic subtotal or total tumor resection performed under image guidance. Specifically, each specimen block chosen for the TMA met the criteria of nonnecrotic, nonirradiated, or chemo-treated glioma tissue. Two separate face cuts were made for each of the specimens used to construct the TMA. H&E staining was done on the face cuts to assist in the identification of the tumor cells. The face cuts were reviewed by two independent experienced neuropathologists (Dr. Ken Aldape, MD Anderson and Dr. David Zagzag, New York University) who designated the areas of core (center of the tumor), edge (interface between tumor core and normal tissue front), and rim (region distal to the edge but still containing notable tumor cells). The TMA was constructed from representative punches of tumor core and invasive rim using an indexed manual arrayer with attached stereomicroscope under the direction of Dr. Galen Hostetter, who also reviewed and verified that the prescribed areas made by the neuropathologists were in agreement before punches were taken for the TMA paraffin block. Every 50th section from the TMA was stained with H&E to confirm tissue morphology and invasive feature of the GBM cells. IHC analysis for Fn14 was done using an Fn14 monoclonal antibody, P4A8 (Biogen Idec, Inc.), as previously described (9). Akt1 and Akt2 IHC analysis was done using anti-Akt1 (Cell Signaling) and anti-Akt2 (Santa Cruz) antibodies, respectively. A scoring system for chromophore was used to capture the outcome: 0, negative; 1, weak; 2, moderate; 3 to 4, strong staining.

Statistical Analysis

Statistical analyses were done using the two-sample *t* test and χ^2 test. Tests for correlation using Pearson's correlation coefficient were calculated using the cor.test function in the R statistical package. $P < 0.05$ was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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