Novel Tumor-Specific Isoforms of BEHAB/Brevican Identified in Human Malignant Gliomas

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Abstract

Malignant gliomas are deadly brain tumors characterized by diffuse invasion into the surrounding brain tissue. Understanding the mechanisms involved in glioma invasion could lead to new therapeutic strategies. We have previously shown that BEHAB/brevican, an extracellular matrix protein in the central nervous system, plays a role in the invasive ability of gliomas. The mechanisms that underlie BEHAB/brevican function are not yet understood, due in part to the existence of several isoforms that may have different functions. Here we describe for the first time the expression of BEHAB/brevican in human brain and characterize two novel glioma-specific isoforms, B/b_{sia} and B/$b_{\Delta g}$, which are generated by differential glycosylation and are absent from normal adult brain and other neuropathologies. B/b_{sia} is an oversialylated isoform expressed by about half the high- and low-grade gliomas analyzed. B/$b_{\Delta g}$ lacks most of the carbohydrates typically present on BEHAB/brevican and is the major up-regulated isoform of this protein in high-grade gliomas but is absent in a specific subset of low-grade, indolent oligodendrogliomas. B/$b_{\Delta g}$ is detected on the extracellular surface, where it binds to the membrane by a mechanism distinct from the other BEHAB/brevican isoforms. The glioma-specific expression of B/$b_{\Delta g}$, its restricted membrane localization, and its expression in all high-grade gliomas tested to date suggest that it may play a significant role in glioma progression and make it an important new potential therapeutic target. In addition, its absence from benign gliomas prompts its use as a diagnostic marker to distinguish primary brain tumors of similar histology but different pathologic course. (Cancer Res 2005; 65(15): 6726-33)

Introduction

Gliomas are the most common primary tumors of the central nervous system (CNS). Malignant gliomas are difficult to treat and nearly impossible to completely remove surgically due to their high invasiveness (1). Despite considerable advances in our understanding of these tumors, low survival rates for patients with gliomas have remained essentially unchanged for 25 years due to tumor recurrence even after extensive resection (2).

Gliomas invade the normal nervous tissue in a uniquely infiltrative and disperse manner and with very rare exceptions, do not extend outside strict CNS boundaries. Conversely, peripheral tumors that metastasize to the brain cannot infiltrate the normal nervous tissue but instead push it aside (3, 4). Glioma cells are thus uniquely able to overcome the normal barriers to cell movement in the CNS. One of the major barriers to cell movement in all tissues is the extracellular matrix (ECM), which in the CNS is composed of a hyaluronic acid scaffold with associated glycoproteins and proteoglycans (5). Typical ECM proteins such as laminin, type-IV collagen, and fibronectin are limited to vascular basal membranes and the *glia limitans* in the adult CNS and are essentially absent from the parenchyma (6). Glioma cells interact with the hyaluronic acid–based ECM through cell surface receptors, including CD44, RHAMM, and the chondroitin sulfate (CS) proteoglycans of the lectican family (7–9).

BEHAB/brevican is a CNS-specific lectican that is expressed in a spatially and temporally regulated manner in the mammalian brain (10, 11). *BEHAB/brevican* mRNA is up-regulated during periods of glial cell motility, such as during prenatal gliogenesis as well as after a stab injury in the adult brain (10). Consistent with a possible role for this protein in glial cell motility, *BEHAB/brevican* mRNA is also dramatically up-regulated in human gliomas as well as in rodent glioma models (12–14). Moreover, BEHAB/brevican up-regulation and subsequent proteolytic processing contribute to the infiltration of glioma cells into normal nervous tissue (15, 16). However, the molecular mechanisms through which these functions are mediated are still poorly understood, due in part to the molecular complexity of this protein.

Several isoforms of BEHAB/brevican have been described in rodent brain, which may interact differently with the cell surface and with other ECM components. These isoforms, which are products of alternative splicing (17), proteolytic cleavage (18, 19), and differential glycosylation of the core protein (11, 20), could also be differentially expressed in human glioma therefore playing specific roles in glioma progression. However, the expression of BEHAB/brevican protein in normal human brain or primary brain tumors has not yet been described.

In the present work, we investigated BEHAB/brevican expression in human primary brain tumors, to determine if the expression pattern of BEHAB/brevican isoforms provides unique insight into the role of this protein in gliomas. We identified two novel tumor-specific isoforms of BEHAB/brevican, which are selectively up-regulated in human gliomas but are absent from normal adult brain and other neuropathologies. We further characterized the underglycosylated isoform named B/$b_{\Delta g}$, which is the major up-regulated form of BEHAB/brevican in high-grade glioma. Our work suggests that B/$b_{\Delta g}$ may play a unique role in glioma progression and may have relevance both as a diagnostic marker and as a cell surface target for immunotherapy.

Materials and Methods

Cell lines and antibodies. The human glioma cell line U87MG (American Type Culture Collection, Manassas, VA) was grown at 5%

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CO_2 in DMEM supplemented with 10% FCS, 50 μg/mL penicillin, and 50 μg/mL streptomycin. The following rabbit polyclonal anti-BEHAB/brevican antibodies, produced against synthetic peptides, were employed in this study: B6, against the CS attachment region (amino acids, AA, 506-529) of rat BEHAB/brevican; B5, against the NH_2 terminus (AA 60-73) of rat BEHAB/brevican; and B_{CRD}, against the COOH terminus (AA 859-879) of human BEHAB/brevican. The NH_2-terminal cleavage product of BEHAB/brevican was detected with a rabbit polyclonal antibody against the cleavage neoepitope QEAVESE (AA 389-395) of rat BEHAB/brevican. These antibodies have been previously described for the detection of BEHAB/brevican in rat brain (18, 20). V5-tagged human BEHAB/brevican was also detected using a mouse monoclonal anti-V5 antibody (Invitrogen, Carlsbad, CA). α-Tubulin was detected with a mouse monoclonal anti-tubulin antibody (Molecular Probes, Eugene, OR).

Human tissue. All studies were done in compliance with the guidelines of the Human Investigations Committee at the Yale University School of Medicine. Pathologically graded fresh-frozen surgical samples of intracranial tumors (20 male, 13 female; ages 13-64 years) were obtained from Yale-New Haven Hospital (New Haven, CT). Samples of normal temporal and parietal human brain cortex (10 male, 10 female; ages 16 gestational weeks to 76 years) were obtained from the Brain and Tissue Bank for Developmental Disorders (University of Maryland, Baltimore, MD). Fresh-frozen surgical samples from epileptic brain tissue (2 male, 1 female; ages 9-50 years) were provided by Dr. D. Spencer (Department of Neurosurgery, Yale University). Postmortem brain cortex samples from Alzheimer's disease patients (3 male, 1 female; ages 78-87 years) were provided by Dr. G. Rebeck (Department of Neuroscience, Georgetown University, Washington, DC).

Subcellular fractionation and Western blotting. Brain and tumor samples were homogenized in 10 volumes of 25 mmol/L Tris-HCl (pH 7.4) containing 0.32 mol/L sucrose and a protease inhibitor cocktail (*Complete*, EDTA-free, Roche, Indianapolis, IN). Because many of the samples assayed contained only a small amount of tumor tissue, homogenates were subjected to a simplified subcellular fractionation protocol by first being centrifuged at 900 × *g* for 10 minutes to separate the nuclear pellet. Subsequently, the post-nuclear supernatant was separated into total particulate and soluble fractions by centrifugation at 100,000 × *g* for 60 minutes, as previously described (20). Aliquots of the subcellular fractions were equilibrated at a final total protein concentration of 1 to 2 mg/mL in CH buffer [40 mmol/L Tris-HCl, 40 mmol/L sodium acetate (pH 8.0)] containing 5 mmol/L EDTA and treated with 0.25 units/mL of protease-free chondroitinase ABC (Seikagaku, Tokyo, Japan) for 8 hours at 37°C. Chondroitinase activity was stopped by boiling the samples in the presence of 1× gel-loading buffer. Samples (10 μg total protein) were electrophoresed on reducing 6% SDS-polyacrylamide gels and analyzed by Western blotting. For semiquantitative analysis, immunoblots were developed by chemiluminiscence and the integrated optical density of BEHAB/brevican isoforms was calculated using the Gel-Pro Analyzer software (v3.1, Media Cybernetics, Silver Spring, MD). Blots were stripped and reprobed for α-tubulin as loading control. Optical density ratios (BEHAB/brevican/tubulin) were compared by Student's *t* test with Welch's correction.

Release of BEHAB/brevican isoforms from brain membranes. To characterize the association of different BEHAB/brevican isoforms with the cell membrane, total membranes (~1 mg total protein/mL) obtained from control and glioma samples were resuspended in 50 mmol/L Tris-HCl buffer (pH 7.4) with or without 10 mmol/L EDTA for 1 hour at 4°C. Alternatively, membranes were resuspended in 100 mmol/L sodium carbonate (pH 11.3) for 30 minutes at 4°C. After incubation, membranes were centrifuged at 20,800 × *g* for 20 minutes. Released BEHAB/brevican was recovered in the supernatant and the membranes containing retained BEHAB/brevican were washed twice with 50 mmol/L Tris-HCl buffer. All samples were finally equilibrated with CH buffer and treated with chondroitinase ABC before protein electrophoresis. For immunoprecipitation studies, membranes were first extracted for 1 hour at 4°C in 50 mmol/L Tris-HCl (pH 7.4) containing 300 mmol/L NaCl and 0.6% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid. Solubilized proteins were immunoprecipitated with the antibody B6 preadsorbed to protein A-Sepharose, according to standard protocols.

Cell culture and immunocytochemistry. A clone containing the complete coding sequence of human BEHAB/brevican (Genbank accession no. BC010571, clone ID 4215184, Invitrogen) was subcloned into pCDNA3.1(+) and pcDNA3.1-V5/6xHis plasmids (Invitrogen). Cells were transfected employing LipofectAMINE 2000 (Invitrogen) according to the manufacturer protocols. Control transfections were done with the pCDNA3.1(+) vector. Transfected cells were changed to serum-free Opti-MEM (Life Technologies, Gaithersburg, MD) for 24 hours before collection. Collected cells were lysed in 25 mmol/L phosphate buffer (pH 7.4) containing a protease inhibitor cocktail and 2 units/mL RNase-free DNase I (Roche). Total membranes were obtained by centrifugation at 20,800 × *g* for 30 minutes and prepared for protein electrophoresis. Culture medium was concentrated by ultradiafiltration and equally processed for SDS-PAGE.

For live immunocytochemical staining, U87MG cells were grown on poly-L-lysine–coated 18-mm glass coverslips for 24 hours before transfection with human *BEHAB/brevican* cDNA. Unfixed, unpermeabilized cultures were repeatedly rinsed with ice-cold DMEM and incubated with the antibodies B6 or anti-V5 at 4°C for 30 minutes. Cells were subsequently rinsed, fixed for 20 minutes in 4% paraformaldehyde in 100 mmol/L phosphate buffer (pH 7.4), and further processed for fluorescence microscopy. To determine which isoform(s) of BEHAB/brevican accounted for the cell surface labeling observed by the live cell staining procedure, transfected cells from parallel coverslips were rinsed with ice-cold DMEM and incubated with DMEM at 4°C for 30 minutes thus mimicking the processing of live-stained cells without addition of antibodies or fixation. These cells were subsequently scraped from the wells and homogenized in 25 mmol/L phosphate buffer (pH 7.4), and the total homogenates were prepared for protein electrophoresis.

Protein deglycosylation. Samples were equilibrated in deglycosylation buffer [20 mmol/L Tris-HCl, 20 mmol/L sodium acetate, 25 mmol/L NaCl (pH 7.0)] containing protease inhibitors, at a protein concentration of ~1 mg/mL, and treated for 8 hours at 37°C with combinations of the following glycosidases: 0.25 units/mL chondroitinase ABC, 20 milliunits/mL *O*-glycosidase (Roche), 100 milliunits/mL sialidase (Roche), and 100 units/mL glycopeptidase F (PNGase F, Calbiochem, La Jolla, CA). Enzyme digestions were stopped by boiling the samples in 1× gel-loading buffer. For denaturing deglycosylation, required for nonexposed N-linked carbohydrates, samples were first equilibrated in 0.1% w/v SDS/0.1 mol/L 2-mercaptoethanol and heated at 95°C for 10 minutes. Subsequently, samples were equilibrated in deglycosylation buffer containing 0.8% v/v NP40 and deglycosylation proceeded in the same conditions as indicated above.

Results

BEHAB/brevican is differentially expressed in normal human brain and primary brain tumors. To examine the expression of BEHAB/brevican protein in normal brain and glioma, tissue was analyzed by Western blot after subcellular fractionation and enzymatic removal of CS chains. Routine treatment of tissue samples with chondroitinase ABC was used to increase BEHAB/brevican immnoreactivity in Western blots, due to the electrophoretic collapse of the isoforms that carry CS into a single band. In normal brain tissue, secreted BEHAB/brevican was detected as a ~160-kDa ("full length") form, as well as ~60- and ~100-kDa cleavage products generated by the disintegrin and metalloprotease with thrombospondin motifs (ADAMTS)-4/Aggrecanase-1 (refs. 18, 19; Fig. 1*B*). In surgical samples from human gliomas, there was an increase in the intensity of the same protein bands that was not unexpected, because our previous work has shown

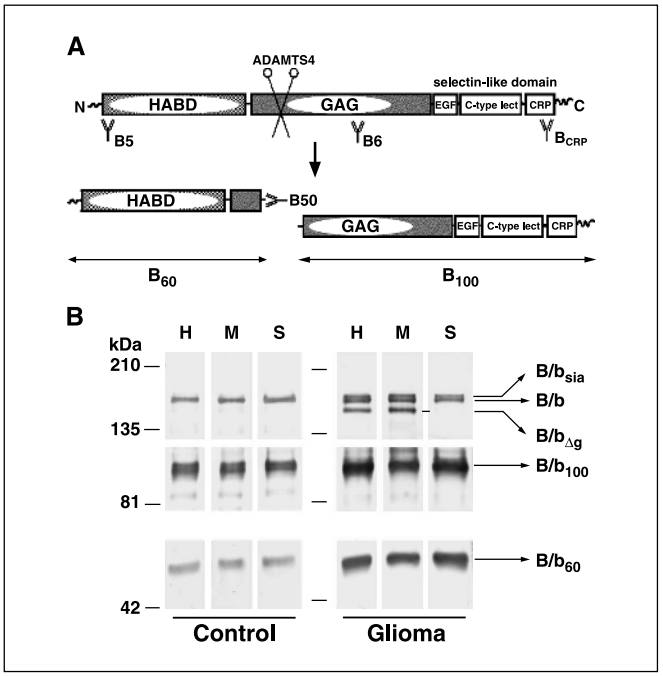

Figure 1. Novel BEHAB/brevican isoforms in human glioma. *A,* structure of full-length BEHAB/brevican, its cleavage products by ADAMTS-4, and location of the epitopes recognized by the antibodies B6, B5, B_{CRP}, and B50. *HABD,* HA-binding domain; *GAG,* CS attachment region; *EGF,* epidermal growth factor repeat; *Lect,* C-type lectin-like domain; *CRP,* complement regulatory protein-like domain. *B,* total homogenate (*H*), membrane-enriched (*M*), and soluble (*S*) fractions from a representative glioblastoma multiforme (*Glioma*) and a normal, age-matched brain cortex (*Control*) were chondroitinased and analyzed by Western blotting. *Arrows, top,* positions of the full-length, ~160-kDa BEHAB/brevican isoform (B/b) and two glioma-specific isoforms: B/b_{sia} and $B/b_{\Delta g}$. *Arrows, middle* and *bottom,* COOH-terminal (~100 kDa) and NH_2-terminal (~60 kDa) cleavage products of BEHAB/brevican.

that *BEHAB/brevican* mRNA expression is dramatically up-regulated in glioma (12, 13). However, the protein analysis disclosed not only an increased expression of the full-length and cleaved forms of BEHAB/brevican but also the presence of additional, unique isoforms in glioma. The most evident novel isoform, named $B/b_{\Delta g}$, migrated at an apparent molecular mass of ~150 kDa and distributed exclusively to membrane-containing fractions. A second isoform, named B/b_{sia}, migrated slightly higher than the ~160-kDa form and distributed both in soluble and particulate fractions of glioma samples.

$B/b_{\Delta g}$ was present in every sample of high-grade glioma, grades 3 (n = 3) and 4 (n = 19), assayed to date, whereas B/b_{sia} appeared in about half of the high-grade gliomas analyzed (Fig. 2*A*). Densitometric analysis of the expression of these isoforms showed that total expression of noncleaved forms of BEHAB/brevican was >4-fold higher in gliomas compared with normal brain tissue (Fig. 2*B*). Similarly, the 60-kDa cleavage product showed a ~5-fold increase in gliomas versus normal tissue. Remarkably, $B/b_{\Delta g}$ alone accounted for roughly half of the total overexpression above control levels for noncleaved BEHAB/brevican, suggesting that a substantial proportion of BEHAB/brevican synthesized in glioma is shunted to the pathway that makes this isoform.

The unique expression of B/b_{sia} and $B/b_{\Delta g}$ in high-grade gliomas lead us to investigate if expression of BEHAB/brevican isoforms correlated with tumor grade. Interestingly, analysis of grade 2 oligodendrogliomas (n = 7) revealed two subsets of samples, one expressing both B/b_{sia} and $B/b_{\Delta g}$ and the other not expressing either of these isoforms (Fig. 2*C*). In this second subset, a faint band was observed in some samples at a position similar to $B/b_{\Delta g}$, but we determined that this band corresponded instead to a minor COOH-terminally cleaved product of BEHAB/brevican (data not shown). The subset of samples that were negative for both B/b_{sia} and $B/b_{\Delta g}$ corresponded to patients diagnosed with low-grade tumors associated with chronic epilepsy, a subclass of gliomas that are notably indolent and

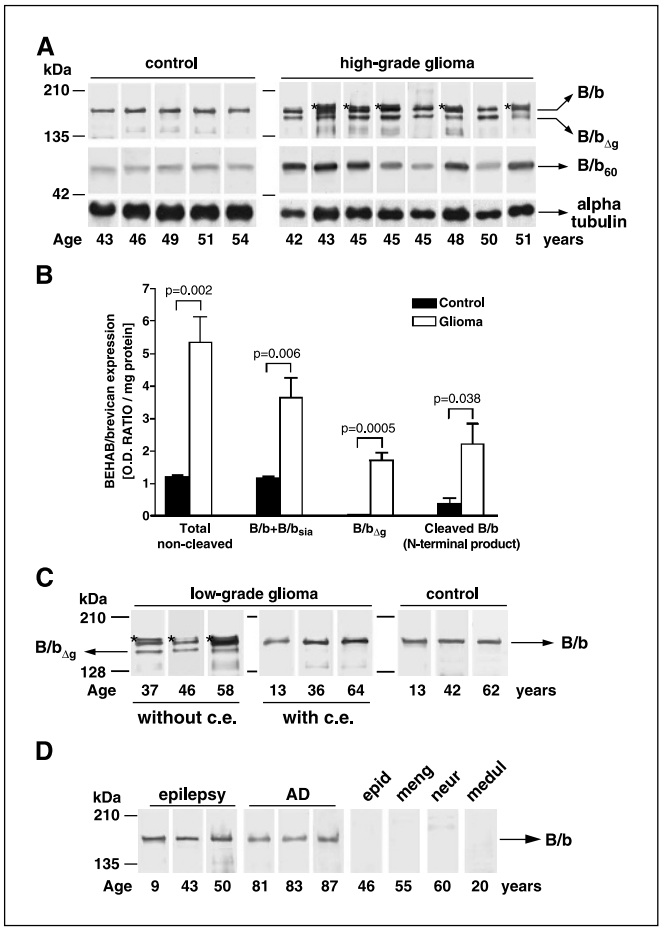

Figure 2. B/b_{sia} and $B/b_{\Delta g}$ are restricted to malignant gliomas. *A,* expression of BEHAB/brevican isoforms in a representative subset of high-grade gliomas (grades 3-4) and age-matched controls. $B/b_{\Delta g}$ was detected in all the high-grade gliomas analyzed. *, position of B/b_{sia}, which was detected in roughly half of the samples analyzed. *B,* BEHAB/brevican expression from the samples shown in (*A*) was quantified by densitometry. *Columns,* averages; *bars,* ±SE. Absorbance from ~160-kDa B/b and B/b_{sia} bands were integrated together because their signals were not always possible to distinguish by chemiluminescence. Total noncleaved B/b = ~160-kDa B/b + B/b_{sia} + $B/b_{\Delta g}$. Cleaved BEHAB/brevican was independently quantified from Western blots of the NH_2-terminal cleavage product. *Optical density (O.D.) ratio* = [BEHAB/brevican O.D.] / [alpha-tubulin O.D.]. The expression of both noncleaved and cleaved BEHAB/brevican was significantly increased in gliomas compared to controls. *C,* expression of BEHAB/brevican in a subset of grade 2 oligodendrogliomas (n = 7) from patients diagnosed with or without tumor-associated chronic epilepsy (*c.e.*). *, position of B/b_{sia}. Neither B/b_{sia} nor $B/b_{\Delta g}$ was detected in the oligodendrogliomas with chronic epilepsy, which have been characterized as gliomas with indolent profile and benign prognosis. Age-matched controls are shown to compare normal BEHAB/brevican expression at similar ages. *D,* expression of BEHAB/brevican in total homogenates from other neuropathologies. Only ~160-kDa BEHAB/brevican was detected in individuals with epilepsy (*epilepsy*) and Alzheimer's disease (*AD*). Consistent with our previous reports that *BEHAB/brevican* mRNA is not detected in nonglial-derived tumors, the protein was not observed in epidermoid tumor (*epid*), meningioma (*meng*), acoustic neuroma (*neur*), and medulloblastoma (*medul*).

benign (21, 22). As discussed below, the expression profile of BEHAB/brevican isoforms in these tumors represents the first known molecular marker that distinguishes these benign tumors from other low-grade gliomas.

In addition, we determined whether B/b_{sia} and $B/b_{\Delta g}$ were unique to gliomas or were also expressed in other neuropathologic conditions, by analyzing samples from other types of intracranial tumors (n = 4) as well as brain cortex from epilepsy (n = 3) and Alzheimer's disease (n = 4). We did not detect $B/b_{\Delta g}$ or B/b_{sia} in any of those samples (Fig. 2*D*).

Previous work from our laboratory has shown that BEHAB/brevican is expressed in a developmentally regulated manner in rodents (20). We therefore analyzed the ontogenetic expression of human BEHAB/brevican in normal tissue to determine whether the glioma-specific isoforms could be detected during human brain development. In none of the samples from normal individuals ages >1 year (n = 14) did we detect either B/b_{sia} or $B/b_{\Delta g}$ (Fig. 3*A*). Only the ~160-kDa isoform of BEHAB/brevican was detected, at a relatively constant level throughout adulthood, in agreement with our previous reports (13). However, at earlier developmental ages (n = 6), from 16 weeks of gestation to 19-day-old infants, we observed a faint band migrating at the position of $B/b_{\Delta g}$ (Fig. 3*B*). This band was barely detectable in total homogenates from cortical tissue but was clearly observed in the membrane-enriched fraction.

B/b_{sia} and $B/b_{\Delta g}$ are generated by differential glycosylation of BEHAB/brevican. Our next step in the characterization of the newly observed glioma-specific isoforms of BEHAB/brevican was to determine the origin of their difference in molecular mass relative to the protein from normal brain tissue. Isoforms of BEHAB/brevican smaller than the full-length secreted protein are known to be produced by alternative splicing, specific proteolytic processing and differential glycosylation, whereas isoforms larger than full-length BEHAB/brevican do not differ in protein sequence but are produced by additional glycosylation with CS chains.

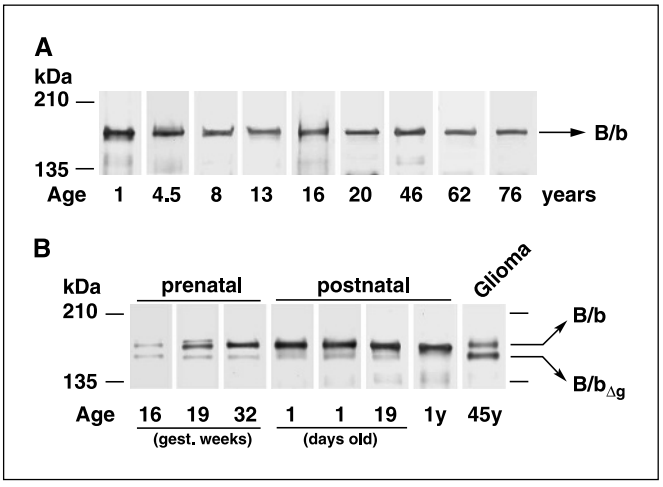

Figure 3. $B/b_{\Delta g}$ is expressed during early human brain development. *A,* total homogenates from human brain cortex at (1-76 years) were processed as in Fig. 2 and qualitatively analyzed. The ~160-kDa B/b band is the only noncleaved form of BEHAB/brevican detected throughout development. *B,* total homogenates from human cortex over prenatal and early postnatal development (16 gestational weeks to 1 year of age), and a representative surgical sample of glioblastoma (*Glioma*), were subjected to subcellular fractionation and the membrane-enriched fractions were treated as in (*A*). $B/b_{\Delta g}$ is detected in the membrane-containing fraction of normal human brain during early development but at much lower levels than in glioma.

We first verified whether $B/b_{\Delta g}$ was smaller than normal BEHAB/brevican due to proteolytic cleavage. BEHAB/brevican was immunoprecipitated from detergent extracts of normal brain and glioma membranes and the immunoprecipitated material was probed with antibodies that detect epitopes located at <5 kDa from the termini of the full-length protein (see Fig. 1*A*). All the antibodies employed recognized both ~160-kDa BEHAB/brevican as well as $B/b_{\Delta g}$ (Fig. 4*A*) and B/b_{sia} (data not shown), indicating that these isoforms were not terminally cleaved products of normal BEHAB/brevican. It bears noting that $B/b_{\Delta g}$ and B/b_{sia} were never detected in immunoprecipitates from control samples, which are highly enriched in BEHAB/brevican, providing further evidence that they are not expressed in normal adult brain.

To further verify whether $B/b_{\Delta g}$ was generated from the same mRNA transcript encoding full-length BEHAB/brevican, we analyzed BEHAB/brevican expression in U87MG cells transfected with full-length human *BEHAB/brevican* cDNA (Fig. 4*B*). These cells do not express endogenous BEHAB/brevican in culture, as shown by the immunoblotting of vector-transfected cells. However, when transfected with *BEHAB/brevican* cDNA, U87MG cells produced both ~160-kDa BEHAB/brevican, secreted to the culture medium, and $B/b_{\Delta g}$, which, as in human glioma samples, localized exclusively to the particulate subcellular fraction. Interestingly, we never observed B/b_{sia} in U87MG or any other of the rat and human glioma cell lines assayed (data not shown).

Because $B/b_{\Delta g}$ and B/b_{sia} did not seem to differ from normal BEHAB/brevican in peptidic sequence, we next explored changes in the glycosylation of BEHAB/brevican to explain the molecular mass of these glioma-specific isoforms. BEHAB/brevican carries N- and O-linked oligosaccharides as well as CS chains. The first indication that $B/b_{\Delta g}$ was less glycosylated than the normal, ~160-kDa BEHAB/brevican resulted from the observation that chondroitinase treatment, which increases the immunoreactivity of ~160-kDa BEHAB/brevican, caused no effect on $B/b_{\Delta g}$ (data not shown) thus suggesting that it lacked CS chains. In addition, further deglycosylation of tissue samples with enzymes that remove N- and O-linked sugars shifted the ~160-kDa BEHAB/brevican band towards the position of $B/b_{\Delta g}$ but did not affect the electrophoretic mobility of the latter, suggesting that $B/b_{\Delta g}$ lacked not only CS but most of, or all, the carbohydrates present in the glycosylated isoform (Fig. 4*C*). Only after protein denaturation followed by deglycosylation with PNGase F did we detect a slight change in the electrophoretic mobility of $B/b_{\Delta g}$ (Fig. 4*D*). These results suggest that $B/b_{\Delta g}$ carries only a few, nonexposed, N-linked carbohydrates per protein molecule thus being an underglycosylated form of BEHAB/brevican.

Results from our deglycosylation assays also showed that the higher molecular mass of the B/b_{sia} isoform was generated by increased sialic acid content on O-linked carbohydrates, because both B/b_{sia} and ~160-kDa B/b collapsed to a single position by SDS-PAGE after treatment with sialidase but not with PNGase F (Fig. 4*E*).

Together, our results indicate that the differences in molecular mass between the normal ~160-kDa isoform of BEHAB/brevican and the glioma-specific B/b_{sia} and $B/b_{\Delta g}$ isoforms are explained by their differential glycosylation.

$B/b_{\Delta g}$ is expressed on the cell surface. Because $B/b_{\Delta g}$ is underglycosylated, a possible explanation for its partitioning with

the membrane fraction is that it is a misfolded form caused by BEHAB/brevican up-regulation, which is then retained in the secretory pathway and does not reach the cell surface. To determine whether B/$b_{\Delta g}$ could be localized on the extracellular surface of glioma cells, U87MG cells transfected with the cDNA for V5-tagged or untagged full-length human BEHAB/brevican were probed with B6 and anti-V5 before fixation. Our results from live cell staining, in which antibodies can only detect extracellularly exposed epitopes, revealed BEHAB/brevican immunoreactivity on the surface of the transfected cells (Fig. 5*A-F*). Further analysis of cells stained for BEHAB/brevican before and after fixation showed that in addition to secreted BEHAB/brevican on the cell surface, the protein was also found intracellularly (Fig. 5*G*). However, this fraction of BEHAB/brevican likely represents the protein progressing through the secretory pathway due to the strong viral promoter-driven overexpression.

Although U87MG cells seem to fully secrete glycosylated BEHAB/brevican to the culture medium (Fig. 4*B*), we needed to determine if the immunoreactivity on the cell surface was solely due to B/$b_{\Delta g}$ or if there was residual ~160-kDa glycosylated BEHAB/brevican that could explain this cell surface labeling. Therefore, cells were processed identically as for immunostaining, except that they were not incubated with antibodies or fixed but were instead collected and homogenized. Western blotting of total homogenates from these cells exclusively detected B/$b_{\Delta g}$ (Fig. 5*H*), confirming that this was the only isoform accounting for the cell surface labeling previously detected by immunocytochemistry.

B/$b_{\Delta g}$ associates with glioma membranes by a mechanism distinct from other BEHAB/brevican isoforms. Because B/$b_{\Delta g}$ partitions with the particulate fraction of glioma and can reach the cell surface, we next explored whether the mechanism of membrane association was the same for B/$b_{\Delta g}$ and glycosylated BEHAB/brevican. First, membranes from normal brain and glioma were treated with sodium carbonate, which releases peripherally associated proteins but not covalently linked or integral membrane proteins. Both glycosylated BEHAB/brevican and B/$b_{\Delta g}$ were released in the same proportion into the soluble fraction (Fig. 6), confirming, as expected, that B/$b_{\Delta g}$ associates peripherally with the cell surface.

All known cell surface ligands of BEHAB/brevican associate to its lectin-like domain by a calcium-dependent mechanism (11). Therefore, we next treated membranes from normal brain and glioma with EDTA to disrupt this binding. EDTA treatment partially released the ~160-kDa isoform in normal tissue and

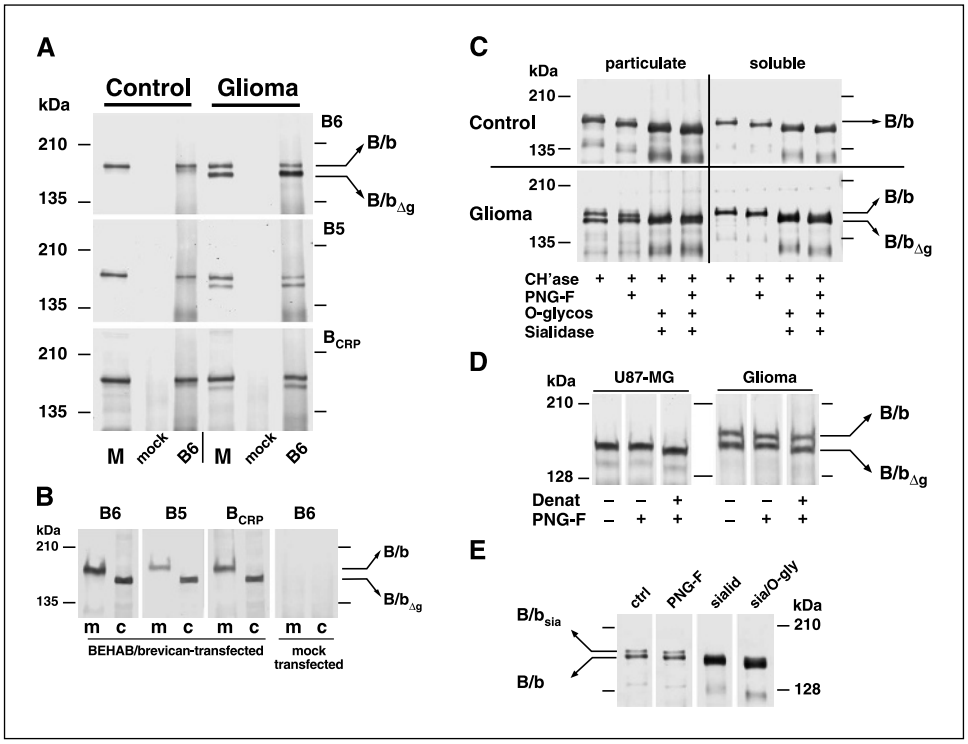

Figure 4. B/b_{sia} and B/$b_{\Delta g}$ are full-length isoforms of BEHAB/brevican produced by differential glycosylation. *A,* solubilized brain membranes (*M*) from control and glioma samples were immunoprecipitated in the absence (*mock*) or presence (*B6*) of B6 antibody. Immunoprecipitated samples were probed with B6, B5, and B_{CRP} antibodies. All antibodies detected ~160-kDa B/b as well as B/$b_{\Delta g}$, indicating that the size difference between these two bands is not generated by protein cleavage. *B,* culture medium (*m*) and cell membranes (*c*) from U87MG cells, transfected with pcDNA3.1 vector (*mock transfected*) or full-length human *BEHAB/brevican* cDNA, were immunoblotted with B6, B5, and B_{CRP}. These cells do not express endogenous BEHAB/brevican in culture, as determined by lack of B6 immunoreactivity in mock-transfected cells (B5, B_{CRP}, identical results omitted for clarity); therefore, all positive detection corresponds to BEHAB/brevican expressed from the transfected cDNA. All three antibodies detected ~160-kDa B/b in the medium as well as B/$b_{\Delta g}$ in cell membranes, indicating that the full-length *BEHAB/brevican* mRNA generates both isoforms. *C,* soluble and particulate fractions from control and glioma samples were deglycosylated in nondenaturing conditions with combinations of chondroitinase ABC (*CH'ase*), PNGase F (*PNG-F*), O-glycosidase (*O-glycos*), and sialidase. Following deglycosylation, the ~160-kDa B/b band collapses towards the position of B/$b_{\Delta g}$, indicating that their difference in molecular mass is due to differential glycosylation. *D,* membranes from a glioma sample and from BEHAB/brevican-transfected U87MG cells were denatured (*Denat*) and treated with chondroitinase and PNGase-F (*PNG-F*). The slight change in B/$b_{\Delta g}$ mobility indicates that it contains N-linked carbohydrates exposed by protein denaturation. *E,* the soluble fraction from a glioma sample expressing B/b_{sia} was chondroitinased (*ctrl*) and additionally deglycosylated in native conditions with PNGase-F (*PNG-F*), sialidase (*sialid*) or sialidase plus *O*-glycosidase (*sia/O-gly*). The mobility shift indicates that B/b_{sia} has additional sialic acid on O-linked carbohydrates because it is affected by sialidase but not by PNGase-F.

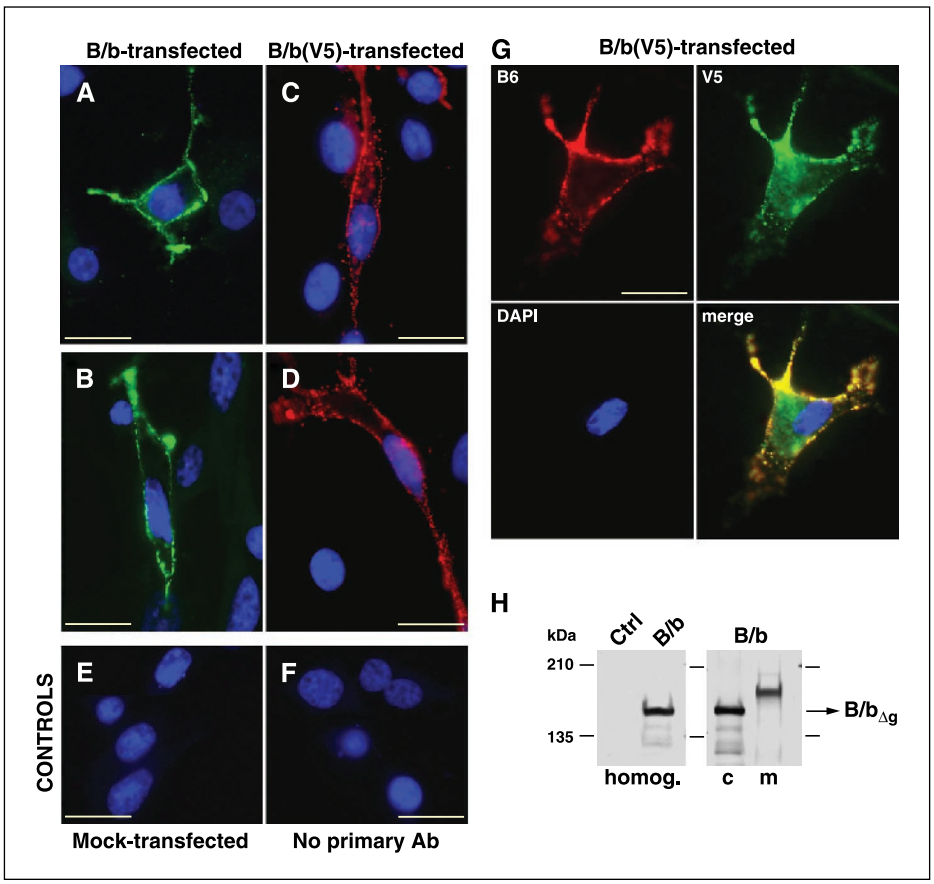

Figure 5. B/b$_{\Delta g}$ is located on the cell surface. U87MG cells were transfected with full-length human BEHAB/brevican, either untagged (*A-B*) or tagged with the V5 epitope (*C-D*). Live cells were stained for 30 minutes at 4°C using the antibodies B6 (*A-B*) and anti-V5 (*C-D*), and further processed for immunocytochemistry. Negative controls included B6-staining of cells transfected with control vector (*E*) and staining of BEHAB/brevican-transfected cells with nonimmune rabbit serum (*F*). Cell nuclei were visualized with 4′,6-diamino-2-phenylindole (*DAPI*). Bar, 25 μm. *G,* U87MG cells transfected with V5-tagged BEHAB/brevican were live stained with B6 antibody, followed by fixation, permeabilization, and staining with V5 antibody. Both antibodies colocalize on the cell surface, as expected. In addition, V5 shows staining of the endoplasmic reticulum, which is an expected result due to protein overexpression under the viral promoter of the pcDNA3.1 vector. *H,* to determine whether only B/b$_{\Delta g}$, or also residual ~160-kDa glycosylated BEHAB/brevican, accounted for the cell surface labeling, BEHAB/brevican-transfected cells (*B/b*) were repeatedly rinsed to mimic the processing of live-stained cells, followed by scraping and homogenization. Only one BEHAB/brevican band was detected in total homogenates (*homog.*) from these cells. By comparing its electrophoretic mobility against BEHAB/brevican from cell membranes (*c*) and culture medium (*m*) of separately transfected cells, this band was identified as B/b$_{\Delta g}$, with no evidence of residual glycosylated B/b. *Ctrl,* total homogenate from vector-transfected cells.

in glioma (Fig. 6), whereas B/b$_{\Delta g}$ was not affected, indicating that it associates with the cell membranes by a unique, calcium-independent mechanism. This mechanism does not likely involve interaction of the NH_2-terminal domain of BEHAB/brevican with hyaluronic acid or CS, because treatment of the membranes with bovine testicular hyaluronidase also failed to release B/b$_{\Delta g}$ from the membrane fraction (data not shown).

Discussion

The diffuse infiltration of glioma cells within the neural tissue relies on their unique ability to interact with the extracellular environment of the CNS. Therefore, molecules selectively expressed in glioma cells that may modify their interaction with the neural environment are of particular interest. Here we described the expression pattern of tumor-specific isoforms of the CNS-specific ECM component BEHAB/brevican in human gliomas. The unique expression profile of BEHAB/brevican in these tumors, together with our previous reports of its proinvasive effect, suggests an important role for this protein in glioma.

Previous studies have shown that *BEHAB/brevican* mRNA is expressed at appreciable levels in normal human brain and significantly up-regulated in malignant gliomas (13, 14). In agreement, we show here the presence of BEHAB/brevican protein in normal brain and its up-regulation in glioma. However, our results indicate that the up-regulation in glioma leads not only to a general increase in the expression of BEHAB/brevican but also to the glioma-specific expression of differentially glycosylated isoforms, B/b$_{sia}$ and B/b$_{\Delta g}$.

B/b$_{sia}$ is present in roughly half of all the high- and low-grade gliomas analyzed, an incidence similar to that of the tumor-specific variant of the epidermal growth factor receptor, EGFR vIII, which is the most typical cell surface marker for high-grade gliomas (1, 23). Apart from the differential glycosylation of B/b$_{sia}$, which includes additional sialic acid on O-linked carbohydrates, all other biochemical properties of this isoform, including subcellular distribution and membrane attachment seem identical to the normal ~160-kDa isoform of BEHAB/brevican (data not shown). The presence of abnormally sialylated cell surface glycoproteins is a typical modification in several tumors, including gliomas, associated with malignant behavior (24, 25). BEHAB/brevican represents a novel substrate for oversialylation, which might have important implications for its function, as well as association with clinical outcome.

The most conspicuous glioma-specific isoform of BEHAB/brevican, B/b$_{\Delta g}$, is a full-length product of *BEHAB/brevican* mRNA that arises from an incomplete or reduced glycosylation of the core protein. B/b$_{\Delta g}$ is absent from the normal adult brain but was found in every sample of high-grade gliomas that we have analyzed to date and can thus be proposed as a novel glioma-specific marker in adult human brain. Indeed, B/b$_{\Delta g}$ is only absent from a restricted subset of low-grade oligodendrogliomas that have been characterized as a unique pathologic entity among primary brain tumors (21, 22). These low-grade, epileptogenic oligodendrogliomas have a predominant cortical localization and uniquely benign pathologic course. The proper identification of this particular

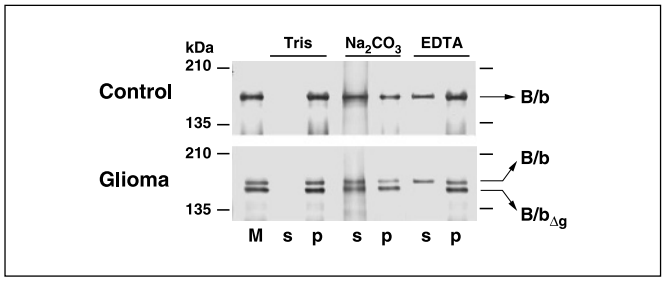

Figure 6. $B/b_{\Delta g}$ associates peripherally with cell membranes in a calcium-independent manner. Total membranes (*M*) from control and glioma samples were resuspended in 50 mmol/L Tris-HCl buffer (pH 7.4) with (*EDTA*) or without (*Tris*) 10 mmol/L EDTA. Parallel samples were resuspended in 100 mmol/L Na_2CO_3 (pH 11.3; *Na_2CO_3*). After incubations, membranes were centrifuged and the resulting supernatant (*s*) and pellet (*p*) were chondroitinased and processed for Western blotting. $B/b_{\Delta g}$ behaves as a peripherally bound protein, being released by sodium carbonate. However, in marked contrast to ~160-kDa B/b, $B/b_{\Delta g}$ is not released from the particulate fraction by EDTA and remains associated in a calcium-independent manner.

subtype of tumors is critical for establishing prospective survival and directing therapy. At present, these tumors cannot be distinguished by histology or chromosomal features from typical low-grade oligodendrogliomas, which have a more aggressive profile and require a distinct clinical approach. $B/b_{\Delta g}$ is the first molecular marker that distinguishes between these indolent and more aggressive low-grade gliomas therefore having potential diagnostic use.

Remarkably, B/b_{sia} and $B/b_{\Delta g}$ are not only absent from normal adult human brain but also from other neuropathologies such as Alzheimer's disease, epilepsy, and several nonglial intracranial tumors. Therefore, their appearance is not likely to reflect a general pathogenic or gliotic process but instead is a result of modifications specific to gliomas. Only $B/b_{\Delta g}$ is expressed at very low levels during the second half of prenatal and first days of postnatal development, a period of intense gliogenesis, and disappears by the first year of age. Expression of $B/b_{\Delta g}$ in gliomas could represent a reactivation of early developmental programs, a mechanism that has previously been implicated in glioma progression (26).

Aberrant glycosylation of cell surface proteins occurs in almost all cancers and can disrupt normal protein-protein interactions thus being a likely mechanism for promoting tumor invasion and metastasis (27, 28). Fully glycosylated BEHAB/brevican is invested with a diverse set of carbohydrates, including N-linked sugars, mucin-type O-linked sugars, and CS chains. Changes in the expression of specific glycosyltransferases or modification of metabolic pathways in glioma can be expected to produce modifications on specific carbohydrates and generate glycovariants such as B/b_{sia}. The origin of $B/b_{\Delta g}$, however, is more difficult to understand, because it seems generated by a mechanism that specifically prevents the addition of several types of carbohydrates to BEHAB/brevican core protein, whereas other proteins are still glycosylated.

Despite being underglycosylated, $B/b_{\Delta g}$ is not a precursor that accumulates in the endoplasmic reticulum but reaches the extracellular surface. The fraction of BEHAB/brevican that is detected intracellularly in transfected cells likely represents an artifact of overexpression. In agreement, primary cultures of rat glial cells and some mouse cell lines naturally express a homologue of $B/b_{\Delta g}$ (see below) that is only detected on the cell surface (20).[3] More importantly, the association of $B/b_{\Delta g}$ with the cell membrane is mediated by a calcium-independent mechanism that is distinct from the binding described for glycosylated forms of BEHAB/brevican and other lecticans (11), suggesting that the underglycosylation of the protein directly affects its biochemical binding properties. Indeed, $B/b_{\Delta g}$ does not interact with the BEHAB/brevican ligand tenascin-R in the absence of calcium,[1] suggesting that the association of $B/b_{\Delta g}$ to the cell membrane likely involves different ligands instead of a novel interaction with previously known receptors.

We have recently described a BEHAB/brevican isoform in the rat brain with similar characteristics to $B/b_{\Delta g}$ but different expression pattern in normal brain (20). Interestingly, although rat $B/b_{\Delta g}$ is expressed in normal brain, it is also the major up-regulated form of BEHAB/brevican in rat experimental gliomas. The up-regulation of this underglycosylated isoform of BEHAB/brevican in both rat and human glioma suggests that regulated glycosylation of BEHAB/brevican may play a significant role in the progression of glial tumors. Glycosylation of the lecticans is precisely regulated in the CNS (29) and many of their inhibitory effects on cell and neurite motility are mediated by their attached carbohydrates (30, 31). Therefore, lack of glycosylation in $B/b_{\Delta g}$ may produce a molecule with very unique functional properties. It has been recently shown that CD44H, another key organizer of the neural ECM, is aberrantly undergycosylated in neuroblastoma and binds defectively to the extracellular hyaluronic acid scaffold (32). It is therefore possible that the overexpression of $B/b_{\Delta g}$ on the surface of glioma cells could promote tumor progression by similarly disturbing the interactions of normal BEHAB/brevican and enabling novel cell-cell interactions that favor invasion.

Finally, selective targeting of cancer cells through specific cell surface antigens is an attractive therapeutic approach that is being currently explored for glioma (33–35). A major hurdle in this approach is the paucity of ideal molecular targets that are both restricted in expression to the tumor cells and are available at the cell surface. The selective expression of $B/b_{\Delta g}$ in glioma, its restricted membrane localization, and its expression in all high-grade gliomas tested to date make it an important new potential target for therapy. In addition, the absence of $B/b_{\Delta g}$ in a specific subset of low-grade, indolent oligodendrogliomas suggests that it could also be used as a diagnostic marker to distinguish primary brain tumors of similar histology but different pathologic course. The potential clinical value of $B/b_{\Delta g}$, together with our previous experimental work showing that BEHAB/brevican promotes glioma progression, further underscore the relevance of this protein as a candidate for novel antitumoral approaches in glioma. Understanding the role of the novel glioma-specific isoforms and the mechanisms of underglycosylation and cell membrane association may reveal additional new therapeutic strategies for glioma therapy.

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[3] M.S. Viapiano and R.T. Matthews, unpublished results.

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