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The Proteoglycan Brevican Binds to Fibronectin after Proteolytic Cleavage and Promotes Glioma Cell Motility*

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The adult neural parenchyma contains a distinctive extracellular matrix that acts as a barrier to cell and neurite motility. Nonneural tumors that metastasize to the central nervous system almost never infiltrate it and instead displace the neural tissue as they grow. In contrast, invasive gliomas disrupt the extracellular matrix and disperse within the neural tissue. A major inhibitory component of the neural matrix is the lectican family of chondroitin sulfate proteoglycans, of which brevican is the most abundant member in the adult brain. Interestingly, brevican is also highly up-regulated in gliomas and promotes glioma dispersion by unknown mechanisms. Here we show that brevican secreted by glioma cells enhances cell adhesion and motility only after proteolytic cleavage. At the molecular level, brevican promotes epidermal growth factor receptor activation, increases the expression of cell adhesion molecules, and promotes the secretion of fibronectin and accumulation of fibronectin microfibrils on the cell surface. Moreover, the N-terminal cleavage product of brevican, but not the full-length protein, associates with fibronectin in cultured cells and in surgical samples of glioma. Taken together, our results provide the first evidence of the cellular and molecular mechanisms that may underlie the motility-promoting role of brevican in primary brain tumors. In addition, these results underscore the important functional implications of brevican processing in glioma progression.

Malignant gliomas are primary tumors of the central nervous system with an almost invariably rapid and lethal outcome. Current treatments for gliomas fail to remove the invasive cells that remain diffusely embedded within normal tissue even after aggressive surgical and postsurgical treatment (1). The dispersion of glioma cells is the major cause of disease progression after initial treatment and, therefore, of therapeutic failure.

The ability of glioma cells to disperse within the mature central nervous system is unusual, because adult neural tissue is predominantly inhibitory to process extension and cell movement (2, 3). One of the major barriers to cellular movement in the central nervous system is the neural extracellular matrix (ECM).[2] This matrix is primarily composed of a scaffold of hyaluronic acid (HA) and associated glycoproteins, with a remarkable absence of fibrillar proteins that support cell motility (2, 4). The inhibitory nature of the neural ECM has been largely attributed to a family of chondroitin sulfate proteoglycans that bind and organize HA within the ECM: aggrecan, neurocan, versican, and brevican, collectively known as lecticans (5–7). It is thought that, to overcome this barrier to movement, glioma cells degrade the normal ECM (8, 9) and secrete mesenchymal matrix components that promote cell adhesion and motility, such as fibronectin and collagens (10–13). However, surprisingly, gliomas also express large amounts of the inhibitory lecticans versican (14) and brevican (15, 16).

Brevican, also known as brain-enriched hyaluronan-binding protein, or BEHAB (17), has been one of the most extensively studied chondroitin sulfate proteoglycans in glioma. This neuron-specific proteoglycan is highly overexpressed in primary brain tumors and in experimental models of glioma (16, 18). Moreover, brevican overexpression increases glioma dispersion (19), whereas brevican knockdown inhibits it.[3] In addition, gliomas exhibit unique brevican isoforms (16), and the complex processing of this proteoglycan seems to be critical for its pro-invasive role in glioma (20, 21). However, despite this evidence, the precise mechanism by which brevican promotes glioma dispersion has remained elusive.

Here, we have determined that brevican secreted and cleaved by glioma cells interacts with the mesenchymal ECM protein fibronectin and increases the levels of this protein on the cell surface to enhance cell adhesion and motility. Our results demonstrate a substrate-dependent motogenic role of brevican and suggest that fibronectin may be a key mediator of this role in glioma cells.

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[2] The abbreviations used are: ECM, extracellular matrix; ADAMTS, a disintegrin and metalloprotease with thrombospondin domains; CAM, cell-surface adhesion molecule; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DPBS, Dulbecco's phosphate-buffered saline; HA, hyaluronic acid; HBSS, Hank's buffered salt solution; NCAM, neural cell adhesion molecule; FBS, fetal bovine serum; aa, amino acids; EGFR, epidermal growth factor receptor; ANOVA, analysis of variance; siRNA, small interfering RNA; RT, reverse transcription.

[3] R. T. Matthews, unpublished data.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—The human glioma cell lines U87MG and U373MG (American Type Culture Collection, Manassas, VA) were grown at 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. The rat glioma cell line CNS-1 was grown in RPMI 1640 medium equally supplemented with FBS and antibiotics. These and other (U118MG and U251MG) glioma cell lines were thoroughly tested and compared, to verify that the effects of brevican were comparable among different lines.

Brevican constructs were detected using the rabbit polyclonal antibodies B5, B6, and B50 that have been previously described (16). B5 binds to the N terminus (aa 60–73) of brevican, B6 binds to the chondroitin sulfate attachment region (aa 506–529), and B50 detects the neoepitope QEA*X*ESE exposed in the N-terminal domain of brevican after cleavage. In addition, the following commercial antibodies were used: mouse monoclonal antibodies against fibronectin, N-cadherin, integrin subunits β1, αV, β3, and Tyr^{759}-phosphorylated β3 (BD Biosciences); mouse monoclonal anti-NCAM and anti-actin (Sigma); rabbit polyclonal anti-epidermal growth factor receptor (EGFR), Tyr^{1068}-phosphorylated EGFR, and Thr^{202}/Tyr^{204}-phosphorylated extracellular signal-regulated kinase 1/2 (Erk1/2; Cell Signaling Systems, Danvers, MA); rabbit polyclonal anti-Erk1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and mouse monoclonal anti-α-tubulin (Invitrogen).

Human Tissue—All studies were performed in compliance with the guidelines of the Human Investigations Committee at The Ohio State University College of Medicine. Pathologically graded fresh-frozen surgical samples of adult gliomas (patients 37–58 years old) were obtained from the Midwestern Division of the National Cancer Institute Cooperative Human Tissue Network (NCI/CHTN).

Constructs, Cell Transduction, and Cell Proliferation Assays—A clone containing the complete coding sequence of human brevican (GenBank™ number BC010571) was first subcloned into the vector pcDNA3.1(+) (Invitrogen). The complete N-terminal fragment of human brevican (Met^1–Glu^{400}), a shorter N-terminal variant (Met^1–Ser^{360}), and the C-terminal fragment (signal peptide Met^1–Ala^{22} plus Ser^{401}–Pro^{911}) were created by PCR and subcloned in the same vector. The "uncleavable" form of human brevican was created by site-directed mutagenesis to change the sequence ^{396}ATESESRGAI^{405} to ATESE<u>NVY</u>AI, as previously described (21). All constructs were subsequently subcloned into the lentiviral carrier vector pCDH1-MCS-EF1-coGFP (System Biosciences, Mountain View, CA). Lentiviruses were produced in H293 cells using the ViraPower (Invitrogen) packaging system. Viruses were collected and titrated according to standard protocols (22) and used to infect all glioma cells at a multiplicity of infection equal to 1. Transduced cells were cultured for 2 weeks and checked for high levels of green fluorescent protein expression before further testing. For proliferation assays, cells were grown in 96-well plates at an initial density of 2,000 cells/well in 200 μl of culture medium. Proliferation was quantified by measuring the reduction of a soluble tetrazolium salt (CellTiter kit, Promega, Fitchburg, WI) according to the manufacturer's instructions.

Cell Adhesion and Motility Assays—48-well plates were precoated for 1 h at room temperature with the following substrates: human fibronectin (5 μg/ml; BD Biosciences), type IV bovine collagen (5 μg/ml; Sigma), type I human laminin (5 μg/ml; Invitrogen), high M_r poly-L-lysine (50 μg/ml; Sigma), and high M_r hyaluronic acid (200 μg/ml; Calbiochem). The plates were subsequently washed with DPBS, and nonspecific binding sites were blocked with 1% bovine serum albumin in DPBS. Stably transduced glioma cells were gently detached by brief exposure to 0.025% trypsin plus 2 mM EDTA in DPBS, further diluted in DPBS, dissociated with a glass Pasteur pipette, washed in culture medium with 10% FBS, and finally resuspended in the same medium for manual cell counting. Cells were plated at 50,000 cells/well on the precoated plates. After 30 min at 37 °C and 5% CO_2, the cells were washed, fixed, and quantified by crystal violet staining as described (23). For haptotactic motility assays, Transwell™ culture inserts (12-mm diameter × 8-μm pore size; BD Biosciences) were precoated on their underside with the same substrates used in cell adhesion assays. Cells were plated inside the inserts at 50,000 cells/well and allowed to migrate during 4 h. Subsequently, the cells on the upper side of the well were removed, and the cells that had migrated to the underside were fixed, stained with 4′,6-diamidino-2-phenylindole, imaged, and quantified by automated nuclei count using the software ImageJ. All experiments were repeated at least three times with 4–6 replicates/experimental condition. Data from adhesion and motility experiments were analyzed by 2-way ANOVA.

RNA Interference, RGD Peptide Competition, and EGFR Inhibition Assays—Two commercially validated siRNA oligonucleotides against human fibronectin (*FN1* gene), known to cause >80% inhibition in cultured cells, were purchased from Qiagen (Valencia, CA). Control siRNAs from the same manufacturer included scrambled versions of the fibronectin siRNAs and a validated, nonsilencing siRNA ("AllStars negative control"). siRNAs were transiently transfected at the rate of 100 pmol/(1.10^6 cells × 3 ml of culture medium), and the cells were collected 48 h post-transfection for adhesion assays and verification of fibronectin levels by Western blotting.

The pentapeptide GRGDS was purchased from Sigma and dissolved at 1 mg/ml in sterile water. Cells freshly resuspended and counted for adhesion assays were incubated with several dilutions of the peptide for 20 min at 37 °C before plating on different substrates.

An inhibitor of EGFR phosphorylation, tyrphostin AG1478, was purchased from Cell Signaling Technologies and dissolved at 1 mM in DMSO. AG1478 was added to the cultures at a final concentration of 150 nM for 6–8 h before preparing the cells for adhesion assays as indicated.

Additional competition assays included treatment of glioma cells with hyaluronidase to remove any potential pericellular coat of HA and preincubation of the cells with purified chondroitin sulfate to compete the effects of brevican. None of these assays resulted in changes in the effects of brevican in glioma cells (data not shown).

Cell Dispersion in Organotypic Cultures—Organotypic cultures of mouse brain slices were essentially performed as described (21). Briefly, postnatal day 1 CD-1 mice (Charles River Laboratories, Wilmington, MA) were decapitated on ice, and their brains were removed into ice-cold HBSS containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml ampothericin. The meninges were quickly removed, and the brains were sectioned coronally into 300-μm slices using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY). Brain slices were dissociated in HBSS and placed on MilliCel membranes (0.4-μm pore size, Millipore, Temecula, CA), suspended inside a 35-mm culture dish over 1 ml of slice medium (Neurobasal-A/HBSS, 70/30 ratio), supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate, 1% FBS, 0.5× B27 supplement (Invitrogen), 0.5× G5 supplement (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Two days before preparing the brain slices, glioma cells were resuspended at 1×10^5 cells/ml and cultured over a 2-mm-thick layer of 1% sterile agarose to form spherical aggregates. These aggregates were individually seeded onto the brain slices with a capillary pipette and cultured for an additional 48–72 h. All assays were performed with CNS-1 cells, because their fast migration rate allows the detection of significant differences over short periods compared with other cell lines, thus ensuring the good structural preservation and survival of the brain slices throughout the assay.

Fluorescent cell aggregates were imaged at low magnification (×4) at 24-h intervals using an inverted microscope. The software ImageJ was used to quantify the cross-sectional area occupied by the glioma cells over time. Brain slice survival was assessed after 72 h by adding 0.5 μg/ml propidium iodide to the slice culture medium and checking the slice for apoptotic cells. Aggregates surrounded by a large number of apoptotic cells in the slice were discarded from further analysis. Experiments were performed in triplicate, using 10 aggregates/experimental condition, and analyzed by two-way ANOVA for repeated measures.

Western Blotting and Quantitative RT-PCR—To analyze changes in cell surface adhesion molecules (CAMs), cells were gently resuspended and dissociated in DPBS as indicated, transferred to fresh culture medium, and plated on uncoated, poly-L-lysine-coated, or fibronectin-coated 60-mm culture dishes at a total density of 5×10^5 cells/dish. After 2 h, nonadhered cells were washed out, and the remaining cells were flash-frozen using ethanol/dry ice. To analyze fibronectin synthesis, cells were plated on uncoated 60-mm dishes, washed twice with serum-free medium Opti-MEM I (Invitrogen), and cultured in Opti-MEM I for periods of 2–16 h before freezing. To determine the effect of brevican on EGFR signaling, the culture medium of parental U87MG cells was replaced with conditioned medium from control or brevican-expressing U87MG cells for 1 h before freezing the cells. To determine the effect of AG1478 on EGFR phosphorylation and fibronectin mRNA levels, cells were incubated with the EGFR inhibitor for 2 h before freezing.

To prepare samples for Western blotting, total extracts of the frozen cells were prepared in 25 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 0.8% (w/v) CHAPS, 10 mM EDTA, and a mixture of protease (*Complete*) and phosphatase (*PhosStop*) inhibitors (both from Roche Applied Science). Cell extracts containing 15 μg of total protein were electrophoresed on reducing 7% SDS-polyacrylamide gels and analyzed by Western blotting. Blots were imaged using a CCD imaging system (UltraLum Omega 12iC) and subjected to a 25% increase in brightness and 15% increase in contrast before densitometric analysis.

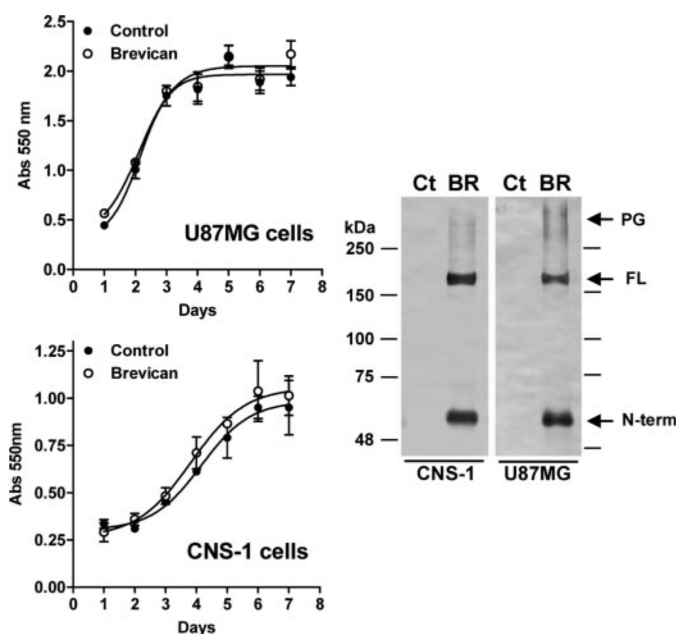

FIGURE 1. **Brevican expression does not affect glioma cell proliferation.** *Left*, human (*U87MG*) and rat (*CNS-1*) glioma cell lines were stably transduced for brevican expression using a lentiviral vector. Two weeks after infection, cells were tested for changes in cell proliferation using a metabolic assay for reduction of tetrazolium as described. Proliferation curves were repeated twice in triplicate for each cell line. Additional controls (not shown) included quantification of Ki67-positive cells as described (24) and crystal violet staining. *Right*, Western blotting of conditioned culture medium revealed a complete absence of brevican expression in control cells, whereas in brevican-expressing cells, we detected the full-length protein (*FL*) and its major N-terminal cleavage product (*N-term*). Film overexposure also revealed the chondroitin sulfate-bearing form(s) of brevican (*PG*). *Ct*, control-transduced cells; *BR*, brevican-transduced cells.

To analyze mRNA expression, frozen cells were extracted in Trizol (Invitrogen), residual DNA was degraded using Turbo-DNA Free (Applied Biosystems, Foster City, CA), and total RNA was processed for quantitative RT-PCR using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions.

Immunocytochemistry—For live immunocytochemical staining, cells were grown on poly-L-lysine-coated coverslips for 48–72 h. Unfixed, unpermeabilized cultures were rinsed twice in cold Dulbecco's modified Eagle's medium and incubated in Dulbecco's modified Eagle's medium, 2% FBS containing anti-fibronectin antibody, for 30 min at 4 °C. Cells were subsequently rinsed, fixed for 10 min in cold 100% methanol, and processed for fluorescence microscopy.

Immunoprecipitation and Dot-blot Assays—Conditioned medium of brevican-expressing cells was subjected to immunoprecipitation with anti-brevican or anti-fibronectin antibodies preabsorbed to protein G (Seize-X kit; Pierce), according to standard protocols. Human glioma samples (250 mg wet

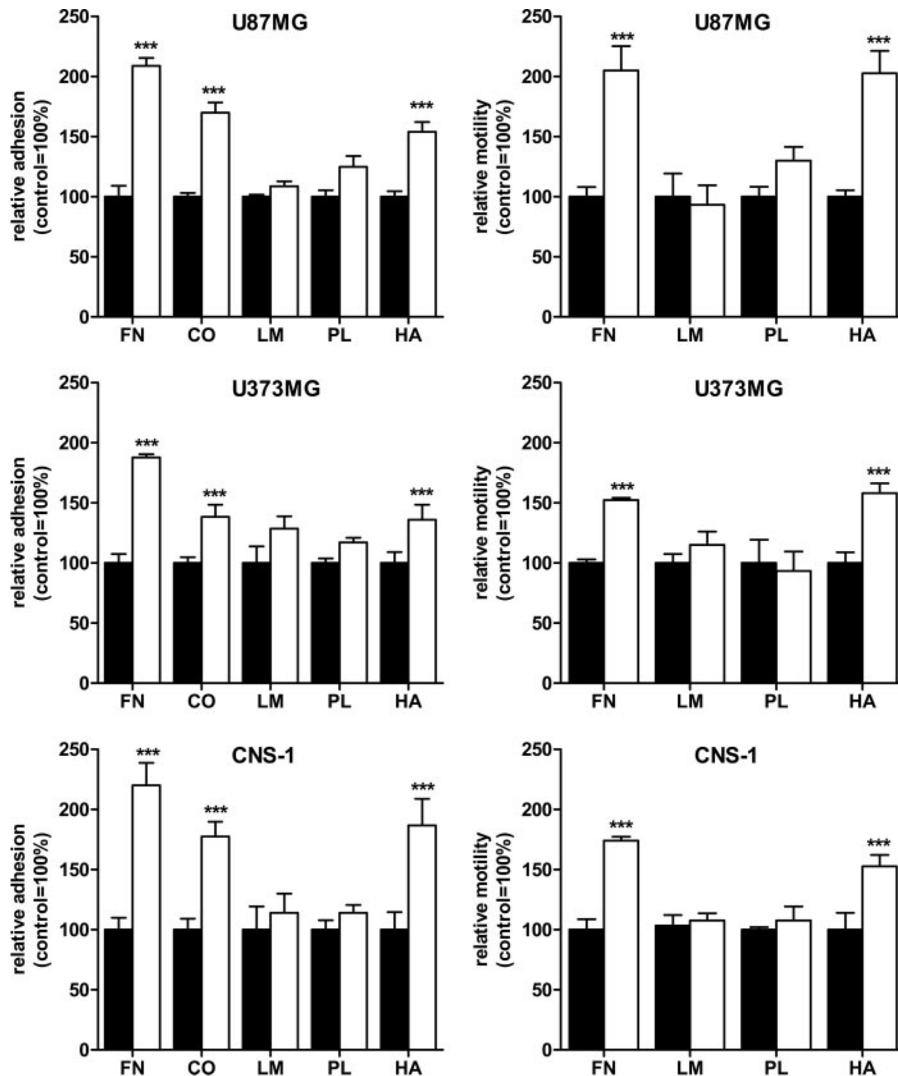

FIGURE 2. **Brevican expression enhances substrate-dependent cell adhesion and motility.** *Left*, human (*U87MG* and *U373MG*) and rat (*CNS-1*) glioma cells stably transduced for brevican expression were plated on multiwell plates precoated with the following substrates: *FN*, fibronectin; *CO*, type-IV collagen; *LM*, type-I laminin; *PL*, poly-L-lysine; *HA*, hyaluronic acid. Cells were subsequently washed, fixed, and quantified by crystal violet staining. *Right*, these cell lines were also plated on Transwell chambers that had been precoated on their underside with the same substrates. Cells that migrated to the underside were quantified by automated nuclei count using ImageJ software. All experiments in both panels were repeated at least three times with 4–6 replicates per experimental condition. Data (mean ± S.E.) were analyzed by two-way ANOVA (***, $p < 0.001$). All of the cell lines assayed here express endogenous fibronectin and can cleave brevican at the ADAMTS cleavage site. *Black bars*, control cells; *white bars*, brevican-expressing cells.

weight) were first homogenized at 10% (w/v) in 25 mM Tris-HCl buffer (pH 7.4), containing 320 mM sucrose and protease inhibitors, and subjected to subcellular fractionation as described (16). The total particulate fraction from each sample was extracted for 1 h on ice in 25 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 0.8% (w/v) CHAPS, and protease inhibitors. Extracts were cleared by centrifugation at 10,000 × *g* for 15 min, and the supernatants were subjected to immunoprecipitation, as indicated.

For dot-blot assays, purified recombinant fibronectin (5 μg in 100 μl of DPBS) or bovine serum albumin (fraction V, 5 μg/100 μl) were dot-spotted in nitrocellulose membranes. Membranes were washed in DPBS, blocked with 1% serum albumin, and incubated overnight with concentrated conditioned medium from control or brevican-expressing cells. Brevican binding was detected using the anti-brevican antibodies B5 and B50 (16) and alkaline phosphatase-conjugated secondary antibodies.

RESULTS

Brevican Promotes Substrate-dependent Cell Adhesion and Motility—The promigratory effects of brevican in gliomas have been described in detail *in vivo* (19, 20, 21), but the precise mechanisms underlying these effects remain unclear. Here, we designed experiments to specifically investigate the mechanism(s) by which brevican enhances glioma dispersion.

Several glioma cell lines express brevican when grafted intracranially but do not express it in culture, probably due to the absence of central nervous system-specific inducing factors (18). To overcome this limitation, we transduced glioma cells with a lentiviral vector to maintain a stable production of this proteoglycan *in vitro*. Stable transduction of brevican did not affect cellular proliferation (Fig. 1) or cell morphology (not shown), in agreement with previous results (19, 21). However, when we tested the ability of these cells to attach to different substrates representative of the neural ECM and the basal lamina of brain blood vessels, we observed a significant, substrate-dependent effect in brevican-expressing cells. Specifically, brevican enhanced glioma cell adhesion to fibronectin, type-IV collagen, and hyaluronic acid but not to poly-L-lysine or laminin (Fig. 2, *left*). Moreover, this substrate-dependent increase in cell adhesion correlated with increased haptotactic motility toward the same substrates (Fig. 2, *right*). These results were verified with additional glioma cell lines, including U118MG and U251MG (data not shown). Interestingly, the expression of brevican did not cause any evident changes in cell adhesion to uncoated wells, explaining in part why the effects of this proteoglycan have remained elusive *in vitro*.

The Motogenic Effects of Brevican Are Cleavage-dependent—Brevican is processed in gliomas by metalloproteases of the ADAMTS family (25, 26), which cleave the full-length protein at a single site. Mutation of this site creates an "uncleavable" form of brevican and abolishes all major processing of this proteoglycan (21). Previous work from our laboratories

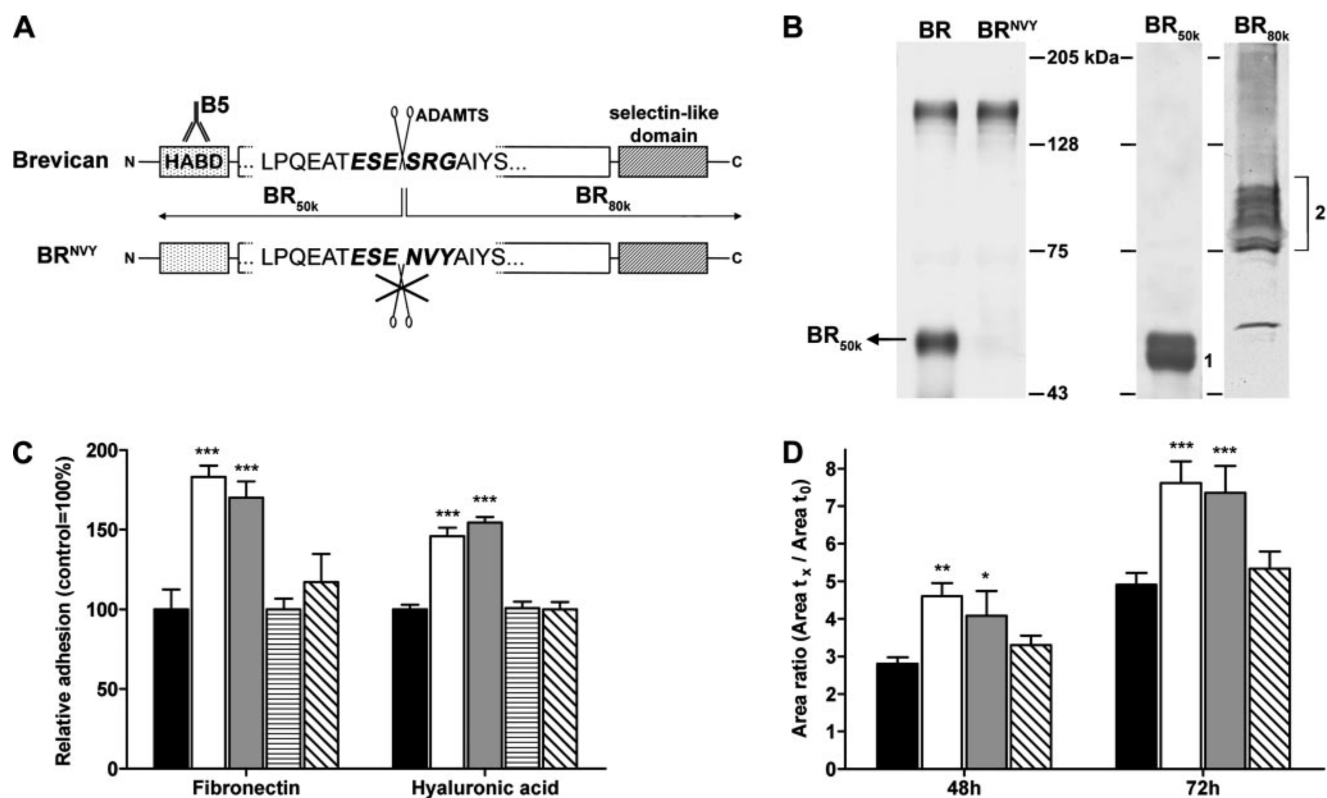

FIGURE 3. **Brevican cleavage is necessary to enhance glioma cell adhesion and dispersion.** *A*, the "uncleavable" form of human brevican (BR^{NVY}) was created by site-directed mutagenesis of the only ADAMTS-mediated cleavage site in the molecule. *HABD*, HA-binding domain. *B*, Western blotting of conditioned culture medium from U87MG cells stably transduced with different brevican constructs. *BR*, full-length brevican; BR^{NVY} is "uncleavable" brevican; BR_{50k} is the N-terminal fragment (aa 1–400); BR_{80k} is the C-terminal fragment (aa 401–911). Stable expression of BR_{50k} yielded the normal product as well as a smaller, less glycosylated variant (*1*), whereas expression of BR_{80k} resulted in a spread of heavily glycosylated variants (*2*). Blots were developed with the antibodies B5 (to detect BR, BR^{NVY}, and BR_{50k}) and B6 (to detect BR_{80k}). *C*, U87MG cells were tested in cell adhesion assays as in Fig. 2. BR_{50k} mimicked the effect of full-length brevican, whereas BR^{NVY} and BR_{80k} were unable to enhance cell adhesion. The same results were obtained with CNS-1 cells (not shown). *Black bars*, control; *white bars*, brevican; *gray bars*, BR_{50k}; *horizontal-crossed bars*, BR_{80k}; *hatched bars*, BR^{NVY}. All experiments were performed in triplicate and analyzed by two-way ANOVA (***, $p < 0.001$). *D*, CNS-1 cells were seeded on brain slices and tested for dispersion as indicated under "Experimental Procedures." Areas occupied by the dispersed cells were compared by two-way ANOVA for repeated measures. Results show a significant enhancing effect of brevican and BR_{50k}, but not BR^{NVY}, on cell dispersion (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The *bars* represent the same constructs indicated in *C*.

has demonstrated that the N-terminal fragment of brevican is sufficient to promote glioma dispersion, whereas the full-length protein has no effects in gliomas *in vivo* (19–21). We thus hypothesized that cleavage could be necessary for the molecular interactions of brevican required to promote cell motility.

To verify if the effects of brevican cleavage on tumor dispersion could be reproduced *in vitro*, we transduced U87MG and CNS-1 glioma cells for stable expression of the cleavage products of brevican and the "uncleavable" form of this proteoglycan (BR^{NVY}). Uncleavable brevican was created by site-directed mutagenesis (Fig. 3, *A* and *B*), using a mutation previously described to abolish the major ADAMTS cleavage site in the related proteoglycan aggrecan (21, 27).

Cells expressing the different brevican constructs were subsequently tested in cell adhesion and dispersion assays. First, we observed that the proadhesive effect of full-length brevican, which is cleaved by ADAMTS proteases in U87MG and CNS-1 cells, could not be reproduced by mutant brevican lacking the ADAMTS cleavage site. In contrast, the N-terminal cleavage fragment of brevican, BR_{50k}, produced the same results as the full-length molecule (Fig. 3*C*). The C-terminal fragment, on the other hand, failed to enhance cell adhesion (Fig. 3*C*) and had no inhibitory effects when co-expressed with the N-terminal fragment (data not shown). Nevertheless, because expression of the C-terminal fragment resulted in the presence of several heavily glycosylated variants not observed *in vivo* (Fig. 3*B*), it is possible that the effects of this fragment could not have been fully reproduced *in vitro*.

Taken together, these results underscored the key role of brevican cleavage and the release of its N terminus as a necessary step for brevican signaling. Thus, we next verified the effects of brevican constructs using an assay for cell dispersion on organotypic brain slices, which mimic the brain cytoarchitecture and its natural barriers to cell movement. Aggregates of glioma cells were placed on cultured brain slices, and the area of cell dispersion was quantified over a 72-h period. Glioma cells expressing normal brevican or its N-terminal cleavage product dispersed over a significantly larger area than control cells (Fig. 3*D*). However, glioma cells expressing BR^{NVY} were indistinguishable from the controls. These results were consistent with the previous assay on cell adhesion and, more importantly, were in agreement with previously reported effects of brevican constructs on tumor progression *in vivo* (21). Overall, these results suggested that brevican was acting as a promotility signal following cleavage in the extracellular space. Therefore, we next

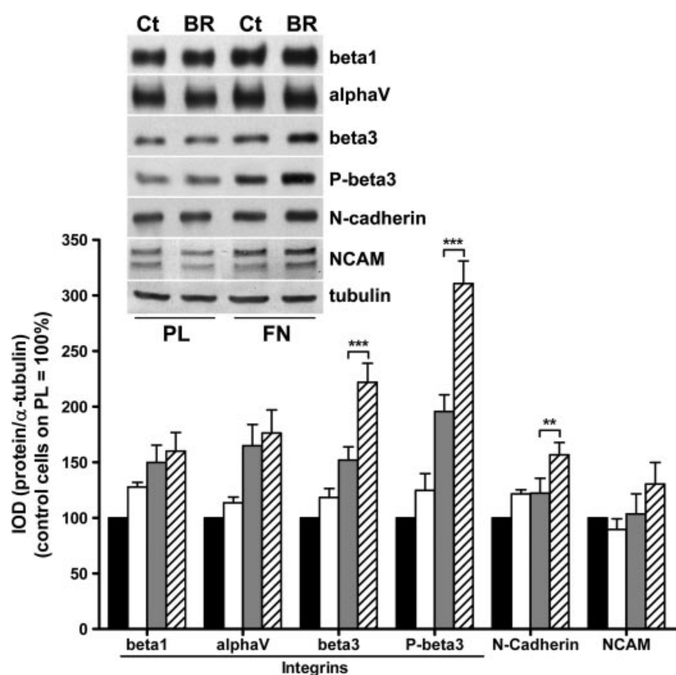

FIGURE 4. **Brevican enhances substrate-dependent expression of CAMs.** Control and brevican-expressing U87MG cells were dissociated, plated on precoated 60-mm dishes for 2 h, and immediately processed to analyze the expression levels of CAMs. The figure shows a representative Western blotting and the analysis of three independent experiments by one-way ANOVA and *post hoc* Tukey-Kramer test (mean ± S.E.; **, $p < 0.01$; ***, $p < 0.01$). Brevican expression did not affect the levels of any of the CAMs analyzed when cells were plated on poly-L-lysine. In contrast, when cells were plated on fibronectin, brevican-expressing cells showed increased levels of β3 integrin, Tyr^{759}-phosphorylated β3 integrin (*P-beta3*), and N-cadherin compared with control cells. Cells plated on uncoated dishes yielded identical results to those plated on poly-L-lysine (not shown). *Ct*, control; *BR*, brevican; *PL*, poly-L-lysine; *FN*, fibronectin. *IOD*, integrated optical density. *Solid black bars*, control cells plated on PL; *white bars*, brevican cells plated on PL; *solid gray bars*, control cells plated on FN; *hatched bars*, brevican cells plated on FN.

focused in the possible mechanisms that could mediate this motogenic effect.

Brevican Induces Up-regulation of CAMs—Brevican and its cleavage products are predominantly soluble in culture conditions (28) and thus unlikely to act as anchoring molecules and directly mediate the enhanced adhesion of the glioma cells to different substrates. However, other proteoglycans of the lectican family can interact directly or indirectly with transmembrane CAMs, such as integrins (29), N-cadherin (30), and the members of the NCAM/L1 family (31), and the domains involved in these interactions are conserved among the lecticans. Thus, we first investigated whether some of these CAMs could be candidate transducers for the observed effects of brevican *in vitro*.

Stable or transient expression of brevican in U87MG and CNS1, followed by cell freezing and processing for Western blotting, did not reveal any significant alterations in the levels of N-cadherin, NCAM, and several integrin subunits (data not shown). Similarly, when brevican-expressing glioma cells were resuspended and plated on poly-L-lysine-coated or uncoated plates, the levels of the different CAMs tested were still undistinguishable from the controls (Fig. 4). However, when the cells were resuspended and plated on fibronectin-coated plates, brevican-expressing cells exhibited modest but significantly higher levels of N-cadherin and the β3 integrin subunit than control cells. Moreover, there was a significant increase in the Tyr^{759}-phosphorylated form of β3 integrin, which has been correlated to increased cell spreading (32, 33). This increase in β3 integrin expression and phosphorylation was additional to the increase caused by cell plating on fibronectin, thus allowing us to differentiate the substrate-dependent from the brevican-dependent effects.

These results matched the effect of brevican on cell adhesion and suggested potential CAMs involved in brevican-enhanced cell attachment. However, they also revealed that the CAMs were not modified by brevican expression alone but required in addition the presence of the ECM molecule fibronectin. This prompted us to explore more closely the possible interaction between brevican and fibronectin as a potential mechanism underlying the effects of brevican in glioma cells.

Brevican Promotes EGFR-dependent Fibronectin Synthesis—Brevican and fibronectin are usually detected at high levels in most clinical specimens of malignant gliomas (13, 16). This co-up-regulation contrasts sharply with normal neural tissue, which lacks fibronectin (4, 34), and with nonneural tumors that metastasize to the brain, which do not produce brevican (18). Preliminary observations[4] had suggested that exposure to brevican could increase fibronectin levels in glioma cells. Thus, we set out to investigate the effect of brevican constructs on fibronectin expression and the possibility of a direct interaction between these two ECM proteins.

Using U87MG cells, we first analyzed the expression of fibronectin in cells stably expressing full-length brevican, BR_{50k}, and BR^{NVY}. Western blotting results showed that cells expressing either normal brevican or its N-terminal fragment exhibited increased levels of fibronectin in cell lysates, whereas cells expressing BR^{NVY} were not significantly different from controls (Fig. 5*A*). Additional tests in the conditioned culture medium (data not shown) also disclosed increased levels of soluble fibronectin in brevican- and BR_{50k}-expressing cells. Results from quantitative RT-PCR (Fig. 5*B*) showed essentially the same results reproduced at the mRNA level, suggesting that the increased levels of fibronectin in brevican- and BR_{50k}-expressing cells were probably due to increased protein synthesis.

To investigate whether the increase of fibronectin in cell lysates corresponded to fibronectin retained on the cell surface rather than intracellularly, we performed live cell staining of control and brevican-expressing cells. Immunocytochemical analysis demonstrated that not only brevican-expressing cells produced more fibronectin than controls, as observed in the Western blots, but also that this protein was accumulated on the cell surface and organized in microfibrillar patterns (Fig. 5*C*). This effect of brevican on the pericellular coat was specific for fibronectin, because we could not detect changes in total HA or pericellular accumulation of HA (not shown).

Previous work from B. Yang and co-workers (35–37), focused on the signaling mechanisms of the lectican versican in glioma cells and neurons, has provided considerable evidence that fragments of this lectican can up-regulate fibronectin syn-

[4] B. Hu and M. S. Viapiano, unpublished observations.

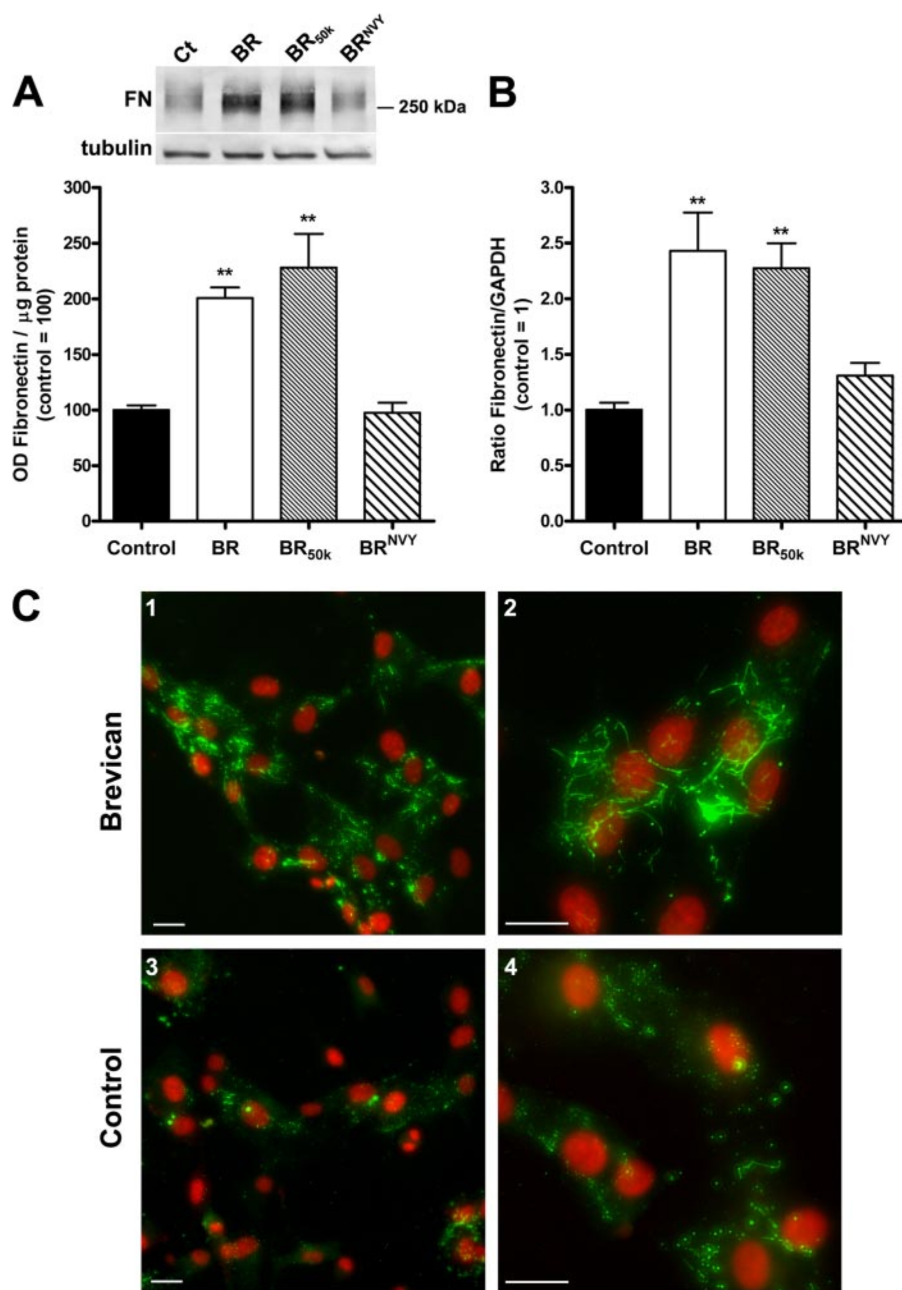

FIGURE 5. **Brevican enhances fibronectin expression on the surface of glioma cells.** U87MG cells stably expressing a control vector (*Ct*), full-length brevican (*BR*), its N-terminal fragment (BR_{50k}), or uncleavable brevican (BR^{NVY}) were plated on uncoated surfaces and cultured on serum-free medium for 16 h. Cells were subsequently processed for Western blotting (*A*) or quantitative RT-PCR (*B*). Brevican- and BR_{50k}-expressing cells showed increased levels of fibronectin compared with control and BR^{NVY}-expressing cells (mean ± S.E.) (**, $p < 0.01$ by one-way ANOVA and *post hoc* Tukey-Kramer test). *C*, live, unpermeabilized U87MG cells were incubated with anti-fibronectin antibodies and subsequently fixed and processed for immunocytochemistry. Fibronectin staining (*green*) was more intense in brevican-expressing cells (*1* and *2*) and showed a predominant microfibrillar pattern, compared with the punctate pattern observed in control cells (*3* and *4*). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (false colored in *red* to enhance contrast). *Bars*, 20 µm.

thesis and can activate the EGFR and Erk1/2. In addition, EGFR and Erk1/2 activation have been shown to cause fibronectin up-regulation in several cell types (38–40). Thus, we decided to explore the effect of brevican on EGFR signaling as a potential mechanism leading to increased fibronectin production in glioma cells.

For these studies, we exposed parental U87MG cells to the conditioned medium of control or brevican- or BR_{50k}-expressing cells. We observed that cells exposed to brevican- or BR_{50k}-containing medium had a significant increase of phospho-EGFR and phospho-Erk1/2 as well as a significant increase in the levels of fibronectin mRNA (Fig. 6*A*). Moreover, treatment of brevican- and BR_{50k}-expressing cells with an inhibitor of EGFR phosphorylation, tyrphostin-AG1478, led to a significant decrease of EGFR phosphorylation (not shown) and reduction of fibronectin mRNA levels (Fig. 6*B*). Furthermore, AG1478 abolished the enhanced adhesion of brevican-expressing cells to fibronectin compared with the controls (Fig. 6*C*). Although these results did not show or suggest a direct interaction of brevican with EGFR, they indicated that EGFR activation was likely involved in transducing the signaling of brevican after its cleavage. In addition, these observations highlighted the possible role of fibronectin as the physical mediator of brevican-enhanced cell adhesion and motility.

Fibronectin Is Necessary for the Proadhesive Effect of Brevican and Associates with the N Terminus of This Proteoglycan—The effects of brevican on fibronectin and β3 integrin up-regulation strongly suggested that fibronectin and its membrane receptors could be necessary components of the brevican-dependent phenotype. To verify this hypothesis, we used siRNA to transiently and effectively reduce the levels of fibronectin to undetectable levels by Western blotting (Fig. 7*A*). After this treatment, brevican-expressing cells became undistinguishable from controls when tested for adhesion (Fig. 7*B*) and motility (not shown), both on fibronectin and HA-coated surfaces. Conversely, an RGD-containing peptide that disrupts integrin-fibronectin association (41) inhibited total cell adhesion but did not affect the enhancing effect of brevican (Fig. 7*C*). These results suggested that the substrate-dependent effects of brevican were critically dependent on cell surface fibronectin but probably not on the association of this molecule to its integrin receptors.

Finally, we investigated the possibility of a direct association between fibronectin and brevican. Co-immunoprecipitation

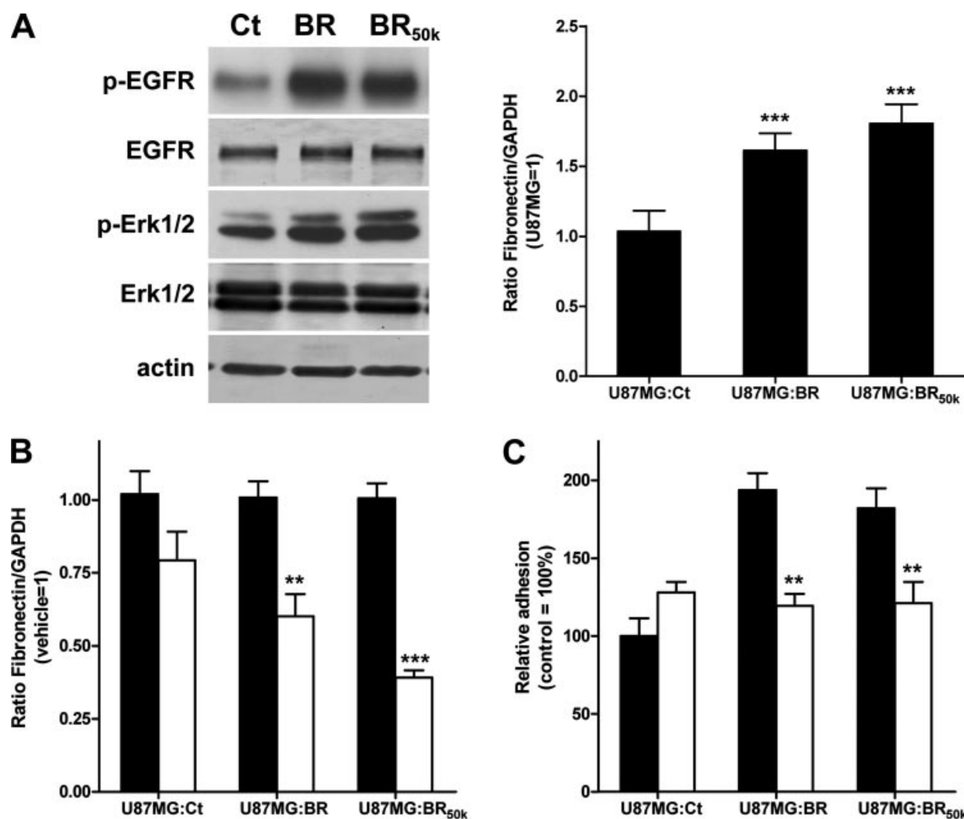

FIGURE 6. **Brevican- dependent increase of fibronectin involves EGFR signaling.** *A*, U87MG parental cells were exposed for 1 h to conditioned medium from control (*Ct*), brevican-expressing (*BR*), and BR_{50k}-expressing (BR_{50k}) cells, and subsequently processed for Western blotting or quantitative RT-PCR. Cells exposed to brevican- or BR_{50k}-containing medium showed increased levels of Tyr^{1068}-phosphorylated EGFR (*p-EGFR*) and Thr^{202}/Tyr^{204}-phosphorylated Erk1/2 (*p-Erk 1/2*) as well as increased levels of fibronectin mRNA (***, $p < 0.001$ by one-way ANOVA). *B*, treatment with the EGFR inhibitor AG1478 for 2 h significantly reduced fibronectin mRNA levels in brevican- and BR_{50k}-expressing cells. *Black bars*, cells treated with drug vehicle; *white bars*, cells treated with 50 μM AG1478. Values (mean ± S.E.) are expressed relative to the fibronectin levels of each cell type treated with vehicle (**, $p < 0.01$; ***, $p < 0.001$ by two-tailed Student's *t* test). *C*, treatment with AG1478 for 6 h abolished the enhancing effect of brevican and BR_{50k} on cell adhesion to fibronectin. *Black bars*, cells treated with drug vehicle; *white bars*, cells treated with 50 μM AG1478. Values are expressed as percentage of control cells treated with vehicle (**, $p < 0.01$; ***, $p < 0.01$ by one-way ANOVA).

assays using conditioned medium from brevican-expressing U87MG glioma cells showed that fibronectin associated to the N-terminal cleavage fragment of brevican but not to the full-length proteoglycan (Fig. 8*A*). In agreement with this result, fibronectin failed to associate to full-length uncleavable brevican, but co-precipitated with the recombinant N-terminal domain of brevican. Moreover, fibronectin co-precipitated with a 40-amino acid shorter version of this N-terminal domain (Fig. 8*B*), suggesting that the association between brevican and fibronectin does not occur at a neoepitope introduced by ADAMTS cleavage but rather at a cleavage-unmasked site(s) located in the N-terminal Ig-like domain or the HA-binding repeats of brevican. Furthermore, co-immunoprecipitation of native brevican and fibronectin from soluble extracts of human glioblastoma specimens showed essentially the same results observed in cultured cells (Fig. 8*C*), suggesting that the N-terminal cleavage fragment of brevican interacts with fibronectin in gliomas *in vivo*.

We also verified the association of brevican and fibronectin using a modified dot-blot assay with purified fibronectin spotted on nitrocellulose membranes. These membranes were incubated with conditioned medium from control or brevican-expressing cells, followed by detection of brevican binding using our anti-brevican antibodies. For this test, we utilized the antibody B5 that detects both full-length brevican and its N-terminal cleavage product and the antibody B50 that only detects the cleavage product (16). Combined use of these antibodies strongly suggested that the N-terminal fragment of brevican could bind directly to fibronectin (Fig. 8*D*). Interestingly, we could not detect this binding in a similar far Western blotting assay using reduced and denatured fibronectin (data not shown), suggesting that the binding of brevican to fibronectin requires the latter to be in its native state.

DISCUSSION

Although cell migration within the neural parenchyma is a common feature of gliomas, it is almost never observed in other tumors that metastasize to the central nervous system, even when those tumors may aggressively invade their tissue of origin (42). At the same time, the migratory ability of gliomas is restricted to central nervous tissue. Gliomas very rarely metastasize and, when implanted experimentally in nonnervous tissue, they grow as encapsulated, noninfiltrative masses (42, 43). This suggests that a combination of glioma-specific molecular mechanisms and the particular composition of the neural microenvironment may underlie the unique ability of these tumors to disperse in the central nervous system.

To overcome the barriers to cell motility, glioma cells degrade the neural ECM (8, 9) and secrete their own matrix components (10–12). Among these, glioma cells produce ECM molecules common to mesenchymal and connective tissues but absent from the adult central nervous system, such as fibronectin and nonfibrillar collagens (44, 45). In addition, and somewhat surprisingly, glioma cells up-regulate some of the lectican proteoglycans that inhibit cell motility in the neural ECM (14–16). This probably results in a uniquely "hybrid" ECM that surrounds the motile glioma cells and differs from the matrices of both normal adult neural cells and tumor cells that metastasize to the central nervous system (45).

Brevican has been repeatedly identified as a highly up-regulated molecule in the ECM of gliomas (45–47). This proteoglycan can be detected in the invasive borders of experimentally induced tumors (48) and is increased in tumors with high infil-

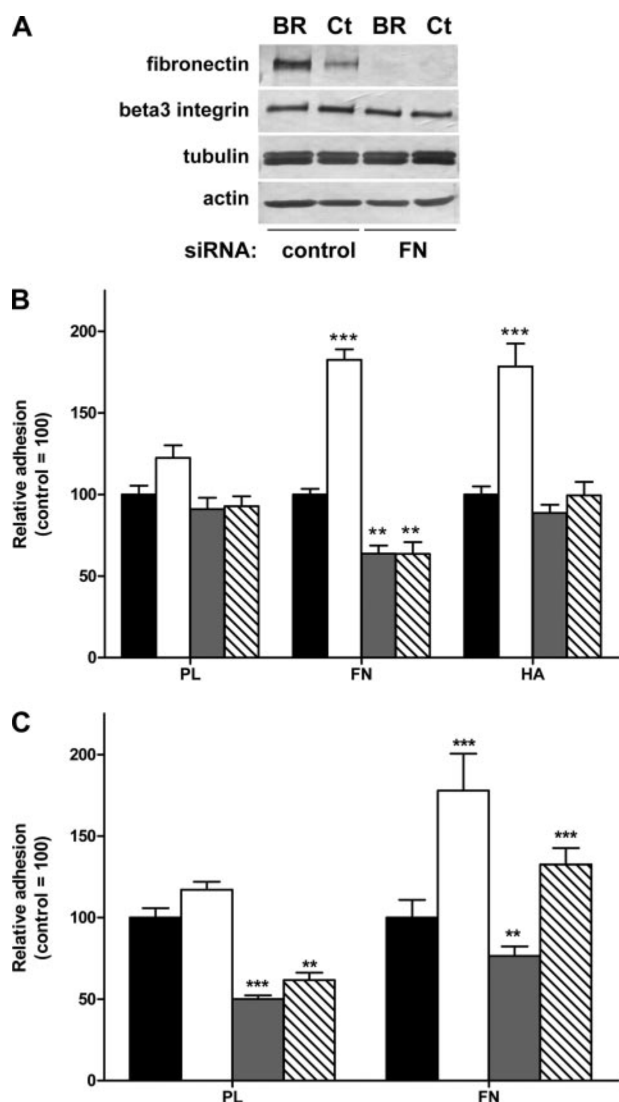

FIGURE 7. **Fibronectin is necessary for brevican-dependent increased cell adhesion.** *A*, U87MG cells were transiently transfected with control or fibronectin-specific (*FN*) siRNA. Fibronectin was undetectable by Western blotting 48 h after siRNA-mediated knockdown. *BR*, U87MG-brevican; *Ct*, U87MG control. *B*, fibronectin knockdown abolished the enhancing effect of brevican on cell adhesion to fibronectin and HA. *Black bars*, U87MG control plus control siRNA; *white bars*, U87MG-brevican plus control siRNA; *gray bars*, U87MG control plus fibronectin siRNA; *hatched bars*, U87MG-brevican plus fibronectin siRNA. Values (mean ± S.E.) are expressed as percentage of control for each substrate. Base-line adhesion was significantly reduced for RNA interference-treated cells plated on fibronectin, suggesting that homotypic fibronectin binding may play a major role for cell adhesion to this substrate. *C*, U87MG cells were dissociated, incubated with 100 μg/ml of the peptide GRGDS for 20 min, and tested for cell adhesion. The peptide reduced cell adhesion to the substrates but did not affect the enhancing effect of brevican. *Black bars*, U87MG control plus vehicle; *white bars*, U87MG-brevican plus vehicle; *gray bars*, U87MG control plus RGD; *hatched bars*, U87MG-brevican plus RGD. Values (mean ± S.E.) are expressed as percentage of control for each substrate. **, $p < 0.01$; ***, $p < 0.001$ by two-way ANOVA.

trative profiles (49, 50). Clinically, brevican up-regulation correlates with poor survival of patients with high grade gliomas (47, 51). Our previous research has identified and characterized glioma-specific isoforms of brevican (16, 28), suggesting that the up-regulation of this proteoglycan by glioma cells is accompanied by changes in its processing and, probably, its molecular interactions.

Brevican belongs to a family of proteoglycans usually described as barrier molecules that prevent cell and neurite motility in the adult nervous system (52). Neurons do not attach well to surfaces coated with brevican and do not extend axons toward a surface containing this chondroitin sulfate proteoglycan (53, 54). *In vivo*, brevican is expressed around the boundaries of the rostral migratory stream (55) and is a major up-regulated component of the glial scar after neural injury (56, 57), limiting axonal extension and probably limiting neuroblast and astrocyte motility (53, 55). Thus, brevican seems to function in the neural matrix predominantly as a stop signal for motile neural cells or extending neurites. This role, however, contrasts with the enhanced dispersion of brevican-expressing glioma cells *in vivo* (19, 21). Therefore, we sought here to identify the possible cellular and molecular mechanisms that could explain the permissive role of brevican in glioma cell migration.

Our first goal was to establish an *in vitro* model to recapitulate some of the proinvasive effects of brevican observed in gliomas. Brevican increases glioma dispersion *in vivo* (19, 20), and this effect is critically dependent on ADAMTS-mediated cleavage (20, 21). We thus hypothesized that, *in vitro*, brevican could affect glioma cell adhesion and/or motility in a cleavage-dependent manner.

Indeed, our results indicate that brevican promotes glioma cell adhesion and motility and that its effects require the cleavage of the full-length protein and the release of the brevican N terminus. More importantly, we determined the need of appropriate extracellular substrate(s) for brevican to promote cell motility, suggesting that this protein may interact with additional extracellular elements to act as a motogenic signal (see below). Taken together, our results strongly suggest that the N-terminal fragment of brevican is responsible for engaging the molecular interactions that enhance motility in brevican-expressing glioma cells.

Next, we focused on identifying changes in CAMs that could correlate to the promigratory role of brevican in glioma cells. All lecticans interact with members of the tenascin and fibulin families, which directly associate to integrins on the cell surface. In addition, the lectican versican binds directly to β1 integrin (58), whereas the lectican neurocan binds to NCAM/L1 molecules (31) and can interact indirectly with N-cadherin via an intermediary cell surface glycotransferase (30). Because these interactions of versican and neurocan are mediated by domains conserved among the lecticans, we hypothesized that expression of brevican could result in altered expression levels of some of these CAMs.

Our initial results indicated that brevican expression alone failed to affect the levels of several integrin subunits, N-cadherin and NCAM. However, when cells were plated on fibronectin, those expressing brevican exhibited increased levels of N-cadherin and the β3 integrin subunit as well as increased Tyr^{759} phosphorylation of β3 integrin. These CAMs have been previously described in primary brain tumors, and the key proinvasive role of integrins in gliomas has been well established (59, 60). Moreover, inhibition of β3-containing integrins has been shown to inhibit glioma growth and progression (61, 62). The proinvasive role of N-cadherin, on the other hand, has been the subject of controversy (recently reviewed in

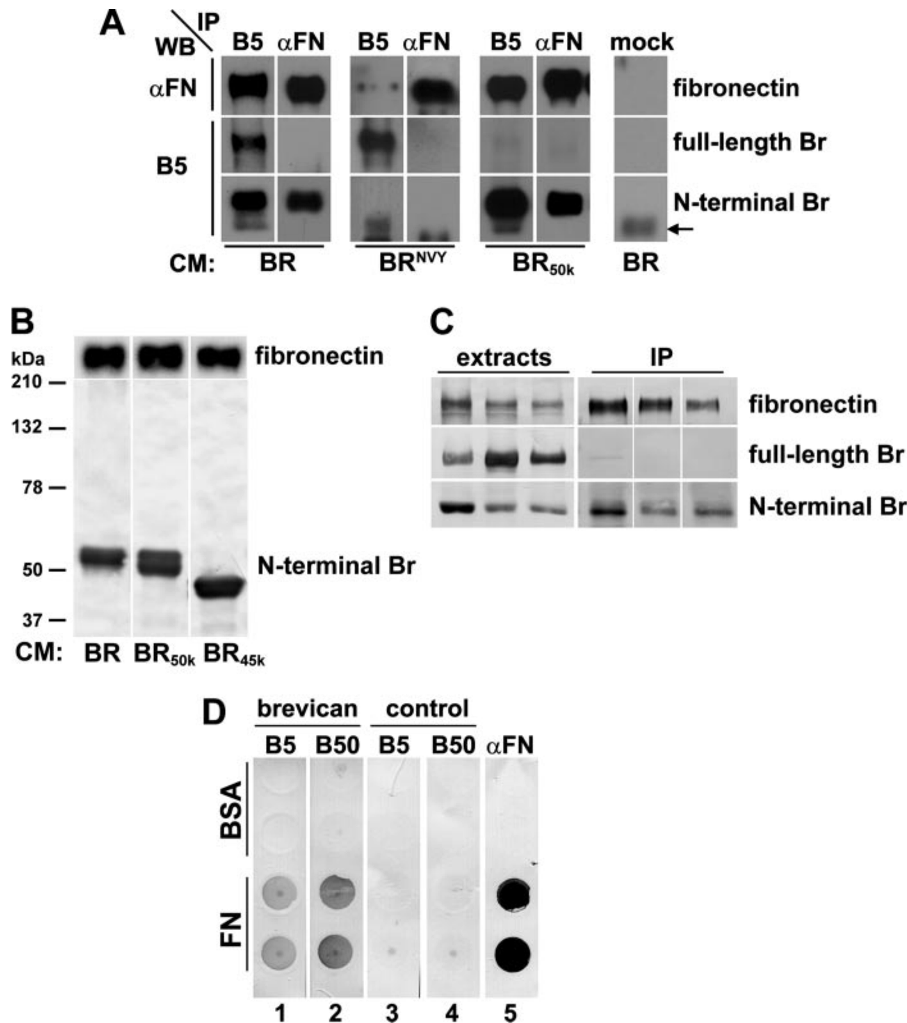

FIGURE 8. **Fibronectin binds to the N-terminal fragment of brevican.** *A*, conditioned medium (*CM*) from U87MG cells stably expressing full-length brevican (*BR*), BR_{50k}, or BR^{NVY} was subjected to immunoprecipitation (*IP*) using anti-brevican (*B5*), anti-fibronectin (*αFN*), and rabbit preimmune (*mock*) antibodies. Samples were subsequently processed for Western blotting (*WB*) with anti-brevican or anti-fibronectin antibodies. Results suggest that fibronectin associates with the N-terminal fragment of brevican but not with the full-length protein. The small amount of fibronectin detected in the *third lane* might have co-precipitated with minor cleavage products of BR^{NVY} that can be detected in overexposed films (not shown). *Arrow*, residual heavy chain of precipitating antibodies. *B*, conditioned medium from U87MG cells transiently transfected with full-length brevican, BR_{50k}, or a shorter N-terminal product (aa 1–360; BR_{45k}) was immunoprecipitated with an anti-fibronectin antibody. Fibronectin co-precipitated with BR_{50k} and BR_{45k} as well as with the cleavage product of brevican but not with the full-length protein. *C*, solubilized extracts from three specimens of glioblastoma multiforme were immunoprecipitated with anti-fibronectin antibody. The results show co-precipitation of the N-terminal fragment of brevican together with fibronectin. *D*, purified fibronectin (*FN*) and bovine serum albumin (*BSA*) were dot-spotted and probed with concentrated conditioned medium from brevican-expressing (*lanes 1* and *2*) or control (*lanes 3* and *4*) U87MG cells. The blots were subsequently developed using anti-brevican B5 and B50 antibodies. The latter detects a neoepitope exposed only in the N-terminal fragment of brevican. The results suggest that the N terminus of brevican binds directly to native fibronectin. An anti-fibronectin antibody was used as positive control (*lane 5*). *Br*, brevican.

Ref. 63), because this protein is increased in high grade gliomas, but *in vitro* assays have failed to show a direct correlation of N-cadherin to glioma invasion.

The observation that brevican can modulate $\beta3$ integrin and N-cadherin expression is the first described effect of this lectican on transmembrane CAMs in gliomas. These results, however, also highlighted that brevican was insufficient for this effect in the absence of additional ECM molecules, such as fibronectin. This prompted us to study the relationship between brevican and fibronectin as the possible underlying mechanism for the effects of brevican in glioma cells.

In agreement with this hypothesis, we first observed that brevican expression induced fibronectin synthesis and accumulation of microfibrils of this molecule on the cell surface. These microfibrils resembled a polymeric arrangement of fibronectin known as "superfibronectin" (64) that is found in the ECM of mesenchymal and epithelial cells. This polymerization, resulting from the homotypic binding of fibronectin units, has been described as a mechanism by which cell surface fibronectin may promote cell attachment to a fibronectin-rich substrate (65) as well as a mechanism of matrix reorganization and tumor cell motility (66, 67).

These results were in agreement with seminal work of Yang and coworkers (29, 68), who had demonstrated that fragments of the lectican versican could also increase fibronectin synthesis in glioma cells, bind directly to fibronectin, and promote cell motility. Additionally, these authors demonstrated that versican isoforms could increase EGFR levels in glioma cells and signal through EGFR activation in neural cells (35–37). This prompted us to investigate whether EGFR signaling could mediate the brevican-dependent increase of fibronectin in our model. Accordingly, we observed that exposure of glioma cells to brevican- or BR_{50k}-containing medium increased the phosphorylation of EGFR and its downstream effector Erk1/2 as well as the levels of fibronectin mRNA in these cells. Moreover, inhibition of EGFR activation in brevican-expressing cells reduced the levels of fibronectin mRNA and abolished the enhanced adhesion of these cells to fibronectin compared with controls. Although these observations do not exclude additional mechanisms that could transduce brevican signaling, they highlight the possible role of EGFR activation for lectican signaling in gliomas.

Our results also demonstrated that the up-regulation of fibronectin was a necessary component of the brevican-induced phenotype. Reduction of fibronectin levels by siRNA abolished the effects of brevican and made brevican-expressing cells undistinguishable from the controls. In contrast, disruption of the fibronectin-integrin association using an RGD peptide did not abolish the enhancing effect of brevican, although it

caused a general reduction in cell adhesion. This suggests that the enhancing effect of brevican could have involved homotypic binding of fibronectin between the cell surface and the substrate, but it did not seem to depend on engagement of integrin receptors. The increase of β3 integrin that we observed could have thus been an independent or complementary event rather than a key element in the signaling cascade initiated by brevican.

Interestingly, the loss of the enhancing effect of brevican for cell adhesion to HA could be simply explained by the knockdown of cell surface fibronectin, because fibronectin and HA have been demonstrated to interact directly (69, 70). This association could explain the motogenic effect of brevican on HA described in the legend to Fig. 2 and also reinforces the possible role of fibronectin as the central mediator of brevican effects in gliomas. It is tempting to speculate whether the introduction of fibronectin in the HA-based neural ECM could be one of the several mechanisms of matrix disruption by migrating glioma cells.

Overall, these results underscored the importance of fibronectin as a key mediator of brevican effect in glioma cells. Furthermore, our co-immunoprecipitation and dot-blot assays indicated a direct interaction of fibronectin with the N-terminal domain of brevican but not (or extremely poorly) with the full-length protein. In addition, association of fibronectin with a shorter version of the N-terminal domain suggested that ADAMTS-cleavage did not likely create a novel binding site for fibronectin but rather unmasked site(s) somewhere else in the N terminus of brevican. Not surprisingly, the shorter version of the N-terminal domain was also able to enhance glioma cell adhesion and motility.[5]

The good agreement of the effects observed here with brevican and those described previously with versican suggests that both lecticans, which are up-regulated in glioma cells, may contribute simultaneously to cell motility via EGFR signaling, interaction with fibronectin, and increase of cell adhesion. It is interesting, however, that some of these effects were observed with different recombinant fragments of versican (35, 36, 68, 71), whereas they seem to be concentrated in the N-terminal domain of brevican. It is not known whether N-terminal versican may bind to fibronectin in a cleavage-dependent manner as we have demonstrated here for brevican. On the other hand, we have observed that fibronectin might interact with a recombinant C-terminal domain of brevican but with much less affinity than with the N-terminal cleavage product.[4]

In sum, our results suggest that brevican effects in glioma cells may involve EGFR signaling, fibronectin-dependent adhesion, and increased expression of CAMs to promote cell motility. This combination of motogenic signals would be unlikely in the normal neural matrix, where fibronectin is absent (4, 34), but it would be possible in the microenvironment of glioma cells, which co-express large amounts of brevican and fibronectin *in vivo* (45). Thus, this interaction could be unique to gliomas and could be a significant factor underlying the distinct ability of these tumors to disperse in the central nervous system. Our results highlight the importance of the lectican-fibronectin interactions as a potential target against glioma spread, and furthermore, they suggest that inhibition of ADAMTS proteases could be an important strategy to disrupt these interactions and limit glioma cell motility.

[5] M. S. Viapiano, unpublished observation.

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