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Reduced Expression of the Hyaluronan and Proteoglycan Link Proteins in Malignant Gliomas*[S]

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Malignant gliomas have a distinctive ability to infiltrate the brain parenchyma and disrupt the neural extracellular matrix that inhibits motility of axons and normal neural cells. Chondroitin sulfate proteoglycans (CSPGs) are among the major inhibitory components in the neural matrix, but surprisingly, some are up-regulated in gliomas and act as pro-invasive signals. In the normal brain, CSPGs are thought to associate with hyaluronic acid and glycoproteins such as the tenascins and link proteins to form the matrix scaffold. Here, we examined for the first time the expression of link proteins in human brain and malignant gliomas. Our results indicate that HAPLN4 and HAPLN2 are the predominant members of this family in the adult human brain but are strongly reduced in the tumor parenchyma. To test if their absence was related to a pro-invasive gain of function of CSPGs, we expressed HAPLN4 in glioma cells in combination with the CSPG brevican. Surprisingly, HAPLN4 increased glioma cell adhesion and migration and even potentiated the motogenic effect of brevican. Further characterization revealed that HAPLN4 expressed in glioma cells was largely soluble and did not reproduce the strong, hyaluronan-independent association of the native protein to brain subcellular membranes. Taken together, our results suggest that the tumor parenchyma is rich in CSPGs that are not associated to HAPLNs and could instead interact with other extracellular matrix proteins produced by glioma cells. This dissociation may contribute to changes in the matrix scaffold caused by invasive glioma cells.

The extracellular matrix (ECM)[2] of the adult central nervous system lacks most fibrous proteins (collagens, fibronectin, and laminins) that are present in the matrices of other tissues and is formed instead by a scaffold of hyaluronic acid (HA) with associated glycoproteins (1). The major family of HA binding matrix glycoproteins in the central nervous system is formed by the chondroitin sulfate proteoglycans of the lectican family (aggrecan, versican, neurocan, and brevican), the last two expressed almost exclusively in neural tissue (2). These proteoglycans bind both to HA and to cell-surface receptors (3), regulating the cross-linking and compressibility of the matrix scaffold and, therefore, modulating many neural processes including cell motility during development, axonal navigation, and the stabilization of synapses (4). The lecticans have been identified as a major class of molecules that restrict cellular and axonal motility in neural tissue and are a major component of the glial scar that forms after neural injury and prevents axonal regeneration (5).

A second family of HA-binding proteins expressed in the central nervous system is formed by small glycoproteins known as HA- and proteoglycan-link proteins (HAPLNs) or, simply, "link proteins." These glycoproteins bind both to HA and to the lecticans, forming ternary complexes (6, 7). The structure of the link proteins is remarkably similar to the N-terminal region of the lecticans, and the highly homologous HA binding domains from HAPLNs and lecticans are indistinctly known as proteoglycan tandem repeats or link-protein modules.

In a striking example of molecular evolution, the genes of the four HAPLNs are located adjacent to the genes of the four lecticans, indicating a common molecular origin by gene duplication (8). Two of the link proteins, HAPLN2 and HAPLN4, have only been detected in neural tissue, and their genes are adjacent to the neural-specific proteoglycans, brevican and neurocan, respectively (8). Both HAPLN2 and HAPLN4, also known as brain-specific link protein (Bral-1) and Bral-2, are up-regulated in the adult central nervous system and match the temporal expression profile of brevican, which is the most abundant CSPG in adult neural tissue (9, 10).

Current evidence suggests that the HAPLNs may be key components in the organization of the HA-based matrix scaffold. HAPLN1, the best studied member of the family, increases the affinity of the lecticans for HA (11, 12) and stabilizes lectican-HA matrix aggregates (6, 13). Moreover, the increased expression of lecticans and HAPLNs in the adult central nervous system correlates temporally and spatially with changes in ECM solubility and with appearance of ECM aggregates around subsets of neurons, known as "perineuronal nets." These changes have been associated with restricted cellular motility and decreased synaptic plasticity (14).

The role of the lectican CSPGs as inhibitors of motility in the adult central nervous system contrasts starkly with their pro-invasive role in the highly aggressive brain tumors known as malignant gliomas. These are the most common primary

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4 and Table 1.

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[2] The abbreviations used are: ECM, extracellular matrix; CSPG, chondroitin sulfate proteoglycan; HA, hyaluronan; HAPLN, hyaluronan and proteoglycan link protein; RT, reverse transcription; ANOVA, analysis of variance; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

tumors of the brain and are characterized by their extensive and diffuse infiltration of the brain parenchyma (15), which makes them impossible to completely remove and facilitates tumor recurrence even after long term therapies. The invasive ability of gliomas is restricted to neural tissue and is not observed in other tumors that metastasize to the brain, suggesting that glioma invasion may be supported in part by unique mechanisms to remodel the neural microenvironment (16).

Two lectican CSPGs, versican and the neural-specific CSPG brevican, are highly up-regulated in gliomas compared with normal brain tissue (3). Although these proteoglycans are thought to inhibit the motility of normal glial cells (17, 18), they instead promote glioma cell adhesion and migration. The underlying molecular mechanisms for this unusual effect are poorly understood, although we and others have demonstrated that these lecticans can activate epidermal growth factor receptor signaling in glioma cells, which leads to an increase of cell-surface adhesion molecules (19). Both brevican and versican can also form adhesive complexes with mesenchymal matrix proteins that are present in the glioma ECM but absent from the normal neural ECM (19, 20).

Although the role of CSPGs in brain tumors is starting to become better defined, their HAPLN partners have never been analyzed in human brain or in neuropathologies. Therefore, we still have a highly incomplete picture of the molecular changes that occur in the tumor ECM and of how those changes could affect critical aspects of glioma biology such as invasion of the surrounding tissue.

We hypothesized that the gain of function of CSPGs in gliomas could be associated with changes in the levels or molecular associations of specific HAPLNs in the ECM of gliomas. Thus, we studied here the expression and biochemical properties of the HAPLN family in human normal brain and glioma tissue. Our results provide the first biochemical characterization of the brain-specific human HAPLN4 and, in addition, show that both neural-specific link proteins HAPLN2 and HAPLN4, which are abundant in adult brain, are virtually absent from the ECM of malignant gliomas.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—The human glioma cell lines U251MG and U87MG (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. Quantitative RT-PCR and Western blotting were used to identify the major CSPGs produced endogenously by these cells. Non-transformed human fetal astrocytes (Clonetics Lonza, Allendale, NJ) were cultured in supplemented astrocyte basal medium provided by the manufacturer. Primary glioma-derived neurospheres prepared from fresh clinical specimens as described (21) were characterized and kindly provided by Drs. Sean Lawler and E. Antonio Chiocca (Department of Neurological Surgery, The Ohio State University). These neurospheres were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 2 μM glutamine, 20 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ), 20 ng/ml basic fibroblast growth factor (Peprotech), and 1× B27 supplement (Invitrogen). Culture medium in all cases was supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin.

Human HAPLNs were detected with mouse monoclonal antibodies against HAPLN1 (clone 8A4, NICHD Developmental Studies Hybridoma Bank, Iowa City, IA), HAPLN2 (Abnova, Taiwan) and HAPLN4 (R&D Systems, Minneapolis, MN). Human CSPGs were detected with antibodies against versican (clone 12C5, NICHD Developmental Studies Hybridoma Bank (DSHB)), phosphacan (clone 3F8 against the core protein, NICHD DSHB), and brevican (rabbit polyclonal B6 and B5 that we have previously characterized (22)). Chondroitin sulfate epitopes were detected after treating CSPGs samples with chondroitinase ABC using the antibodies anti-chondroitin-4-sulfate (clone BE123, Millipore, Temecula CA) and anti-6-sulfate (clone 3B3, Seikagaku, Japan). Actin and the V5-epitope tag were detected with mouse monoclonal antibodies from Sigma-Aldrich and Invitrogen, respectively.

Human Tissue Processing—All studies involving human tissue specimens 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 specimens of high grade adult gliomas (patient age range, 37–74 years old) were obtained through the NCI Cooperative Human Tissue Network. Human brain cortex tissue (parietal and temporal) from age-matched controls and other developmental stages was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). Tissues were individually homogenized in 20 mM Tris-HCl, pH 7.4, containing 320 mM sucrose and a mixture of protease inhibitors (Complete, Roche Applied Science). Total homogenates were subjected to subcellular fractionation as previously described (22) and further processed for protein electrophoresis.

Constructs and Cell Transfection—Clones containing the complete coding sequence of human HAPLN4 (NM_023002), human brevican (NM_021948), and the HA synthase HAS2 (NM_005328) were subcloned by PCR into the vector pcDNA3.1-V5.6xHis (Invitrogen) to produce V5/His-tagged proteins. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. cDNA dosage was controlled by quantitative RT-PCR, and cells were selected for further experiments when their stable expression levels were comparable with those in neural tissue (for HAPLN4) or glioma tissue (for brevican) as previously described (23). To confirm that protein overexpression was compatible with normal endoplasmic reticulum (ER) function, we analyzed the expression of the ER stress sensor protein GRP78 and observed that this protein was not increased in the transfection conditions we used for our experiments.[3] Control transfections were performed with the original pCDNA3.1 vector or, alternatively, with pDsRed2 (BD Biosciences) carrying the cDNA for red fluorescent protein. Transiently transfected cells employed for biochemical experiments were routinely changed to serum-free Opti-MEM culture medium (Invitrogen) 24 h after transfection followed by medium collection

[3] H. Sim, B. Hu, and M. S. Viapiano, unpublished observations.

24–48 h later. Stably transfected cells were selected with Geneticin (G418, Invitrogen) as previously described (23).

Cell Adhesion and Motility Assays—48-well plates were precoated for 2 h at room temperature with the following substrates: human fibronectin (5 μg/ml, BD Biosciences), high M_r poly-L-lysine (50 μg/ml, Sigma-Aldrich), and high M_r hyaluronic acid (200 μg/ml, Calbiochem,). Nonspecific binding sites were subsequently blocked with 1% bovine serum albumin in Dulbecco's phosphate-buffered saline. Glioma cells were dissociated in Dulbecco's phosphate-buffered saline, 2 mM EDTA, washed in fresh culture medium, and plated on the precoated well (at 50,000 cells/well) for 30 min. Adhered cells were fixed and quantified by crystal violet staining as described (19).

To analyze cell migration, we used a radial-dispersion assay to mimic cell dispersion away from the tumor bulk (24), a predominant feature of malignant brain tumors. Glioma cells were resuspended at a density of 100,000 cells/ml and seeded on 1% agar plates for 48 h to form floating aggregates. Aggregates were manually applied to the center of individual precoated wells, and cells were followed by time-lapse microscopy to analyze detachment and migration as described (25). The dispersion index (total area occupied by cells at each time divided by the original area of the cell aggregate) was plotted against time and analyzed by two-way ANOVA for repeated measures. As independent confirmation of cell motility, we tested the cells in a "wound-healing" assay using culture inserts with preformed gaps of 500 μm (Ibidi GmbH, Germany). Cells were plated at 60,000 cells/insert and allowed to form monolayers around the gaps overnight. Inserts were subsequently removed, and the residual gap area was measured at 0 and 24 h to quantify cell dispersion. Results were analyzed by one-way ANOVA. All experiments in both motility models were repeated three times with three to six replicates per experimental condition.

Protein Solubilization from Brain Membranes—Microsomal fractions obtained from subcellular fractionation of human brain specimens were pooled and used as source of "total brain membranes" at a protein concentration of 1–2 mg of total protein/ml. Total membranes were resuspended in 25 mM Tris-HCl buffer, pH 7.4, in the presence or absence of 10 mM EDTA or 1% w/v Triton X-100 for 1 h at 4 °C. Alternatively, the membranes were resuspended in 100 mM sodium carbonate buffer, pH 11.0, for 30 min at 4 °C.

To test whether HAPLN4 solubilized with Triton X-100 effectively partitioned with the aqueous phase, membranes were instead solubilized with cold 2% w/v Triton X-114 (Sigma-Aldrich) as previously described (26). The resulting solution was warmed at 37 °C, causing the separation of aqueous and detergent-containing phases. All samples were subsequently diluted to reduce the Triton X-114 concentration before preparation for protein electrophoresis.

To investigate the effect of glycosamino-glycan removal on protein solubilization, membranes were resuspended in 50 mM Tris-HCl buffer, pH 7.0, containing 10 mM sodium acetate and protease inhibitors (CH buffer) and treated with 1 milliunits/μl protease-free chondroitinase ABC (EC 4.2.2.4 Seikagaku) or 0.5 milliunits/μl hyaluronidase from *Streptococcus dysgalactiae* (EC 4.2.2, Seikagaku) for 4 h at 37 °C. To investigate the possible involvement of a glycosylphosphatidylinositol anchor in HAPLN4 retention, membranes were resuspended in CH buffer and treated with 1 milliunit/μl protease-free chondroitinase ABC and 1 units/ml phosphatidylinositol-specific phospholipase C (EC 3.1.4.10, Sigma-Aldrich) for 8 h at 37 °C.

After any of these incubations the suspended membranes were centrifuged at 25,000 × *g* for 15 min. Released proteins were recovered in the supernatant, and the membranes were washed twice in 25 mM Tris-HCl and re-centrifuged as before. Samples that had not been pretreated with chondroitinase were then further treated with 1 milliunit/ml chondroitinase ABC for 4 h at 37 °C to improve the resolution of CSPGs in blots. Samples were finally processed for protein electrophoresis.

Western Blotting and RT-PCR—Subcellular fractions from brain tissue were prepared for electrophoresis using standard protocols. Cultured cells were first lysed in 25 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% w/v CHAPS, and a mixture of protease inhibitors (Complete). Conditioned, serum-free, culture medium was concentrated and, if required, treated with chondroitinase ABC as previously described (22). Cell and tissue samples containing 15–20 μg of total protein were electrophoresed on 4–10% gradient polyacrylamide gels and analyzed by Western blotting.

Samples processed for mRNA analysis were snap-frozen immediately after dissection or culture followed by extraction in Trizol (Invitrogen). Residual DNA was degraded using TurboDNA Free (Applied Biosystems, Foster City, CA), and total RNA was processed for RT-PCR PCR using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. When possible, primers were designed to span gene introns and, thus, detect contamination with genomic DNA. Primers are listed in supplemental Table 1.

Microarray Meta Analysis—mRNA expression data for the members of the lectican and HAPLN families were obtained from 295 grade II-IV glioma specimens and 30 controls stored in the NCI Repository for Molecular Brain Neoplasia Data (REMBRANDT, rembrandt.nci.nih.gov, accessed between January and December 2008). Expression values were collected for "unified gene" probe sets corresponding to the different splice forms of each gene (27). Values were plotted as the -fold level (*i.e.* log 2 ratio) of each tumor to control samples and analyzed by one-way ANOVA for each gene. To normalize the expression of each gene using neuronal markers, values were recalculated relative to the expression level of the genes *ENO2* (neuron-specific enolase) and *TUBB4* (neuron-specific β-tubulin III) for each sample.

RESULTS

HAPLN4 Is the Predominant Member of the Link Protein Family in Human Brain Cortex—Previous studies have confirmed the expression of the link proteins HAPLN1, -2, and -4 in rodent neural tissues (10, 28, 29), but no comparison of these link proteins has yet been made in human central nervous system. To determine which members of the HAPLN family were expressed in human brain cortex, we first compared their expression by quantitative RT-PCR in adult tissue (Fig. 1*A*). Our results indicated that the neural-specific HAPLN4 was the largely predominant member of this family in human brain cortex, in agreement with what has been observed in rat and mouse

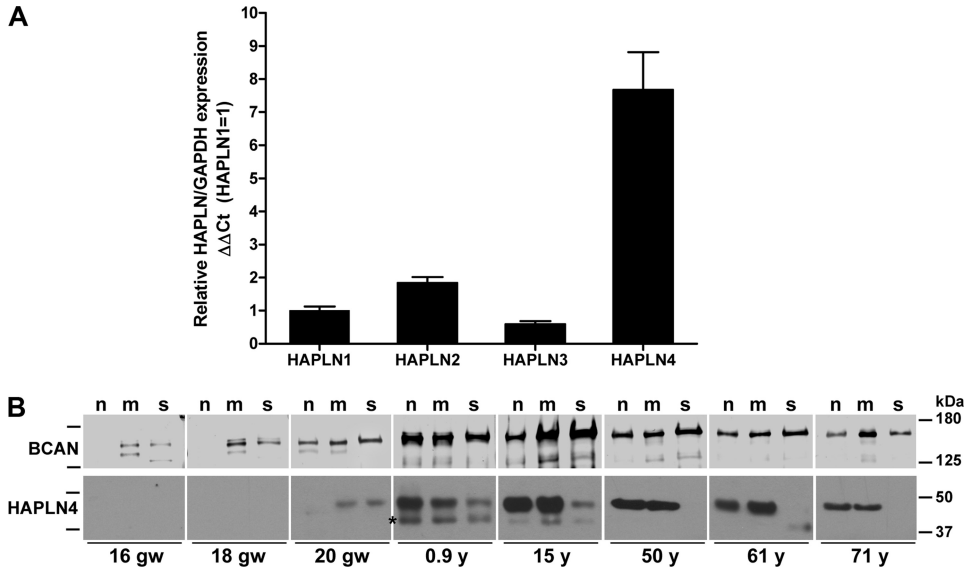

FIGURE 1. **HAPLN4 is the predominant member of the link protein family in adult human brain.** *A*, comparison of HAPLN mRNA expression by quantitative RT-PCR using total RNA from four independent samples of normal human brain cortex (range, 45–65 years old). Measurements were repeated two times in triplicate for each individual tissue sample. HAPLN4 was in all cases largely predominant over all other members of the family. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as normalization control. *B*, total homogenates from human brain cortex (16 gestational weeks (*gw*) to 71 years (*y*) old) were separated by subcellular fractionation into high density nuclear (*n*), membrane-containing (*m*), and soluble (*s*) fractions. Equal amounts of total protein from each fraction (15 μg) were treated with chondroitinase ABC and processed for Western blotting to detect HAPLN4 and the lectican brevican. The *asterisk* indicates a possible cleavage product of HAPLN4. Note the strong association of HAPLN4 to membrane-containing fractions in all postnatal stages.

brain. Processing of additional tissue samples revealed that the ranking HAPLN4 ≫ HAPLN2 > HAPLN1 ≫ HAPLN3 was consistent in different regions of the brain and spinal cord (not shown). This prompted us to focus mostly on HAPLN4 as the most abundant, but largely unexplored, member of this family in human central nervous system.

Western blotting analysis of the developmental profile of HALPN4 in human brain cortex showed a low expression of this protein in fetal tissue, which increased significantly during the first postnatal year (Fig. 1*B*). HAPLN4 was not found at developmental ages earlier than 19 weeks of gestation, and not all 19-week samples yielded positive results (not shown), suggesting that this could be the starting point for expression of the protein.

In rodents, the postnatal time-course of HAPLN4 follows closely that of brevican, the predominant lectican in adult neural tissue (2). In agreement, brevican and HAPLN4 followed very similar postnatal expression profiles in human brain cortex, including a peak during adolescence and late fall in adulthood that has been previously observed for brevican in human but not in rat brain (30). However, our results indicated that brevican protein appeared in human brain at earlier developmental stages than HAPLN4 (Fig. 2*B*), matching the early embryonic expression of this lectican in the rat (26, 31). Remarkably, although brevican started as a largely soluble protein and remained partially soluble throughout human brain development (22), HAPLN4 became aggregated and/or strongly membrane-associated within the first year of postnatal life, and there was virtually no soluble form of this protein in the adult brain (see further analysis below).

Expression of Brain-specific HAPLNs Is Strongly Reduced in High Grade Gliomas—Next, we compared the expression of the link proteins in normal adult brain *versus* high grade malignant gliomas, which are known for their high expression of two members of the lectican family, versican and brevican (3). For this, we first analyzed microarray data from glioma specimens stored in the NCI Repository for Molecular Brain Neoplasia Data (REMBRANDT), which is used to identify specific patterns of gene expression in primary brain tumors (27). This analysis disclosed a strong decrease in mRNA levels for the neural-specific link proteins HAPLN2 and HAPLN4 in high grade gliomas, a notorious contrast against the up-regulation of the lecticans versican and brevican in the same types of tumors (Fig. 2*A*). When the expression patterns were expressed relative to the levels of two neuron-specific markers in each sample (supplemental Fig. 1), both HAPLN2 and HAPLN4 remained unchanged in tumor *versus* non-tumor tissue, suggesting that their decrease matched the death of adult neurons in tissue invaded by glioma.

To validate our analysis of microarray data, we quantified the expression of the proteins HAPLN1, -2, and -4 in total homogenates of normal brain cortex and glioma tissue by Western blotting (Fig. 2, *B*–*C*). Results indicated that both HAPLN2 and HAPLN4 were virtually absent from the tumor parenchyma compared with normal brain. Remarkably, HAPLN1, which had not appeared significantly down-regulated at the mRNA level, was also strongly reduced at the protein level. We further analyzed and characterized the expression of CSPGs and HAPLNs in glioma cell lines and found that although expression of some CSPGs was maintained, link proteins were absent in glioma cells (supplemental Fig. 2). However, we were able to detect both HAPLN2 and HAPLN4 mRNA in short term primary cultures of normal human astrocytes (Fig. 2*D*). Moreover, we detected HAPLN4 by RT-PCR (not shown) and Western blotting (Fig. 2*E*) in several cultures of primary glioma-derived neurospheres, which have been characterized as a source of glioma initiating cells that can express both neuronal and glial markers (21). Even though the detected levels were much lower than in adult brain, the results suggested that HAPLNs might not be completely restricted to adult neurons and could be expressed in minor populations of glial/glioma cells.

Expression of HAPLN4 Increases the Motility of Glioma Cells—Because lecticans and HAPLNs co-localize and are thought to associate in the ECM scaffold of normal neural tissue, our results suggested a remarkable dissociation between these two kinds of molecules in the parenchyma of malignant gliomas.

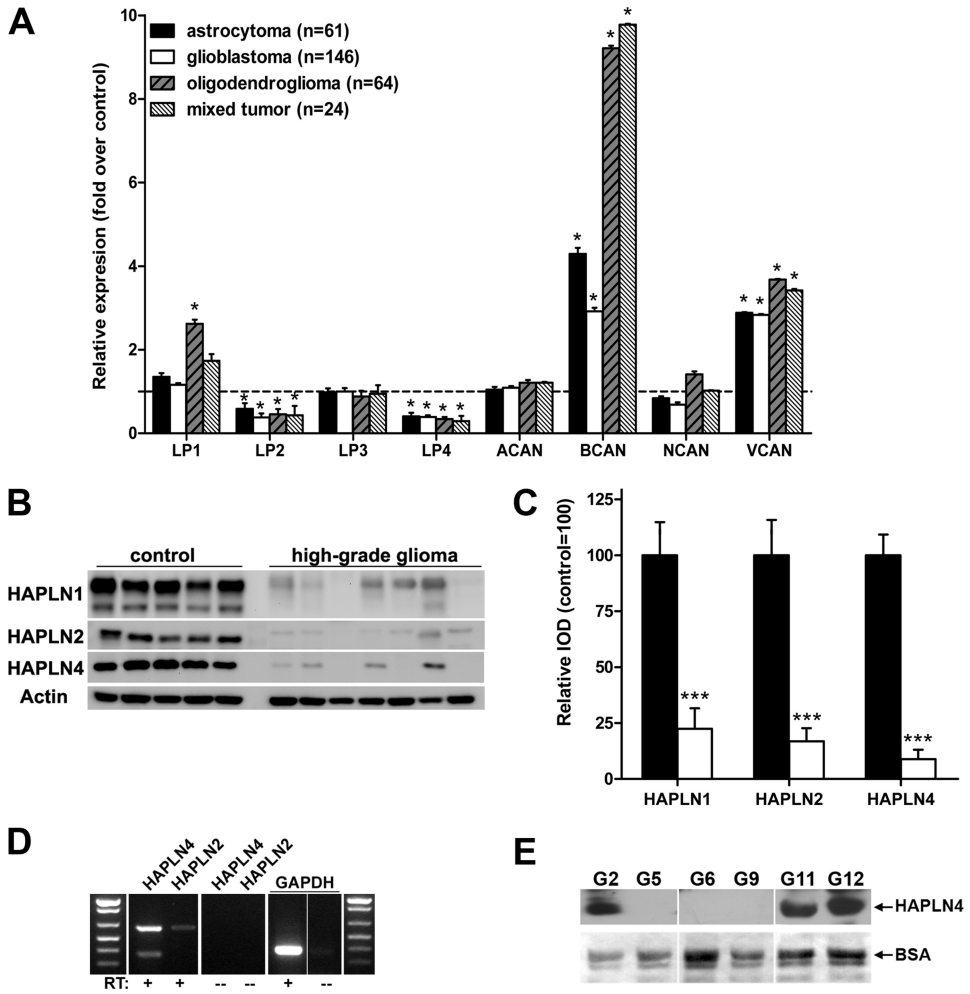

FIGURE 2. **Expression of the HAPLNs is highly reduced in malignant gliomas.** *A*, comparison of mRNA expression levels for the members of the link protein (*LP*) and lectican families in different types of high grade gliomas using microarray data from the NCI Repository for Molecular Brain Neoplasia Data. Each *column* represents the level of expression (-fold-over-control, mean ± S.E.) for each tumor type compared with normal brain tissue. Results for each gene were analyzed by one-way ANOVA (* indicates significant differences versus control, $p < 0.01$ or lower). The results show strong reduction of the expression of HAPLN2 and HAPLN4 that contrasts with the up-regulation of brevican and versican in gliomas. *BCAN*, brevican; *ACAN*, aggrecan; *NCAN*, neurocan; *VCAN*, versican. Data were renormalized using neuronal markers to test the correlation of HAPLN2 and HAPLN4 to the abundance of neurons in the tumor parenchyma (see supplemental Fig. 1). *B*, total homogenates from high grade gliomas (two grade III astrocytomas and seven grade IV glioblastomas, of which five are shown) and age-matched controls were probed for HAPLN1, HAPLN2, HAPLN4, and actin as loading control. *C*, quantification of normalized results from *B*; HAPLN2 and HAPLN4 are expressed at very low levels in the tumor parenchyma (*white bars*) compared with normal brain (*black bars*). *IOD*, integrated optical density. *D*, detection of HAPLN2 and HAPLN4 by RT-PCR using total RNA from low passage cultures of human fetal astrocytes. The same procedure yielded negative results with five different human glioma cell lines tested (U87MG, U118MG, U251MG, U373MG, and A172, not shown). *RT*, presence (+) or absence (−) of reverse transcriptase in the mixture. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *E*, detection of HAPLN4 by Western blot in the conditioned medium from primary glioma-derived neurospheres (identified as *G2* to *G12*). Cultures with detectable expression of HAPLN4 also expressed high levels of brevican (not shown). *BSA*, bovine serum albumin.

We hypothesized that the absence of HAPLNs could correlate to the pro-motility gain-of-function of CSPGs in gliomas. Thus, we next asked whether introduction of HAPLNs in glioma cells could affect cell behavior by itself or modulate the effects of the lecticans secreted by glioma cells.

Previous studies by our laboratory have shown that up-regulation of the lectican brevican in glioma cells increases cell adhesiveness and motility and could underlie in part the ability of these cells to invade the neural parenchyma (19). We then hypothesized that HAPLNs might disturb the pro-adhesive/pro-migratory effects of brevican and perhaps impair cell adhesion directly, as previously observed for HAPLN1 (32). To test this hypothesis, we generated stable transfectants in several glioma cell lines that are used as typical models to study the molecular mechanisms of glioma cell adhesion and migration.

Expression of HAPLN4 alone or in combination with brevican did not affect significantly cell morphology (not shown) or proliferation *in vitro* (Fig. 3*A*). However, when we tested the adhesive ability of glioma cells overexpressing HAPLN4, we found that they had enhanced attachment to substrates enriched in the glioma ECM, such as HA and fibronectin (19) (Fig. 3*B*). We have already reported a pro-adhesive effect of brevican on these substrates and shown that it is likely caused by brevican-dependent up-regulation and recruitment of fibronectin to the surface of glioma cells (19). However, we did not observe an increase of fibronectin on cells overexpressing HAPLN4 (not shown), suggesting a different pro-adhesive mechanism. In agreement, co-expression of HAPLN4 and brevican resulted in partially additive effects and increased glioma cell adhesion on all substrates tested (Fig. 3*B*).

Because increased adhesion may result in either increased or decreased motility, we next tested whether HAPLN4 expression in glioma cells could specifically affect cell motility. We used a radial-dispersion model in which a cell aggregate is plated on a precoated surface and acts as a "tumor bulk" from where cells detach and migrate radially (Fig. 4*A*) (24). Cell migration in this model is a result of cell-substrate *versus* cell-cell adhesive forces, mimicking the detachment and invasive properties of malignant brain tumor cells (33). Results from these assays indicated that expression of HAPLN4 resulted in a net increase in cell motility (measured as the increase in area occupied by migrating cells) on surfaces coated with HA but not on uncoated surfaces, in agreement with our adhesion tests (Fig. 4*B*). Again, co-expression of HAPLN4 and brevican caused a partial additive effect on cell motility, indicating that introduction of HAPLN4 not only did not disrupt but in fact enhanced the motogenic role of the lec-

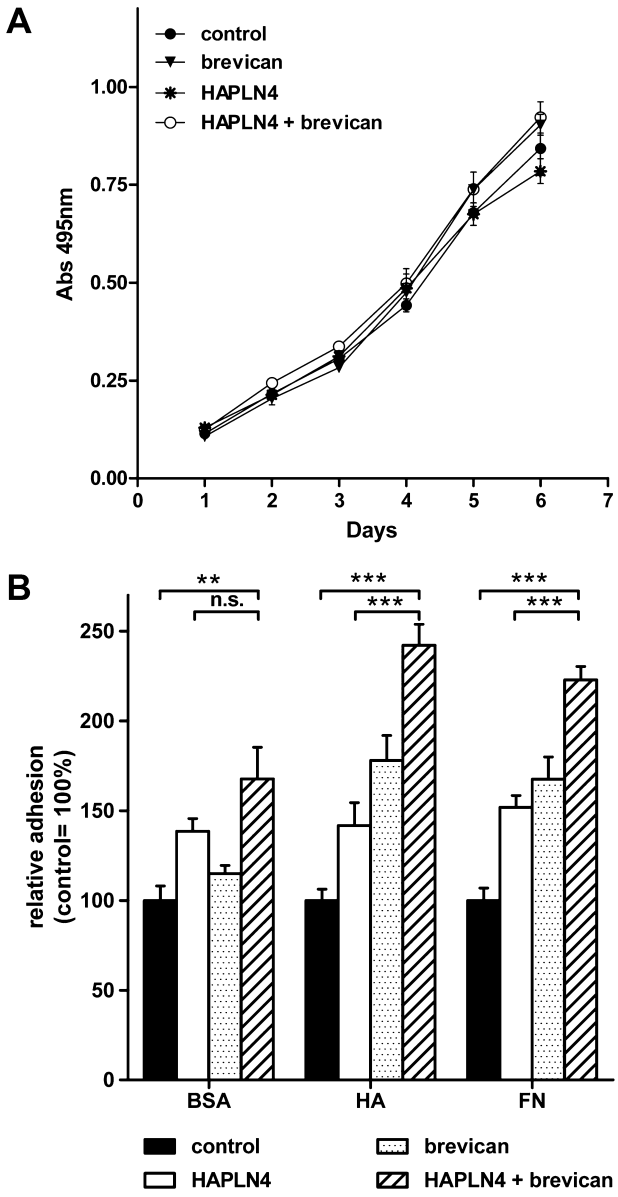

FIGURE 3. **HAPLN4 increases glioma cell adhesion in a substrate-dependent manner.** *A*, U251MG human glioma cells were transiently transfected for expression of HAPLN4 and/or brevican and cultured in multiwell plates for 6 days to analyze cell proliferation rates. No significant differences were observed in overall cell morphology (not shown) or cell proliferation. *B*, same cells as in *A* were plated on multiwell plates coated with fibronectin (*FN*) or high M_r HA or left uncoated and blocked with albumin (*BSA*) as described under "Experimental Procedures." Adherent cells were fixed and quantified by crystal violet staining. All experiments were repeated at least 3 times with 3–6 replicates per condition. Data (mean ± S.E.) were analyzed by two-way ANOVA and Bonferroni's post-hoc test (**, $p < 0.01$; ***, $p < 0.001$). Essentially the same results were observed with the human glioma cell line U87MG and rat glioma cell line CNS-1 (not shown). Results on poly-L-lysine-coated wells were undistinguishable from those on uncoated surfaces. *n.s.*, not significant.

tican. The pro-migratory effects of brevican and HAPLN4 (although not their additive effect) were confirmed using an independent wound-healing assay (supplemental Fig. 3).

HAPLN4 Retention in Brain Cell Membranes Cannot Be Replicated in Cultured Glioma Cells—Although the functional effects of HAPLN expression have never been previously studied in tumor cells, the motogenic effect observed with HAPLN4 was somewhat surprising because HAPLNs have been shown to reduce cell adhesion in other cell types (32) and have been proposed as restrictive to cell and axonal motility in the central nervous system (34). Thus, we investigated whether the expression of HAPLN4 in our glioma cell cultures actually reproduces the expression profile of the native protein, in particular its distinguishing, strong association with cell membranes that we had previously observed in neural tissue (Fig. 1).

Partition of secreted ECM proteins with "insoluble" microsomal-containing fractions is commonly observed in adult neural tissue and is thought to reflect the aggregation and membrane-association of matricellular complexes during neural development and maturation (26, 35). However, most ECM proteins can still be found in both soluble and particulate subcellular fractions. Indeed, we observed that HAPLN1 and HAPLN2 as well as the lecticans brevican and versican, distributed as both soluble and membrane-associated proteins in the same brain tissue specimens in which HAPLN4 was found only associated to membranes (Fig. 5*A*).

Strikingly, transient overexpression or stable expression of HAPLN4 in glioma cell lines resulted in this protein being largely secreted to the culture medium (Fig. 5*B*). The remaining HAPLN4 detected in cell lysates lacked attached carbohydrates[4] and could not be detected on the cell surface by live-cell staining but was detected intracellularly (Fig. 5*C*). This suggested that secreted HAPLN4 in cultured glioma cells was largely soluble rather than retained on the cell surface, in contrast to the previous description of the native protein (10).

To determine whether the lack of association of HAPLN4 to glioma cell membranes was due to the absence of its possible molecular partners, *i.e.* lecticans or HA, we studied the soluble *versus* cell-associated distribution of HAPLN4 in U251MG glioma cells after co-expression with the lectican brevican and/or the HA synthase enzyme HAS2 (Fig. 6*A*). We confirmed that HAS2 expression resulted in large pericellular coats of HA using a standard particle-exclusion assay with fixed red blood cells (36) (supplemental Fig. 4). However, our results indicated that neither expression of brevican, HAS2, nor a combination of both proteins altered the relative proportions of HAPLN4 in the culture medium and cell lysates.

Therefore, to further characterize the association of native HAPLN4 to brain cortex membranes, we assayed different extraction conditions from native tissue (Fig. 6*B*). HAPLN4 was resistant to mild solubilization conditions unless the membranes themselves were solubilized with detergents. Because HAPLN4 has a hydrophobic C-terminal sequence that could be a potential lipid-anchoring site (see "Discussion"), we also tested the possibility of an integral association of this protein with cell membranes. However, HAPLN4 remained in the aqueous phase after solubilization with Triton X-114 and was not affected by phospholipase C, suggesting that no lipid anchors were attached to it. Moreover, we were able to extract HAPLN4 with alkaline carbonate buffer, which finally suggested a strong but still peripheral association to neural cell membranes.

Most notoriously, HAPLN4 was highly resistant to treatment of cell membranes with the glycosaminoglycan-degrading

[4] H. Sim and M. S. Viapiano, unpublished observations.

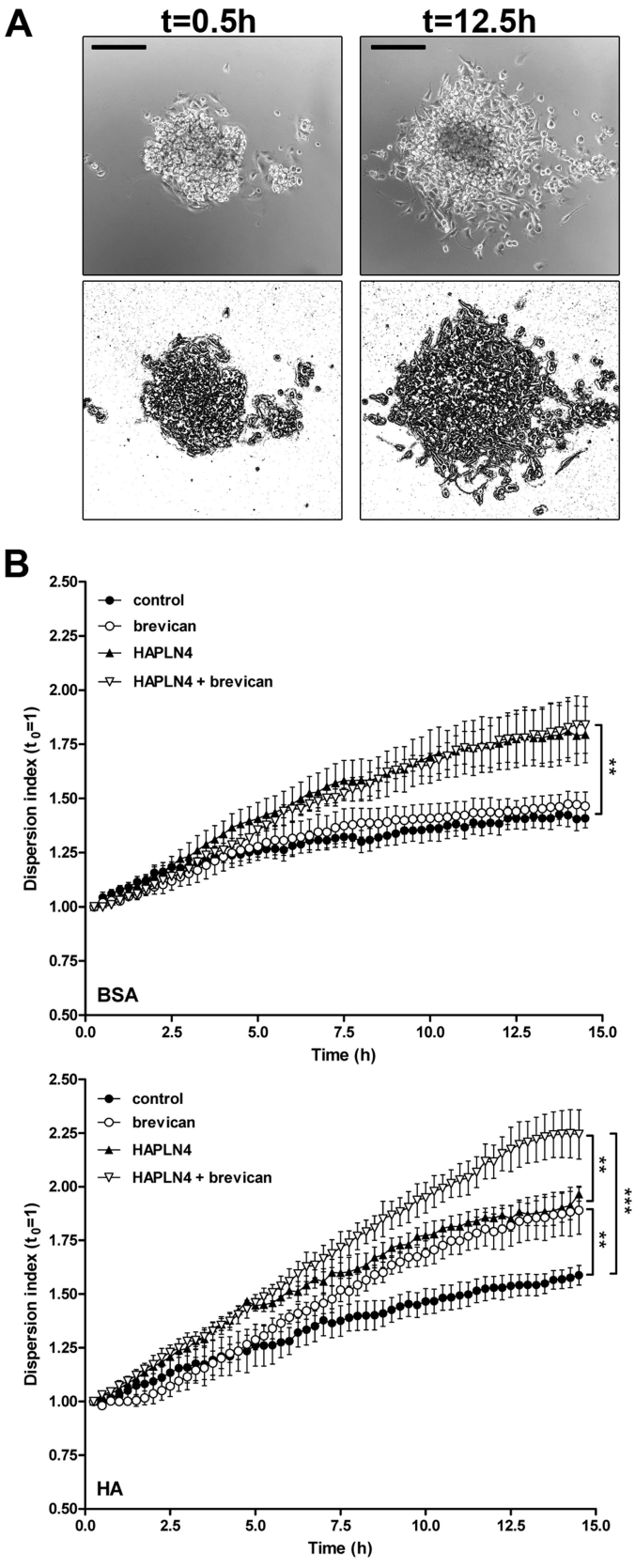

FIGURE 4. **HAPLN4 increases glioma cell motility.** *A*, representative images of U251MG cells detaching from a cell aggregate and migrating on a pre-coated culture well. Images were captured by time-lapse microscopy every 15 min for a total time of 14 h. Pictures in the *second row* show the result of applying contrast- and edge-enhancing algorithms to measure the total area of dispersion, as previously described (33). *B*, the average dispersion index (*i.e.* area occupied by cells at each time point divided by area occupied by the original aggregate) was plotted against elapsed time for glioma cells migrating on non-coated (*BSA*, *upper graph*) or HA-coated (*HA*, *lower graph*) surfaces. Brevican increased net cell motility, in agreement with our previous observations (19). HAPLN4 did not affect the motility of cells plated on non-coated surfaces but promoted cell motility independently of brevican when cells were plated on HA-coated surfaces. Total areas covered by migrating cells at the end of the experiment, a measure of net cell migration, were compared by two-way ANOVA for repeated measures (**, $p < 0.01$; ***, $p < 0.001$). *Bars* = 200 μm. Additional results in supplemental Fig. 2 confirmed the independent effects of brevican and HAPLN4 on cell motility using a traditional wound-healing assay.

enzymes chondroitinase ABC (Fig. 6*B*) and hyaluronidase (from *Streptococcus dysgalactiae*) (Fig. 6*C*), which readily solubilized other HA-binding proteins such as HAPLN1, brevican, and versican. Taken together, these results argued for a strong HA-independent and lectican-independent association of HAPLN4 to brain membranes that could not be reproduced in glioma cells.

DISCUSSION

Although the first link protein (HAPLN1) was identified shortly after the first aggregating proteoglycan (aggrecan) in the early 1970s, the properties and functions of these HA-binding glycoproteins have remained much less investigated than those of their lectican partners. The major function identified for these proteins, as stabilizers of HA-lectican complexes and, therefore, of ECM aggregates, is certainly critical for survival and normal development of the skeleton and cartilaginous tissue (37). However, the high expression of HAPLNs in the central nervous system, the existence of neural-specific HAPLNs that follow the expression pattern of neural-specific lecticans, and the observed association of HAPLNs with lecticans in perineuronal nets and nodes of Ranvier strongly suggest additional functions of these glycoproteins in neural development, synaptic plasticity, and the organization of the neural extracellular matrix (34, 38).

Here, we have focused on the expression and biochemical properties of human HAPLN4, the predominant member of this family in the adult mammalian brain. This protein has high homology to the other members of the family but exhibits two differences worth noting; it contains a 10-amino acid long glycine-rich insertion within the first HA binding domain, which could affect its HA binding activity, and has a 30-amino acid-long, unique C-terminal sequence. This C-terminal sequence is a hydrophobic, low complexity motif rich in glycine, tryptophan, and proline and has ~95% identity between human, bovine, rat, and mouse HAPLN4, suggesting a strong conservation of function. Two prediction algorithms (Koh-GPI by N. Fankhauser and P. Maeser at University of Bern, Switzerland; and Big-PI predictor at the Research Institute of Molecular Pathology GmbH, Vienna, Austria) assigned a high probability of cleavage in that sequence for the addition of a glycosylphosphatidylinositol anchor, although our solubilization results suggest that this is not the case in native HAPLN4.

Previous studies of HAPLN4 have described the expression of this protein in different regions of rodent central nervous system, including brain cortex (mouse), cerebellum (mouse and rat), and spinal cord (rat) (10, 34, 39). These descriptions have

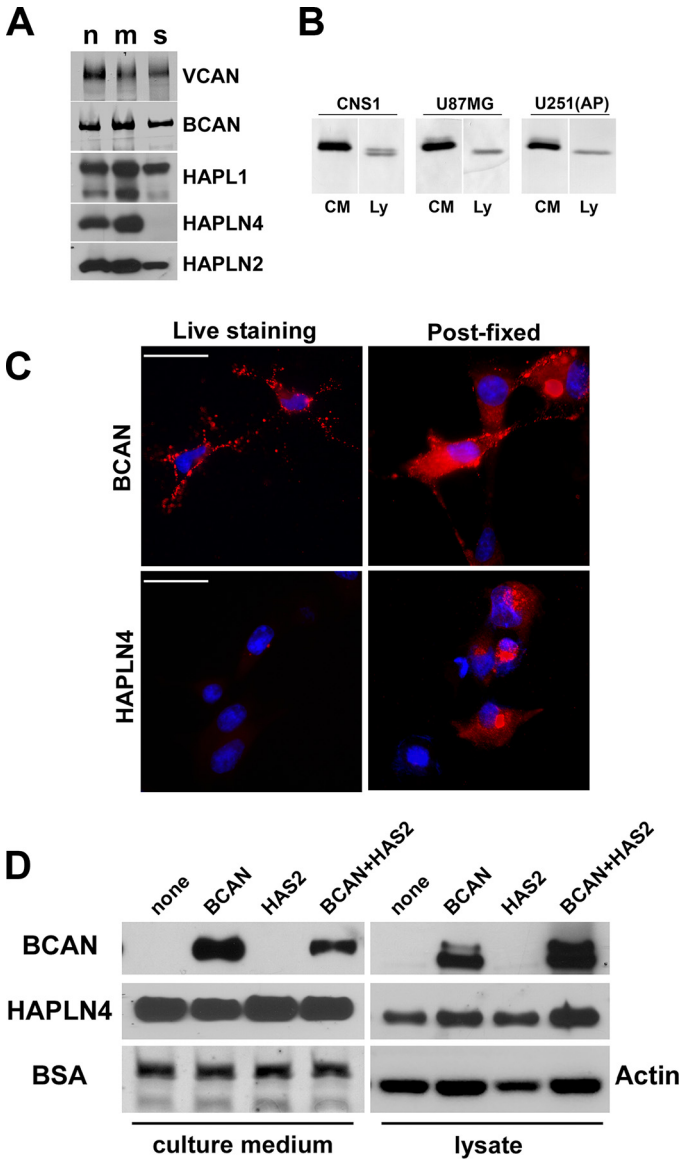

FIGURE 5. **HAPLN4 does not associate to the surface of cultured glioma cells.** *A*, subcellular fractionation of human adult brain cortex followed by probing of the nuclear-enriched (*n*), membrane-enriched (*m*), and soluble (*s*) fractions with antibodies against versican (*VCAN*), brevican (*BCAN*), HAPLN1, HAPLN2, and HAPLN4. Only HAPLN4 appeared tightly associated to membrane-containing fractions, whereas all the other ECM proteins distributed in the soluble and particulate fractions. *B*, transient transfection of HPLN4 in cultured human (U87MG and U251MG) and rat (CNS-1) glioma cell lines followed by preparation of conditioned medium (*CM*) and total cell lysates (*Ly*) for Western blotting; equal amounts of total protein (10 μg) were loaded for all samples. HAPLN4 appeared largely secreted to the medium, and little protein was found in the cell lysates. *C*, U251MG cells, transiently transfected with brevican or HAPLN4, were cultured on glass coverslips and processed for immunofluorescence as previously described (22). Primary antibodies were added to unfixed, unpermeabilized cells (*Live staining*) or to post-fixed and permeabilized cells (*Post-fixed*). Both proteins were detected in the cells after fixation, but only brevican was detected associated to the cell surface of non-permeabilized cells. *D*, U251MG cells expressing HAPLN4 alone (*none*) or co-expressed with brevican (*BCAN*), HAS2, or brevican+HAS2 (*BCAN+HAS2*), were collected and processed for Western blotting as in *B*. None of the co-expressed proteins significantly affected the distribution of secreted HAPLN4. Brevican accumulated in the cell lysates in presence of HA synthesized by HAS2; formation of HA pericellular coats after HAS2 transfection was verified by particle-exclusion assay (see supplemental Fig. 2). Equal protein loading in culture media and lysates was controlled by detection of total albumin in the medium and actin in the cell lysates. Western blots show more intense bands than in *B* because they were developed by quantitative chemiluminescence and exposed for maximum detection of each protein. *BSA*, bovine serum albumin.

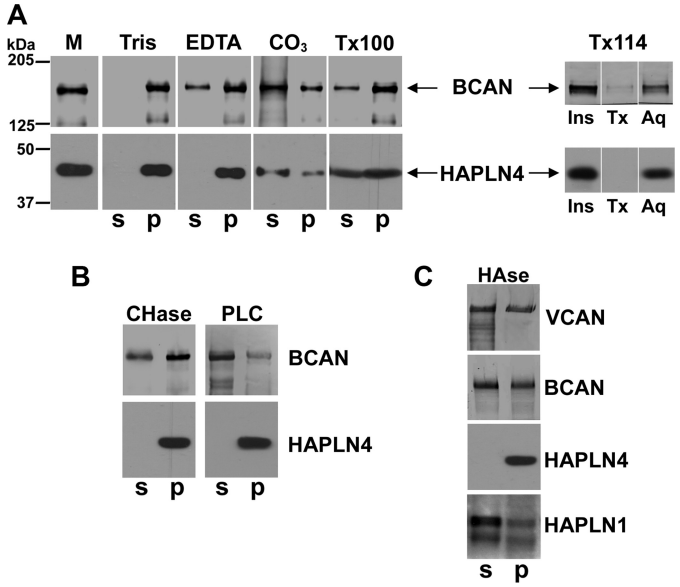

FIGURE 6. **HAPLN4 is strongly associated to cell membranes of neural tissue.** *A*, total membranes (*M*) obtained from subcellular fractionation of adult human brain cortex were resuspended in 25 mM Tris-HCl buffer, pH 7.4 (*Tris*), Tris buffer containing 10 mM EDTA (*EDTA*), Tris buffer containing 0.5% w/v Triton X-100 (*Tx100*), or 100 mM sodium carbonate buffer, pH 11.0 (CO_3). After incubation, membranes were centrifuged, and the resulting supernatant (*s*) and pellet (*p*) were processed for Western blotting. To verify whether released HAPLN4 was effectively water-soluble, membranes were extracted with 2% Triton X-114 as indicated under "Experimental Procedures" and subsequently separated by temperature-dependent partition in an insoluble pellet (*Ins*), aqueous phase (*Aq*), and Triton-containing phase (*Tx*). All the results suggested that HAPLN4 was strongly, but peripherally, associated to cell membranes. *B*, brain cortex membranes resuspended in Tris-HCl buffer containing 10 mM sodium acetate were treated with chondroitinase ABC (*CHase*) or chondroitinase plus phosphatidylinositol-specific phospholipase C (*PLC*). After incubation, membranes were processed as indicated in *A*. *C*, brain cortex membranes resuspended in Tris-HCl buffer were treated with hyaluronidase (*HAse*) for 4 h, separated into supernatant (*s*) and pellet (*p*) fractions as indicated, and processed for Western blotting for versican (*VCAN*), brevican (*BCAN*), HAPLN4, and HAPLN1. Only HAPLN4 was insensitive to hyaluronidase treatment.

indicated a late appearance of HAPLN4, from postnatal day 7 to postnatal day 20 depending on the central nervous system region and methodology used, followed by maintenance of the protein levels throughout adulthood (29). In contrast, we detected HAPLN4 as early as mid-gestation in the human brain cortex (Fig. 2). This pattern matched the prenatal expression of brevican, which is also expressed earlier in human than in rodent brain development (22), suggesting that the neural-specific components of the brain ECM appear at earlier developmental stages in humans. In addition, HAPLN4 levels decreased in the mature brain cortex, again following the temporal profile of brevican in human cortex, which differs slightly from the one observed in the mature central nervous system of the rat.

Both HAPLN4 and brevican switched progressively during development from a highly soluble subcellular localization to a membrane-associated localization, likely reflecting their gradual accumulation in aggregates and further insolubilization (26). However, although other lecticans and HAPLNs remained partially soluble in adult brain cortex, HAPLN4 became completely membrane-associated and could not be detected in the soluble fraction even after attempted enrichment by immunoprecipitation (not shown).

The initial characterization of HAPLN4 in normal brain led us to investigate the expression of this and other HAPLNs in malignant gliomas because these tumors are thought to remodel the neural ECM as glioma cells disperse away from the tumor mass (16). This remodeling is in part a result of overexpression of ECM proteins by the tumor cells, including matrix proteins specifically produced by neural cells as well as mesenchymal proteins not found in nervous tissue (16, 40). Some members of the lectican family that are abundant in normal brain (*e.g.* brevican) are even more highly expressed in malignant gliomas and directly promote glioma invasion in the brain (3). We, thus, investigated whether the HAPLNs would perhaps be expressed in these tumors following the expression pattern of their putative lectican partners.

However, our results indicated that none of the HAPLN members were up-regulated in malignant gliomas, and in fact, both HAPLN2 and HAPLN4 that predominate in normal brain were strongly reduced in the tumor parenchyma (Fig. 3). This decrease mirrored that of neuronal-specific markers, suggesting that it could have been largely caused by the neuronal death in tissue invaded by the tumor. Interestingly, pre-cancer inflammation in peripheral tissues causes increased methylation of the genomic region containing HAPLN4 (41), raising the possibility that this ECM protein could have also been down-regulated in neural cells by inflammatory events occurring during tumor infiltration. Finally, it is worth mentioning that, although adult neurons are thought to be the major source of the neural link proteins (29), we could detect HAPLN expression in cultures of fetal astrocytes and primary glioma-derived neurospheres, suggesting that these proteins are also expressed, albeit in low amounts, by subsets of cells from the glial lineage.

The decrease of the neural HAPLNs in the tumor parenchyma suggests that lecticans such as versican and brevican, which are highly expressed in gliomas, would be largely non-associated to link proteins and could perhaps interact with matrix or cell-surface ligands absent in normal brain and expressed in these tumors. For example, we have shown that brevican associates with fibronectin produced by glioma cells and that this mesenchymal ECM protein is necessary for the pro-migratory effect of brevican in glioma cells (19).

The absence of HAPLNs in gliomas prompted us to investigate the effect of reintroducing these proteins in glioma cells. Expression of HAPLN1 has been shown to disrupt cell adhesion (32), which led us to hypothesize that HAPLN4 could have a similar effect on glioma cells. However, introduction of HAPLN4 in cultured glioma cells did not perturb the pro-adhesive and pro-migratory effects of the lectican brevican and moreover increased net adhesion and migration of glioma cells in a manner partially additive to that of brevican.

Although we do not have a conclusive explanation for this general increase in adhesion/motility, we have observed that expression of HAPLN4 results in increased expression of β1-integrin in U251MG cells (data not shown). Interestingly, we had previously demonstrated that brevican increases the expression of β3- but not β1-integrin in glioma cells (19). It is possible that co-expression of HAPLN4 and brevican may have resulted in joint up-regulation of several classes of integrins, resulting in the observed increase of cell adhesion and motility. These results led us to investigate more closely whether HAPLN4 in glioma cells showed the same properties of native HAPLN4 in normal brain.

A distinctive feature of native HAPLN4 in brain tissue was its strong association with membrane-containing subcellular fractions, contrasting with the more widespread distribution of all other neural ECM proteins tested. On the other hand, HAPLN4 secreted by cultured glioma cells was almost exclusively soluble, even in cells stably expressing this protein and cultured undisturbed for several days.

Most ECM proteins expressed in cultured cells are secreted to the culture medium and appear largely soluble but can also be detected in membrane-containing fractions. For example, brevican retains a cell surface-associated isoform in cultures (22, 26), and this proteoglycan released to the medium can be induced to accumulate on the surface of glioma cells by the addition of exogenous fibronectin of HA (Fig. 5).[3] In contrast, HAPLN4 remained largely soluble even after co-expression with a lectican (brevican) and a HA source (the HA synthase HAS2), which are considered the necessary components for the formation of cell-associated aggregates in the neural ECM (29, 39). These results suggested that, at least in our culture conditions, HAPLN4 had little, if any, interaction with both brevican and cell-surface-associated HA. Attempts to co-immunoprecipitate HAPLN4 and brevican from the conditioned medium of glioma cells were unsuccessful.[4]

In comparison, native HAPLN4 in brain tissue was not only strongly associated to cell membranes but also difficult to solubilize and was only extracted after membrane solubilization with non-ionic detergents or harsh alkaline treatment with sodium carbonate. Mild treatments that detached other membrane-associated HAPLNs or lecticans did not work with HAPLN4. In particular, HAPLN4 could not be released by enzymatic treatment that degraded HA or chondroitin sulfate, which partially released all other HAPLNs and lecticans tested. This suggests that, although HAPLN4 could associate with HA in adult brain tissue (10), it also seems to be retained on neural cell membranes by a strong, HA-independent interaction. This result differs from the previous observation by Bekku *et al.* (10) that HAPLN4 immunoreactivity decreases in tissue sections after hyaluronidase treatment, probably because of solubilization of the protein. Our different observation could be largely a result of using different tissue sources and methods to evaluate the association of HAPLN4 to neural tissue. Moreover, we have observed that long term treatment of tissue samples with hyaluronidase reduces overall protein content because of residual protease activity in the enzyme preparation,[4] which prompted us to use shorter incubation times and an excess of protease inhibitors in our experiments.

In sum, our results demonstrate that HAPLN4 is the predominant member of the HAPLN family in human brain and discloses the significant reduction of the neural HAPLNs in the parenchyma of high grade malignant gliomas. In addition, we have shown that HAPLN4 exhibits a strong, HA-independent association to membranes of neural cells, which is not reproduced in glioma cells *in vitro* and may in part underlie the unusual motogenic effect of recombinant HAPLN4 in cultured cells. Overall, our results strongly suggest that the glioma ECM

is rich in lecticans that are not associated to HAPLNs, in stark contrast with the proposed association of these molecules in the normal neural ECM (34). This dissociation may contribute to the matrix remodeling caused by glioma cells as they invade the neural tissue.

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