Brain-enriched hyaluronan binding (BEHAB)/brevican cleavage in a Glioma Cell Line Is Mediated by a Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS) Family Member*

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Brain-enriched hyaluronan binding (BEHAB)/brevican is a brain-specific extracellular matrix protein containing a cleavage site between Glu395-Ser396, which bears remarkable homology to the "aggrecanase" site in the cartilage proteoglycan aggrecan. Expression of BEHAB/brevican is dramatically increased in human gliomas, notoriously invasive tumors. Recently, we showed that the rat 9L gliosarcoma cell line, which does not express BEHAB/brevican and forms non-invasive tumors when grown as intracranial grafts, can form invasive tumors when transfected with a 5' cDNA fragment of BEHAB/brevican, but not when transfected with the full-length cDNA. In marked contrast, the highly invasive CNS-1 glioma cell line expresses and cleaves BEHAB/brevican protein when grown as an intracranial graft. These results suggest that both synthesis and cleavage of BEHAB/brevican protein may play a role in the invasiveness of gliomas. We report here, using an antibody developed to the neoepitope created by BEHAB/brevican cleavage at the Glu395-Ser396 site, that the CNS-1 cells are able to cleave the protein in vitro. We characterized the CNS-1-derived cleavage activity by assaying its ability to cleave BEHAB/brevican proteoglycan, and determined that the enzyme is a constitutively expressed, secreted activity. Using a variety of protease inhibitors, reverse transcriptase-polymerase chain reaction, and specific antibodies, we determined that this activity is likely to be a member of the ADAMTS family of metalloproteinases, specifically ADAMTS4. These results suggest a novel function for ADAMTS family members in BEHAB/brevican cleavage and glioma and indicate that inhibition of ADAMTS in glioma may provide a novel therapeutic strategy.

BEHAB1 is a brain-specific, extracellular matrix protein (1), which was independently cloned in another laboratory and named brevican (2). BEHAB/brevican is the newest member of the lecitin family of chondroitin sulfate proteoglycans, a family that also includes aggrecan, versican, and neurocan. Like all lecitics, the BEHAB/brevican protein contains a hyaluronan-binding domain in its N terminus and an epidermal growth factor-like repeat, a C-type lectin-like domain, and a complement regulatory protein-like domain in its C terminus (see Fig. 1A). A glycosylphosphatidylinositol-linked isoform of this gene has also been described (3, 4). BEHAB/brevican exists, somewhat unusually, both as a proteoglycan and as a non-glycosylated core protein (5). The BEHAB/brevican protein contains a single known cleavage site between Glu395-Ser396, which bears striking homology to the "aggrecanase" cleavage site in aggrecan (6), the major cartilage proteoglycan. The 23 amino acids surrounding the cleavage site in BEHAB/brevican are 50% identical to the cleavage site in aggrecan. In the normal adult rat brain, full-length BEHAB/brevican protein (145 kDa) and both 90- and 50-kDa cleavage products are detected (2, 7).

The function of BEHAB/brevican in the brain is poorly understood, however, it is clear that its expression is developmentally regulated (4, 8, 9). Additionally, a growing body of evidence suggests that BEHAB/brevican expression is high at times and in places where glial cells are highly motile. The highest level of BEHAB/brevican expression during brain development is in the ventricular zone, coincident with the peak in gliogenesis in each brain region that has been examined (8). Additionally, BEHAB/brevican is up-regulated in glial cells immediately adjacent to the site of an experimentally induced brain injury, coincident with increases in markers of reactive gliosis (10). Finally, BEHAB/brevican expression is dramatically increased in primary tumors (gliomas) of the central nervous system (11), an unusually invasive tumor cell type.

The remarkably invasive growth pattern of gliomas makes these tumors extremely difficult to control and nearly impossible to remove surgically. In every sample of human glioma assayed to date, including oligodendroglioma, all grades of astrocytoma, and gliosarcoma, a high level of BEHAB/brevican mRNA is detected (11). In contrast, BEHAB/brevican mRNA is either undetectable or detected at very low levels in tumors of non-glial origin and is detected at relatively low levels in normal human brain (11). A glioma’s ability to express and modify an extracellular matrix molecule such as BEHAB/brevican with thrombospondin motifs; RT-PCR, reverse transcriptase-polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; TPEN, N,N,N',N'-tetakis(2-pyridylmethyl)ethylenediamine.

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‡The abbreviations used are: BEHAB, brain-enriched hyaluronan binding; MMP, matrix metalloproteinase; FBS, fetal bovine serum; TIMP, tissue inhibitors of metalloproteinase; APMA, p-aminophenylmercuric acetate; ADAMTS, a disintegrin and metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; APMA, p-aminophenylmercuric acetate; ADAMTS, a disintegrin and metalloproteinase; RT-PCR, reverse transcriptase-polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; TPEN, N,N,N',N'-tetakis(2-pyridylmethyl)ethylenediamine.
might contribute to an alteration in the extracellular environment that facilitates invasion (12).

Recently we showed that the 9L gliosarcoma cell line, which does not express BEHAB/brevican and forms non-invasive tumors when grown as intracranial grafts, can form invasive tumors when transfected with a 5’ fragment of a BEHAB/brevican cDNA (7). Interestingly, 9L cells transfected with the full-length BEHAB/brevican cDNA did not grow as invasive tumors. Analysis of the protein produced by the full-length transfected 9L cells revealed that the predicted full-length protein is produced, but is not cleaved. Conversely, intracranial grafts of the invasive CNS-1 glioma cell line express high levels of BEHAB/brevican mRNA and also cleave the full-length protein, producing 50- and 90-kDa cleavage products (7). The suggestion from this study is that both synthesis and cleavage of the BEHAB/brevican protein may play critical roles in the invasiveness of gliomas.

Here we report the characterization and identification of the activity that cleaves BEHAB/brevican. When grown as intracranial grafts, CNS-1 cells express BEHAB/brevican, cleave the full-length protein, and aggressively infiltrate the surrounding normal brain tissue (11). However, expression of BEHAB/brevican by these cells requires a brain-specific inducing factor, because CNS-1 cells do not express BEHAB/brevican either in culture or when grown as subcutaneous grafts. To determine whether the CNS-1 cells constitutively produce a BEHAB/brevican cleavage activity, we transfected these cells with the full-length cDNA and assayed their ability to cleave the resultant protein in vitro. To assay cleavage specifically at the predicted cleavage site, we developed an antibody to the neoepitope produced by BEHAB/brevican cleavage at Glu395-Ser396. Our results show that the enzyme responsible for BEHAB/brevican cleavage is a secreted activity, constitutively expressed by CNS-1 cells. The pattern of inhibition of this activity is consistent with it being a member of the ADAMTS family of metalloproteinases (13–15). Further characterization of this activity by RT-PCR, Northern blot analysis, and immunodepletion indicates that the CNS-1 cell-derived activity responsible for BEHAB/brevican cleavage at the Glu395-Ser396 cleavage site is ADAMTS4.

**EXPERIMENTAL PROCEDURES**

**Cell Transfections—** Rat CNS-1 glioma cells were transfected with full-length cDNA encoding the secreted form of rat BEHAB/brevican (generously provided by Dr. Yu Yamaguchi, Burnham Institute) by electroporation. The cDNA was cloned into the EcoRI site of the eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA). Transfectants were selected in RPMI with 10% FBS and 1 mg/ml G418. After 10–14 days, G418-resistant colonies were isolated and their media assayed by Western blot for the presence of BEHAB/brevican protein. The 9L gliosarcoma cell line was transfected by a similar method, as described previously (7). All cell lines were maintained in RPMI with 10% FBS and 500 μg/ml G418 was added to maintain expression in transfected cells.

**Antibodies—** The B50 antibody was designed to recognize the neoepitope formed by the cleavage of BEHAB/brevican using the strategy employed by Hughes and colleagues (16, 17) in making cleavage site-specific antibodies to link protein and to aggrecan. The peptide used for antibody production represents the last 7 amino acid residues in the new C terminus (QEAVERSE) produced by cleavage of BEHAB/brevican protein at the Glu395-Ser396 cleavage site (Fig. 1A). The peptide was designed with two glycine spacers and a cysteine residue added to the N terminus. Rabbits were immunized with the antigen (CGQGEAVERSE) coupled to the carrier protein ovalbumin by the N-terminal cysteine (Zymed Laboratories Inc., San Francisco, CA). Antiserum from immunized rabbits was affinity purified, and specific immunoreactivity was confirmed by blocking with the specific peptide. Antibody B5 recognizes a peptide within the N-terminal fragment of BEHAB/brevican (amino acids 60–73, HLRPPFSRSAAPG) and antibody B6 recognizes a peptide within the C-terminal fragment (amino acids 506–529, SPSPPRPRVHOGPAPETLIQPPREGS) (Fig. 1A), and have been previously described (7). A rabbit polyclonal antibody to the peptide VMAD-VDEEP, which recognizes amino acids 393 to 403 of ADAMTS4 was made as described previously (13).

**CNS-1-derived Cleavage Activity—** We first determined that CNS-1 cells transfected with full-length BEHAB/brevican can cleave the full-length protein; we next wanted to determine the cells’ role in regulation of this activity. Non-transfected, parental CNS-1 cells were plated and maintained in RPMI with 10% FBS until they reached an approximately 50% confluence. The media was removed and replaced with OPTI-MEM (Life Technologies) without FBS for 48 h. After 48 h the media was collected and concentrated in a stirred cell with a 30,000 kDa cut-off filter (Amicon). The cells were rinsed twice in Dulbecco’s phosphate-buffered saline, collected by scraping, pelleted by centrifugation, resuspended in 100 mM Tris-HCl (pH 7.2), and lysed by sonication. The resulting homogenate was centrifuged at 2,000 × g for 10 min to remove debris and unlysed cells. The supernatant was removed and centrifuged at 100,000 × g for 45 min. The resulting pellet represents a crude membrane preparation, and the supernatant represents the cell lysate. All fractions, media, pellet (membrane), and lysate, were tested for cleavage activity as described below.

**Preparation of BEHAB/brevican Substrate—** Rat BEHAB/brevican proteoglycan was isolated as follows. Adult Lewis rats were deeply anesthetized, sacrificed by decapitation, and their brains quickly removed and homogenized in 9 volumes of cold homogenization buffer (100 mM NaCl, pH 7.2). The homogenate was then centrifuged at 10,000 × g for 15 min to remove the bulk of the insoluble material. The supernatant was centrifuged at 150,000 × g for 45 min to remove any remaining insoluble material. The remaining supernatant was considered to be the soluble fraction and was the source of the substrate for subsequent assays. The BEHAB/brevican proteoglycan was further enriched in the soluble fraction by Q-Sepharose anion exchange chromatography. The soluble fraction was brought to 1% CHAPS, equivalent to our starting buffer (100 mM NaCl, 1% CHAPS, pH 7.2), and was loaded on a 5 ml of Fast Flow Q-Sepharose column (Amersham Pharmacia Biotech). The column was washed with 7 column volumes of the starting buffer. Protein was eluted from the column with a continuous NaCl gradient, from 50 mM NaCl (100% starting buffer) to 1 M NaCl (100% elution buffer) over a total volume of 100 ml. The column was subsequently washed with 5 volumes of the elution buffer. Protein elution was monitored by UV absorbance at 280 nm. BEHAB/brevican full-length proteoglycan eluted as a relatively broad peak starting when the NaCl concentration reached about 0.7 M and continuing until the concentration reached approximately 0.95 M. All samples containing BEHAB/brevican proteoglycan determined by Western blot, were concentrated and buffer-exchanged (100 mM Tris, pH 7.2) in a stirred cell with 100,000 molecular weight cut-off filter. Samples were then used in the cleavage assay as described below.

**Cleavage Assay—** All cleavage assays were carried out in a volume of 15 μl. In each reaction 3 μg of the adult rat soluble BEHAB/brevican proteoglycan was used as substrate and 10 μg of the CNS-1-derived proteoglycan was used as inhibitor. Cleavage was monitored by the influence on cleavage activity (data not shown). The most effective buffer was 100 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl2, and 2% glycerol (pH 7.4), which therefore became our standard activity buffer. Glycerol enhanced cleavage in every buffer tested, perhaps by stabilizing protein-protein interactions. The time course (between 2 and 48 h) and temperature dependence (between 4 and 55 °C) of substrate cleavage were examined but cleavage was typically assessed after 15 h at 37 °C unless otherwise noted. Cleavage of the substrate was stopped by boiling samples in a standard gel-loading sample buffer. These samples were then analyzed by gel electrophoresis and Western blotting.

**Inhibitor Studies—** For all inhibitor studies we used the standard activity buffer described above except we minimized the amount of Ca2+, decreasing its concentration to 3 mM. EDTA, EGTA, dipicolonic acid, 1,10-phenanthroline, TPEN, phosphoramidon, and 4-aminobenzyl-Gly-Pro-d-Leu-o-Ala-hydroxyamic acid were purchased from Sigma. Recombinant TIMP-1 and TIMP-2 were purchased from Chemicon. The hydroxyamic acid inhibitors XS303 (3S-[3R,8 Z]-2-[N-(2,6-dimethylphenyl)aminomethyl]-3-[2-hydroxyethylhydroxyamino]-2-[2-4-hydroxyamino]-1-methyl-2-oxazolidine-4-one hydrochloride) and SE206 (2S,2R,4S)-3-oxo-10-oxa-5-hexyl-2(3H)-methylcarboxamidone) (Tocris) were synthesized at DuPont as described previously (18, 19). All other inhibitors were purchased from Roche Molecular Biochemicals. The effect of all inhibitors was evaluated after incubation at 37 °C for 15 h. Samples were analyzed by Western blot.

**Stimulation of Latent MMP Activity—** To determine if the CNS-1-derived activity was latent, we preincubated CNS-1 cell homogenates with metalloproteinase inhibitors alone or in combination with 200 μg/ml of the MMP-1 activator lysyl oxidase (LOX). After preincubation for 2 h at 37 °C, the activity was assayed by Western blot. One key finding of this study was that both synthesis and cleavage activity are inhibited by the metalloproteinase inhibitors, but that the latent activity can be significantly increased by pretreatment with lysyl oxidase. The latent activity was also blocked by the specific ADAMTS inhibitor ADAMTS4, indicating that the CNS-1-derived activity is of the ADAMTS family. The activity of the CNS-1-derived activity was not inhibited by the gelatinases A and B, indicating that the latent activity is not of the gelatinase family. Finally, the CNS-1-derived activity was not inhibited by the human telopeptidase inhibitor, indicating that the latent activity is not of the human telopeptidase family.
BEHAB/Brevican Cleavage in Glioma-derived Cells

Cells—Total RNA from rat brain, CNS-1 cells, and 9L cells was isolated in the Trizol by standard methods. 1 μg of total RNA was then reverse transcribed using oligo(dT). PCR amplification was performed on the transcribed substrate using sequence-specific primers designed to recognize both rat and human ADAMTS42 (5′-primer sequence ACCGAC-CTCTTAAAGAGCTT and 3′-primer sequence GCATCACTGTTAGTAT-TCACCA). The resulting PCR-amplified product was cloned into TOPO pCR 2.1 TA-cloning vector by following manufacturers instructions (Invitrogen). Clones were subsequently sequenced by the HHHM/W.M. Keck Biotechnology Resource Lab at Yale University using the fluorocently labeled dideoxynucleotide chain termination method.

Northern Analysis of ADAMTS4—25 μg of total RNA from rat brain, CNS-1 cells, and 9L cells was denatured in 2.2 M formaldehyde, 50% formamide, 1 × MOPS buffer at 65 °C for 15 min. The RNA was electrophoresed on a 1.0% agarose-formaldehyde gel with 1 × MOPS at 25 V. The gel was briefly neutralized in transfer buffer and RNA blotted to Zetaprobe (Bio-Rad) by capillary transfer. After transfer filters were washed briefly in 2 × SSC and baked to immobilize RNA. The cloned ADAMTS4 fragment obtained by RT-PCR was used as the hybridization probe and labeled by random priming. A cyclophilin-specific probe was also used to adjust for relative loading and RNA quality. Hybridization conditions were identical to those previously described (1).

RESULTS

Protein Analysis in Transfected Cells—9L cells transfected with full-length BEHAB/brevican cDNA produced only one immunoreactive band at approximately 145 kDa on Western blots as previously reported (Fig. 1B, lane 4) (7). In contrast, CNS-1 cells transfected with the full-length cDNA produced 145- and 90-kDa immunoreactive bands when assayed with antibody B6, 145- and 50-kDa bands with antibody B5 (Fig. 1B, lane 2). An antibody that specifically recognizes the neoepitope revealed by cleavage at Glu395-Se5306 was generated and named antibody B50. Fig. 1B shows the specificity of the antibody B50. It does not recognize full-length BEHAB/brevican protein pro-
duced by either the 9L or CNS-1 cells and recognizes only the 50-kDa cleavage product produced by CNS-1 cells transfected with the full-length BEHAB/brevican cDNA. Because of its selectivity and specificity antibody B50 was used almost exclusively in subsequent experiments.

Preparation of BEHAB/Brevican Proteoglycan as Substrate—Since BEHAB/brevican is a relatively abundant proteoglycan in the adult rat brain (1, 5), a rat brain proteoglycan fraction was used as the substrate for our assays. BEHAB/brevican protein exists in both full-length and cleaved forms in the adult rat brain (5, 7). Fig. 2 shows a Western blot of a soluble rat brain preparation digested with chondroitinase and stained with antibodies B5 and B50, demonstrating that both the full-length and 50-kDa cleavage products are present in this preparation. Even in this complex mixture of proteoglycans antibody B50 recognized only a single, 50-kDa band. Using Q-Sepharose anion-exchange chromatography we isolated the BEHAB/brevican proteoglycan-containing fraction from this soluble rat brain preparation. This provided a preparation that was relatively enriched in full-length BEHAB/brevican proteoglycan (Fig. 2). Furthermore, the 50-kDa N-terminal cleavage product contains no glycosaminoglycan attachment sites and, accordingly, eluted before the proteoglycan fraction. Thus, the soluble proteoglycan fraction derived from the adult rat brain is highly enriched in full-length BEHAB/brevican and does not contain the 50-kDa cleavage fragment (Fig. 2). This substrate, therefore, permitted us to monitor cleavage activity by following the generation of the 50-kDa product.

Localization of Cleavage Activity—The ability of the transfected CNS-1 cells to cleave BEHAB/brevican indicated that these cells express a constitutively active proteolytic enzyme in culture. We next determined the cellular location of this activity. We compared the ability of conditioned media from CNS-1 cells, CNS-1 cell lysates, and CNS-1 cell membranes to cleave BEHAB/brevican. As shown in Fig. 3, the cell lysate does not contain activity, the membrane fraction contains only a low level of activity, and the conditioned media contains the highest level of activity. Accordingly, we used concentrated CNS-1-conditioned media as the source of activity in all subsequent studies.

Characterization of the CNS-1 Cell Media-derived Activity—A variety of different buffers at several concentrations were assayed to determine the most effective conditions for cleavage (data not shown). The concentrations of Ca\(^{2+}\), Zn\(^{2+}\), and NaCl all proved to be important in modulating activity. Cleavage increased with increasing Ca\(^{2+}\) concentrations from 1 to 20 mM (Fig. 4A). There was very little, if any, cleavage without the addition of Ca\(^{2+}\). The Zn\(^{2+}\) concentration was also significant. High levels of Zn\(^{2+}\) (above 500 \(\mu\)M) were inhibitory even in the presence of Ca\(^{2+}\) (Fig. 4B). Cleavage proceeded without added Zn\(^{2+}\), however, Zn\(^{2+}\) was present in low concentrations in our activity preparation. The lack of stimulatory effect of exogenously added Zn\(^{2+}\) does not suggest that Zn\(^{2+}\) is not necessary for enzyme activity. The concentrations of other divalent cations such as Mg\(^{2+}\) had little effect on cleavage (data not shown).

Effects of Protease Inhibitors—Initial studies were designed to determine which class of protease is responsible for cleavage of BEHAB/brevican protein. Cleavage assays were carried out in the presence of class-specific protease inhibitors: 20 mM EDTA (metalloproteinase inhibitor), 150 \(\mu\)M bestatin (aminopeptidase inhibitor), 50 \(\mu\)M E64 (cysteine proteinase inhibitor), 1 \(\mu\)M pepstatin (aspartic proteinase inhibitor), 4 mM Pefabloc SC (serine proteinase inhibitor), 0.5 \(\mu\)M aprotinin (serine proteinase inhibitor), or 10 \(\mu\)g/ml leupeptin (cysteine proteinase inhibitor). As shown in Fig. 6A, only EDTA inhibited cleavage of the substrate required almost 48 h (Fig. 5C).

To further investigate and characterize the metalloproteinase responsible for cleavage of the BEHAB/brevican protein we evaluated the ability of a variety of different specific metal chelators to inhibit cleavage. We tested EDTA, a chelator of divalent cations, EGTA, a Ca\(^{2+}\) chelator, and 1,10-phenanthroline, TPEN, and dipicolonic acid, all Zn\(^{2+}\) chelators, for inhibition of BEHAB/brevican cleavage. The inhibition profile of all chelators was similar. They all inhibited cleavage completely at concentrations of 2 mM (Fig. 6B). All metal chelators showed roughly similar inhibition profiles; producing nearly complete inhibition at concentration of 1 mM or higher (data not shown). As would be expected due to the presence of 3 mM Ca\(^{2+}\) in our
activity buffer, EGTA was the least effective, becoming ineffective as an inhibitor below concentrations of approximately 2 mM (data not shown). These results suggest that both the Zn$^{2+}$
and Ca$^{2+}$ concentrations are important for activity of this proteolytic enzyme, consistent with it being a metalloproteinase.

Having determined that the BEHAB/brevican cleavage activity is likely to be a metalloproteinase requiring both Zn$^{2+}$ and Ca$^{2+}$, we used more specific inhibitors of metalloproteinases to define a family to which this protease might belong.

Phosphoramidon (1 mM) and 4-aminobenzyl-Gly-Pro-D-Leu-D-Ala-hydroxamic acid (10 mM), inhibitors of a subset of metalloproteinases, showed no inhibition of BEHAB/brevican substrate cleavage in our assay (Fig. 6C).

TIMP-1 and TIMP-2, physiological inhibitors of MMPs, were also tested in our assay. At concentrations of 250 nM TIMP-2 showed no inhibition and TIMP-1 only slight inhibition of cleavage (Fig. 6C).

Furthermore, APMA, an activator of latent MMPs, not only did not stimulate activity but actually inhibited activity at its highest concentrations (2 and 1 mM) (Fig. 6D). APMA at all concentrations activated latent MMPs in our media as assayed by gelatin zymography (data not shown).

To further understand which family of metalloproteinase may be responsible for BEHAB/brevican cleavage, we tested two hydroxamic acid-based inhibitors with different inhibition profiles. XS309 is a nanomolar inhibitor of a broad range of MMPs, but is much less potent against ADAMTS family metalloproteinases (14). In contrast, SE206 is a potent inhibitor of MMPs, but in addition is also a nanomolar inhibitor of the ADAMTS family metalloproteinases (15). As shown in Fig. 7, XS309 did not inhibit the CNS-1 cell-derived BEHAB/brevican cleavage activity at concentrations as high as 10 $\mu$m. However, SE206 was a potent inhibitor of this cleavage activity, showing strong inhibition at concentrations as low as 100 nM. These experiments again suggest that MMPs do not mediate BEHAB/brevican cleavage at the Glu$^{395}$-Ser$^{396}$ site in CNS-1 cells. Furthermore, these data strongly suggest that ADAMTS family members mediate cleavage.

Cleavage of Recombinant BEAHB/Brevican with Recombinant Human ADAMTS4—Earlier work on aggrecan cleavage at the aggrecanase site suggested that cleavage is mediated, in part, by the ADAMTS family member, ADAMTS4 (13). Since the Glu$^{395}$-Ser$^{396}$ cleavage site in BEHAB/brevican is remarkably similar to the aggrecanase cleavage site, preliminary experiments were performed to determine if ADAMTS4 was capable of cleaving at this site. We found that recombinant human ADAMTS4 cleaved the rat recombinant BEHAB/brevican, resulting in the generation of the characteristic 90-kDa B6-reactive fragment after incubation for 16 h (Fig. 8). The specificity of cleavage at the Glu$^{395}$-Ser$^{396}$ site is demonstrated by staining with the B50 antibody (Fig. 8). Furthermore, staining with B6 revealed only one cleaved product suggesting that cleavage of BEHAB/brevican by ADAMTS4 occurs at only the Glu$^{395}$-Ser$^{396}$ site.

Expression of ADAMTS4 in CNS-1 Cells—As a first step in exploring whether ADAMTS4-mediated BEHAB/brevican cleavage in CNS-1 cells, we first determined if it was expressed.
in CNS-1 cells. To investigate this, we used RT-PCR with ADAMTS4-specific primers to amplify fragments of CNS-1 cell cDNA. Using this strategy we amplified a band of 449 base pairs, identical in size to the product observed by PCR amplification of a human ADAMTS4 clone and rat brain using the same primers (Fig. 9A). To confirm that this PCR product was ADAMTS4, the product was cloned and sequenced. The determined sequence was more than 90% identical to human ADAMTS4 and identical to the partial rat sequence, indicating that it was the rat homologue of this protease (data not shown). The high level of identity between human ADAMTS4 and the sequenced PCR product confirms that CNS-1 cells express ADAMTS4. Furthermore, 9L cells, which are incapable of cleaving BEHAB at the Glu395-Ser396 cleavage site, did not display an amplified band and do not express ADAMTS4 (Fig. 9A, lane 4).

In order to investigate the level of ADAMTS4 expression in CNS-1 cells, a Northern blot was performed using a rat-specific ADAMTS4 probe. The ADAMTS4 probe specifically hybridized to a single band of RNA in CNS-1 cells, corresponding to approximately 4.4 kilobases (Fig. 9B). This is consistent with human ADAMTS4 RNA, which is also approximately that size (15). The ADAMTS4 probe also hybridized to a similarly sized band (4.4 kilobases) on rat brain RNA, however, with much lower affinity. No probe hybridized to 9L cell RNA, consistent with the RT-PCR results (Fig. 9B). The high level of expression in CNS-1 cells relative to rat brain further implicates ADAMTS4 in the cleavage of BEAHAB/brevican at the Glu395-Ser396 cleavage site and suggest a possible role in gliomas.

Immunoprecipitation of ADAMTS4 in CNS-1 Cells—Having determined that both ADAMTS4 is capable of cleaving BEHAB/brevican and is expressed in CNS-1 cells, we next attempted to determine if this protease was responsible for cleavage of BEHAB/brevican by CNS-1 cells. To investigate this, the CNS-1 cell-derived activity was immunoabsorbed with an ADAMTS4-specific rabbit affinity purified polyclonal antibody coupled to protein-G Sepharose. Immunodepletion led to nearly complete loss of the BEHAB/brevican cleavage activity, while immunodepletion with ADAMTS1-specific antibody did not (Fig. 10). These results strongly indicated that ADAMTS4 is responsible for cleavage of BEHAB/brevican at the Glu395-Ser396 cleavage site by CNS-1 cells in vitro.

**DISCUSSION**

BEHAB/brevican is markedly up-regulated in primary tumors (gliomas) of the central nervous system (10). BEHAB/brevican protein is cleaved at a single known site, which our previous experiments have suggested to be an important factor for the function of this protein (7). The protease responsible for this cleavage has not yet been identified. Therefore, the four goals of the present study were: first, to establish a source of the BEHAB/brevican cleavage activity; second, to develop a method that would allow for the evaluation of cleavage specifically at the Glu395-Ser396 site; third, to obtain a biochemical characterization of this activity; and fourth, to identify the protease responsible for this cleavage.

Intracranial tumors derived from grafts of the CNS-1 cell line in rats are similar to naturally occurring human gliomas in that they are invasive, express high levels of BEHAB/brevican mRNA, and cleave the full-length protein at the Glu395-Ser396 site (7, 11). CNS-1 cells, however, do not express BEHAB/brevican in culture (11). By transfecting these cells with a
cDNA encoding full-length BEHAB/brevican protein, we were able to determine that the CNS-1 cells express a constitutively active cleavage activity in vitro. Furthermore, the majority of the cleavage activity is present in the media from CNS-1 cells. While most of the activity is detected as a soluble, diffusible enzyme, a CNS-1 cell membrane preparation also shows a low level of cleavage activity. The conditioned media from the CNS-1 cells contains substantially more cleavage activity and so it is used as the source of cleavage activity in our assays.

Using established methods we developed antibodies to the new C terminus revealed by cleavage of BEHAB/brevican at the Glu395-Ser396 site (16, 17). Antibodies made to a neo-epitope of aggrecan revealed by cleavage are highly specific for their respective sites (16, 17, 20). The B50 neoepitope antibody also shows a remarkably high level of selectivity for the new C terminus. The B50 antibody does not recognize full-length BEHAB/brevican protein, and only recognizes the 50-kDa cleavage fragment. Furthermore, this antibody has remarkable specificity, recognizing only the single 50-kDa band in a mixture as complex as whole rat brain soluble homogenates (Fig. 2). The specificity of the B50 antibody permits us to monitor cleavage at this Glu395-Ser396 site, and therefore allows us to establish an effective assay for characterization of the BEHAB/brevican cleavage activity.

Using a BEHAB/brevican-enriched soluble proteoglycan fraction derived from the adult rat brain, we developed a sensitive cleavage assay by monitoring the generation of the 50-kDa cleavage product with the B50 antibody by Western blot. As can be seen with the B6 antibody full-length BEHAB/brevican was nearly completely cleaved after 16 h and only one cleavage product was noted, the expected 90-kDa band. Staining with the B50 antibody reveals that cleavage is at the expected Glu395-Ser396 site.

**Fig. 8. Digestion of BEHAB/brevican by ADAMTS4** Rat recombinant BEHAB/brevican (40 μg) was incubated with human recombinant ADAMTS4 (8.3 μg/ml) for 0 or 16 h (lanes 1 and 2, respectively) at 37 °C in a buffer containing 50 mM Tris-HCl, 10 mM CaCl2 and 100 mM NaCl (pH 7.5). The assay was quenched with 20 mM EDTA, and the products of digestion which represented 20 μg of starting material were then analyzed by Western blot with the B6 or B50 antibodies. As can be seen with the B6 antibody full-length BEHAB/brevican was nearly completely cleaved after 16 h and only one cleavage product was noted, the expected 90-kDa band. Staining with the B50 antibody reveals that cleavage is at the expected Glu395-Ser396 site.

**Fig. 9. Expression of ADAMTS4 in CNS-1 cells.** A, PCR using primers designed to both human and rat ADAMTS4 was performed on a human ADAMTS4 clone as a control (lane 1). RT-PCR was then performed on mRNA from rat brain (lane 2), CNS-1 cells (lane 3), and 9L cells (lane 4). PCR products were then run on a 1% agarose gel. PCR products from the human ADAMTS4 clone, rat brain, and CNS-1 cells were all identical in size, 449 base pairs (bp). No PCR-amplified band was found in 9L cells. The 500-base pair fragment of DNA ladder is denoted as an indication of relative size. Bands were excised gel and products cloned and sequenced. Sequencing revealed that these products (for rat brain and CNS-1 cells) are the rat homologue of ADAMTS4. B, Northern hybridization using a rat ADAMTS4-specific probe demonstrated low expression in rat brain, no expression in 9L cells, but relatively high expression in CNS-1 cells. The lower blot was hybridization with a cyclophilin-specific probe and shows that approximately equal amounts of RNA were loaded from each sample. kb, kilobases.

Inhibitor studies also indicate that the activity derived from the CNS-1 conditioned media is a metalloproteinase. The divalent cation chelator and metalloproteinase inhibitor EDTA inhibits BEHAB/brevican cleavage, whereas inhibitors of cysteine, serine, aspartyl, or amino class proteases do not. The BEHAB/brevican cleavage activity is not inhibited by phosphoramidon or 4-aminobenzyl-Gly-Pro-o-Leu-d-Ala-hydroxamic acid, both inhibitors of a subset of metalloproteinases. TIMP-1 slightly inhibits the cleavage activity, suggesting that this activity may represent a MMP. However, the TIMP-1 concentration required for only partial inhibition of the BEHAB/brevican cleavage activity is 50 times higher than that divalent cation, Mg2+, has little effect on the activity. The dependence of this activity on Zn2+ and Ca2+ suggest that it is a metalloproteinase.
reported for complete inhibition of MMPs (21, 22). TIMP-2 shows no inhibition of the BEHAB/brevican cleavage activity. These results suggest that the activity described here is a metalloproteinase but is not a typical MMP. This conclusion is further supported by the finding that APMA, an activator of latent MMPs, not only does not stimulate activity, but also at its highest concentrations actually inhibits BEHAB/brevican cleavage. In addition, the hydroxamic acid inhibitor, XS309, a broad nanomolar MMP inhibitor that is a very weak inhibitor of the aggrecanases (ADAMTS4 and ADAMTS5), did not inhibit cleavage of BEHAB/brevican at the Glu395-Ser396 cleavage site, even at micromolar concentrations. In contrast, SE206, a broad nanomolar MMP inhibitor that is also a nanomolar inhibitor of aggrecanases, strongly inhibits the activity at concentrations as low as 100 nM. These studies indicate that BEHAB/brevican cleavage at the Glu395-Ser396 site in CNS-1 cells is not mediated by an MMP, but likely by an ADAMTS family member.

The CNS-1 cell-derived BEHAB/brevican cleavage activity we report here shows many similarities to aggrecanase, a protease that cleaves the cartilage proteoglycan, aggrecan. Proteolytic degradation in the intraglomerular domain of aggrecan, including at the aggrecanase site, is thought to be pathologically important in the progression of arthritis. The BEHAB/brevican Glu395-Ser396 cleavage site shares a high degree of similarity to the aggrecanase site in aggrecan. The six amino acids surrounding the cleavage site in aggrecan are ESE/SRG, while for BEHAB/brevican the sequence is ESE/SRG. Additionally, 23 amino acids around these cleavage sites are 50% homologous between the two molecules (6). Furthermore, our characterization of the activity in CNS-1 cells described here is remarkably similar to recent characterizations of the aggrecanase activity (13–15, 23–25). We found nearly identical optimal NaCl concentrations, Ca2+ concentrations, temperature, and pH for the activity described as was found for aggrecanase (14). In addition, the inhibition profiles for both activities are nearly identical (13–15, 23, 25).

Recently two aggrecanases were cloned and revealed to be members of the ADAMTS protein family, ADAMTS4 and ADAMTS5 (originally named ADAMTS11) (13, 15). The remarkable similarity between the activity described here and aggrecanase encouraged us to investigate whether ADAMTS4 could be responsible for BEHAB/brevican cleavage in CNS-1 cells. Preliminary experiments showed that ADAMTS4 was capable of cleaving BEHAB/brevican specifically at the Glu9395-Ser9396 cleavage site. Using RT-PCR, we show that CNS-1 cells constitutively express ADAMTS4 while 9L cells do not. Furthermore, we found that expression of ADAMTS4 in CNS-1 cells is considerably higher than found in normal rat brain by Northern blot. In addition we found that the cleavage activity was completely inhibited by immunodepletion with an ADAMTS4-specific antibody. Accordingly, our results demonstrate that ADAMTS4 is the predominant protease responsible for BEHAB/brevican cleavage at Glu9395-Ser9396 in our preparation.

Previous work has shown that BEHAB/brevican expression is dramatically up-regulated in gliomas. Furthermore, it has been suggested that not just production, but also cleavage of BEHAB/brevican, may be critical in mediating the invasiveness of gliomas. In the present study we have described a cellular source of the BEHAB/brevican cleavage activity, and developed a sensitive and specific assay to monitor and characterize this activity. Using this assay, we were able to identify a protease capable of cleaving BEHAB/brevican, ADAMTS4. In addition, we have determined that ADAMTS4 is responsible for the majority of BEHAB/brevican cleavage in CNS-1 cells, in vitro. These results suggest a novel and important role of an ADAMTS family member in cleavage of BEHAB/brevican and suggest a novel role for ADAMTS4 in the central nervous system and, potentially, in the progression of glioma. Development of specific inhibitors of ADAMTS family members, and accordingly, of BEHAB/brevican cleavage may provide a new therapeutic strategy for the treatment of glioma.

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