ADAMTS4 (aggrecanase-1) cleaves human brain versican V2 at Glu^{405}–Gln^{406} to generate glial hyaluronate binding protein

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Human brain tissue from cerebellum and hippocampus was obtained between 2 h and 24 h *post mortem* and, after extraction in the presence of proteinase inhibitors, proteoglycans were purified by anion-exchange chromatography. The versican component was characterized by Western analysis with antibodies to the N-terminal peptide (LF99), the N-terminal globular domain (12C5) and the two GAG (glycosaminoglycan) attachment regions (anti-GAG-α and anti-GAG-β). The results indicated that versican V2 is the major variant in all brain samples, and that it exists as the full-length form and also as at least six C-terminally truncated forms. The major immunoreactive species present is a 64 kDa product, which we identified by biochemical and immunological analysis as the brain protein previously termed GHAP (glial hyaluronate binding protein) [Perides, Lane, Andrews, Dahl and Bignami (1989) J. Biol. Chem. **264**, 5981–5987]. Immunological analysis of purified human GHAP using a new anti-neoepitope antiserum (JSCNIV) showed that its C-terminal sequence is NIVSFE^{405}, and digestion of human cerebellum proteoglycans with ADAMTS4 (aggrecanase-1, where ADAMTS, a disintegrin and metalloproteinase with thrombospondin-1-like motifs) indicated that GHAP is a product of cleavage of versican V0 or V2 at the Glu^{405}–Gln^{406} bond. Since human cerebellum extracts contained multiple forms of ADAMTS4 protein on Western analysis, these data suggest that one or more members of the 'aggrecanase' group of the ADAMTS family (ADAMTS 1, 4, 5 and 9) are responsible for turnover of versican V2 in the adult human brain.

Key words: ADAMTS, aggrecanase, extracellular matrix, hippocampus, neoepitope antibody, versican V2.

INTRODUCTION

In 1989, an abundant 60 kDa glycoprotein was isolated from human brain white matter and given the name GHAP (glial hyaluronate binding protein) [1]. It appeared to be similar to, but distinct from, a hyaluronate binding protein identified previously in the brains of multiple species and called hyaluronectin [2]. Human GHAP was identified as a 60 kDa species that was substituted with both N-linked and O-linked oligosaccharides and which showed sequence similarities to cartilage aggrecan and link protein. It was localized primarily to the cerebral white matter [1]. Further analysis of brain extracts identified a large aggregating proteoglycan [3] which appeared to co-localize with GHAP and which had an N-terminal sequence identical with that predicted from the cDNA of a fibroblast product named versican [4]. The finding that brain versican could be digested with MMPs (matrix metalloproteinases) to generate a glycoprotein with the same electrophoretic mobility, immunoreactivity and peptide mapping profile as native GHAP [5] firmly established GHAP as a proteolytic degradation product of versican. The demonstration [6] that versican exists in multiple splice variants in various tissues (V0, V1, V2, V3, Vint) was followed by the important observation that the major variant present in mature brain is versican V2 [7]. Further it is now known that versican V2 is co-localized with hyaluronan and a brain-specific link protein, Bral1, at the nodes of Ranvier in the developing and adult CNS (central nervous system) [8].

While the available literature suggests that GHAP is the hyaluronate-bound N-terminal fragment of versican V0 or V2, the exact relationship between the proteoglycan form(s) of versican V0 or V2 and GHAP is not clear, and the proteinase(s) primarily responsible for the formation of native GHAP have not been described. Earlier data suggesting that GHAP is generated from versican by MMP-mediated cleavage [5,9] were based on the invalid assumption that brain versican is the V1 variant.

With the knowledge that versican V2 is the primary brain versican variant [7], and the availability of V0- and V2-specific antibodies, we decided to examine the forms of versican V2 core glycoprotein in human brain extracts. In the process we have shown using a novel anti-neoepitope antibody (JSCNIV) that native GHAP is generated from versican V2 core by digestion with ADAMTS4 (a disintegrin and metalloproteinase with thrombospondin-1-like motifs 4), and that the cleavage site is at the Glu^{405}–Gln^{406} bind in the GAG-α domain of versican V2. The data suggest that an ADAMTS proteinase cleaves versican V2 in human brain *in vivo* in a manner similar to the ADAMTS-mediated cleavage of aggrecan in cartilage and spinal cord [10,11], brevican in brain [12] and versican V0 and V1 in aorta [13].

EXPERIMENTAL

Materials

Endo-β-galactosidase, keratanase II and chondroitinase ABC (protease-free) were from Seikagaku (Falmouth, MA, U.S.A.). SDS/4–12 %-PAGE gels were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Nitrocellulose was from Bio-Rad

Abbreviations used: AD, Alzheimer's disease; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-1-like motifs; AEBSF, 4-(2-aminoethyl) benzenesulphonyl fluoride; CNS, central nervous system; CS, chondroitin sulphate; GAG, glycosaminoglycan; GHAP, glial hyaluronate binding protein; MMP, matrix metalloproteinase; PD, Parkinson's disease; PMI, post-mortem interval.

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(Hercules, CA, U.S.A.). Hyperfilm and ECL® (enhanced chemiluminescence) reagent were from Amersham (Piscataway, NJ, U.S.A.). Recombinant human ADAMTS4 was kindly provided by Dr Elisabeth Morris (Wyeth Inc., Boston, MA, U.S.A.). Recombinant MMP-3 was from Merck, Inc. Sialidase, O-Glycanase and N-Glycanase were purchased from Prozyme (San Leandro, CA, U.S.A.).

Preparation and sources of antibodies

Antibodies JSCNIV and JSCVMA were raised in rabbits against the ovalbumin-conjugated peptides CGGNIVSFE and CVMAHVDPEEP respectively (Research Genetics, Huntsville, AL, U.S.A.). The antibodies were affinity purified against the immunizing peptide using a Sulfolink column (Pierce, Rockford, IL, U.S.A.). LF99 was a gift from Dr Larry Fisher (NIDCR, Bethesda, MD, U.S.A.) and was raised against 13 amino acid residues of versican, L^{21}HKVKVGKSPPVR^{33}, a sequence conserved in all human versican isoforms. 12C5 (ascites fluid) is a monoclonal antibody raised against purified human GHAP [14]. Antibodies specific for the GAG-α domain (residues Lys^{349} to Arg^{764}; accession number AAA67565) and the GAG-β domain (residues Gly^{357} to Asp^{567}; accession number CAA34128) of versican, where GAG is glycosaminoglycan, have been described [6,15]. Anti-(human ADAMTS4) (JSCYNH) was the same as that described previously as anti-YNHRTD [13]. The anti-neoepitope antiserum (JSCDPE) to the C-terminus of ADAMTS4-digested versican V1 is the same as anti-DPEAAE described previously [13].

Source and PMI (post-mortem interval) of human brain samples

Normal human hippocampus was obtained by G. P. from four individuals (PMI unknown) with no evidence of brain pathology. In addition, two samples each were taken from different individuals affected with AD (Alzheimer's disease) (patient A, PMI = 6 h; patient B, PMI = 9 h) or PD (Parkinson's disease) (patient C, PMI = 6 h; patient D, PMI = 11 h) obtained at autopsy and snap frozen by Dr Denis Dickson (Mayo Clinic, Jacksonville, FL, U.S.A.). Human cerebellum samples were also obtained from an individual with PD (patient E, PMI = 5 h) and two individuals with AD (patient F, PMI = 2 h; patient G, PMI = 6 h) by Dr Denis Dickson. Human sample usage for these studies was approved by the Institutional Review Boards at the University of South Florida, Harvard Medical School and the VA Medical Center in West Roxbury, MA, U.S.A.

Purification of versican from human brain tissues

Purification was based on methods described previously [16,17]. Human hippocampus samples were thawed and washed immediately in ice-cold PBS containing protease inhibitors {5 mM EDTA, 0.1 mM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride], 5 mM iodoacetic acid, 0.3 M aminohexanoic acid, 15 mM benzamidine and 1 μg/ml pepstatin} and then finely sliced. The tissues were then extracted overnight at 4 °C in ice-cold sucrose buffer (0.3 M sucrose, 4 mM Hepes, 0.15 M NaCl, 5 mM EDTA, 0.1 mM AEBSF, 5 mM iodoacetic acid, 0.3 M aminohexanoic acid, 15 mM benzamidine and 1 μg/ml pepstatin, pH 7.5) at 1 ml of sucrose buffer per 35 mg of dry weight tissue and clarified by centrifugation at 12 000 ***g*** for 30 min at 4 °C. The clear supernatant was loaded on to a 0.5 ml bed volume column of DE52-cellulose in Poly-prep chromatography columns packed and equilibrated in 50 mM Tris/HCl, 0.15 M NaCl and 0.1 % CHAPS, pH 8.0. The column was washed with 4 ml of the same buffer, the unbound material (flow through) was collected and the column was eluted sequentially with 4 ml of 50 mM Tris/HCl, 6 M urea, 0.25 M NaCl and 0.1 % CHAPS, pH 8.0, and then 4 ml of 50 mM Tris/HCl, 1.5 M NaCl and 0.5 % CHAPS, pH 8.0. The flow-through pool, the 0.25 M NaCl wash and the 1.5 M NaCl wash were each dialysed extensively against water at 4 °C, and portions representing 3.5 mg dry weight of tissue were examined by Western analysis.

Human cerebellum samples were prepared as described in [17]. Briefly, the tissue (2 g wet weight) was homogenized with a glass–Teflon pestle and extracted in 9 vol. of ice-cold extraction buffer (0.3 M sucrose, 4 mM Hepes, pH 8.0, 0.15 M NaCl and 5 mM EDTA containing proteinase inhibitor cocktail III (1 mM AEBSF, 0.8 μM aprotinin, 0.05 μM bestatin, 15 μM E-64, 20 μM leupeptin and 10 μM pepstatin; Calbiochem-Novabiochem Co., La Jolla, CA, U.S.A.). The extract was centrifuged at 500 ***g*** for 10 min, the pellet was discarded, and the supernatant was centrifuged again at 40 000 ***g*** for 30 min. This supernatant was applied to a 8 ml bed volume of DEAE-Sepharose Fast Flow (Pharmacia) pre-equilibrated with 50 mM Tris/HCl, 0.15 M NaCl, 0.1 % Triton X-100 and 5 mM EDTA, pH 8.0. The column was washed with 4 column volumes of the equilibration buffer, 4 column volumes of 50 mM Tris/HCl, pH 8.0, 0.25 M NaCl, 0.1 % Triton X-100 and 5 mM EDTA, 4 column volumes of 50 mM Tris/HCl, pH 8.0, 0.25 M NaCl, 6 M urea, 0.1 % Triton X-100 and 5 mM EDTA, and finally with 4 column volumes of 50 mM Tris/HCl, pH 8.0, 1 M NaCl and 5 mM EDTA. All buffers contained Proteinase inhibitor cocktail III from Calbiochem-Novabiochem.

Digestion by ADAMTS4 and deglycosylation of GHAP and cerebellum proteoglycan

A sample of 60 μl of purified cerebellum extract (from 3.7 mg wet weight of tissue) was incubated at 37 °C for 21 h in digestion buffer (20 mM Tris, pH 7.5, 100 mM NaCl and 10 mM $CaCl_2$) with or without 350 nM ADAMTS4. Digested samples and 2 μg of purified GHAP (from human cerebral white matter [1]) were then digested at 37 °C for 3 h with chondroitinase ABC (protease-free; 20 m-units), dialysed against water for 5 h at 4 °C, dried, resuspended in 50 mM sodium phosphate, pH 5.0, and treated with 5 m-units of sialidase for 45 min and 2.5 m-units of O-Glycanase for 1 h at 37 °C. The samples were then denatured by addition of 50 mM sodium phosphate, pH 7.5, 0.1 % SDS and 50 mM 2-mercaptoethanol and heating at 100 °C for 5 min; they were then cooled, and 0.75 % Triton X-100 and 8 m-units of N-Glycanase were added, followed by further incubation for 1 h at 37 °C.

Western analysis

Samples were dried and resuspended in equal volumes of Tris/glycine/dithiothreitol/SDS sample buffer (2×; Invitrogen) and 6 M urea, boiled for 5 min, and then loaded on 4–12 % (w/v) Tris/glycine SDS gels (Invitrogen) and transferred to nitrocellulose (Bio-Rad) for Western analysis with chemiluminescent detection (Amersham) as described [18]. Peptide blocking experiments were performed by adding the immunizing peptide to the primary antibody solution (5 μM CYNHRTDLFKSFPGP and 5 μM CVMAHVDPEEP or 10 μM of CGGNIVSFE) and rocking for 1 h at room temperature before the addition of the membrane.

Mutant recombinant substrates

Wild-type and mutated poly-His-tagged fusion proteins containing amino acids 1344–1554 of the human versican V0 core

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protein [6] were prepared as described in [15]. Specific mutations were introduced by PCR and verified by DNA sequencing. For digestion by ADAMTS4, these recombinant substrates were dialysed extensively against water for 6 h to remove PBS and then incubated at a final substrate concentration of 28 μM with ADAMTS4 (60 nM) for 1 h at 37 °C in digestion buffer (20 mM Tris, pH 7.5, 100 mM NaCl and 10 mM $CaCl_2$) or terminated immediately with 10 mM EDTA.

Enzyme digestions

Recombinant preparations of ADAMTS4 or MMP-3 were incubated with purified human cerebellum proteoglycan (fraction eluted with 1 M NaCl buffer and corresponding to the total versican in 1.2 mg wet weight of tissue) at 37 °C in digestion buffer (20 mM Tris, pH 7.5, 100 mM NaCl and 10 mM $CaCl_2$) overnight. Digests were then deglycosylated in 50 mM Tris, pH 7.6, 50 mM sodium acetate and 10 mM EDTA with chondroitinase ABC (protease-free; 8 m-units) for 2 h at 37 °C.

Detection of ADAMTS4 in human cerebellum

Human cerebellum samples (from AD and PD patients) were extracted at 4 °C for 20 h in ice-cold buffer (0.3 M sucrose, 4 mM Hepes, 0.15 M NaCl, 5 mM EDTA, 0.1 mM AEBSF, 5 mM iodoacetic acid, 0.3 M aminohexanoic acid, 15 mM benzamidine and 1 μg/ml pepstatin, pH 7.5) at 111 mg wet weight of tissue per ml. The extracts were clarified by centrifugation at 8000 ***g*** for 30 min at 4 °C. Portions of these extracts (corresponding to 0.7 mg wet wt of tissue) and autoproteolysed recombinant human ADAMTS4 (100 ng) were taken for Western analysis with a combination of anti-(human ADAMTS4) antibodies (JSCYNH and JSCVMA, both at 1:2000 dilution).

RESULTS

Versican core protein species in human brain

In order to examine the nature of the naturally occurring versican core protein species in post-mortem human brain, portions of hippocampus from normal (PMI unknown), AD (PMI,9 h) and PD (PMI,6 h) individuals were extracted and fractionated on DE52-cellulose (see the Experimental section for details) to generate an unbound fraction (fraction 1), a 0.25 M NaCl eluate (fraction 2) and a 1.5 M NaCl eluate (fraction 3). After digestion with chondroitinase ABC, these products were examined by Western analysis with antibody LF99 (Figure 1). Since this antibody was raised against the conserved N-terminal 13-residue peptide sequence (LHKVKVGKSPPVR) of human versicans, it was expected to detect molecules containing the N-terminal peptide of versican V0, V1 [13] and V2 [7]. In all samples there were multiple species of variable abundance (labelled 1–11 in Figure 1), with the majority of the immunoreactive material recovered in fractions 1 and 2 in each case. All of the three samples analysed (normal, AD and PD) contained a major LF99-reactive product which ran at approx. 64 kDa (species 7) and a less abundant 85 kDa product (species 6). Also quantitatively important were species 10 and 11, which did not bind to DE52 and presumably represent N-terminal fragments of the versican G1 domain. Similar results with LF99 (not shown) were obtained with three other normal human hippocampus samples (PMIs unknown) and different AD (PMI,6 h) and PD (PMI,11 h) samples.

To characterize these multiple species further, portions of the 0.25 M NaCl eluates were examined (with and without chondroitinase ABC treatment) by Western analysis with antibodies specific for the GAG-β domain (results not shown) or the GAG-α domain (Figure 2). The anti-GAG-β antibody, which reacted strongly with versican V1 from aorta [13], did not react with any of the LF99-positive species in brain, suggesting that these species were derived from versican V2 and that neither versican V0 or V1 was present in these samples. In contrast with the absence of GAG-β-positive species, the anti-GAG-α antibody reacted strongly with species 2–8 (Figure 2), confirming the high abundance of versican V2 in human brain [7].

Interestingly, none of the major V2 species (2–8) presented with altered electrophoretic migration after chondroitinase treatment, suggesting that they were not substituted with CS (chondroitin sulphate). On the other hand, there was some evidence for an increased abundance and slight shift of band 1 in the AD and PD samples at approx. 300 kDa following chondroitinase treatment, consistent with CS substitution of the full-length V2 versican.

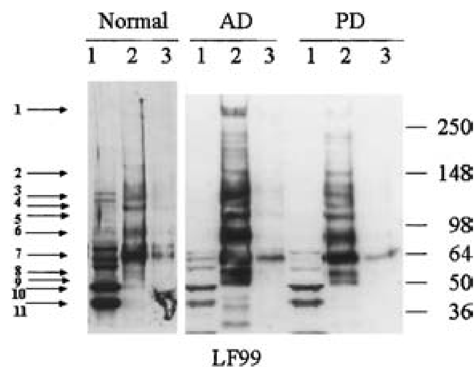

Figure 1 Characterization of versican species found in human hippocampus

DE-52-purified proteoglycans (representing 3.5 mg wet weight of tissue) from samples of normal (PMI unknown), AD (PMI,9 h) and PD (PMI,6 h) hippocampus were run on SDS/4–12 %-PAGE for Western analysis with an antiserum to the N-terminus of versican (LF99, at 1:1000 dilution). The DE52 fractions shown are: 1, flow through; 2, 0.25 M NaCl elution; 3, 1.5 M NaCl elution. Arrows labelled 1–11 indicate multiple species of variable abundance.

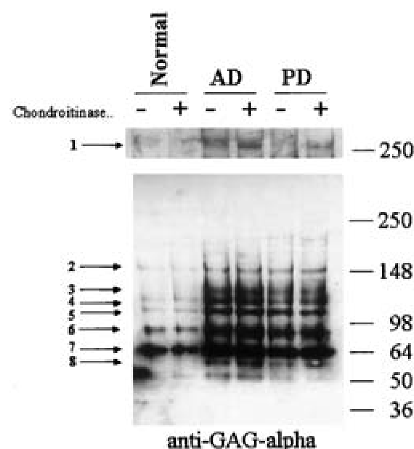

Figure 2 Effect of chondroitinase ABC treatment on purified proteoglycan

The 0.25 M NaCl fractions from normal, AD and PD hippocampus (as shown in Figure 1) were either treated (+) or not (−) with chondroitinase ABC (protease free) and run on SDS/4–12 %-PAGE. Western analysis was with an antiserum to recombinant GAG-α domain (1:1000). The upper panel shows a darker exposure of the high-molecular-mass GAG-α-reactive species (species 1) that was not detected by the low exposure shown in the lower panel. Arrows labelled 1–8 indicate multiple species of variable abundance.

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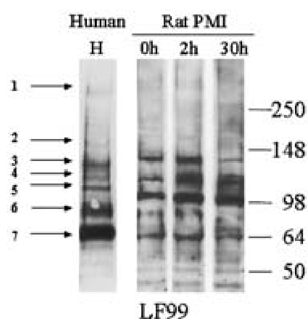

Figure 3 Effect of PMI on production of versican species

Rat hippocampus was isolated at various PMIs (0, 2 and 30 h), and proteoglycan was extracted and purified as described for human hippocampus samples. Western analysis (LF99, diluted 1:3000) of the 0.25 M NaCl eluant from human normal hippocampus (H) and rat hippocampus (0, 2 and 30 h PMI) is shown. Arrows labelled 1–7 indicate multiple species of variable abundance.

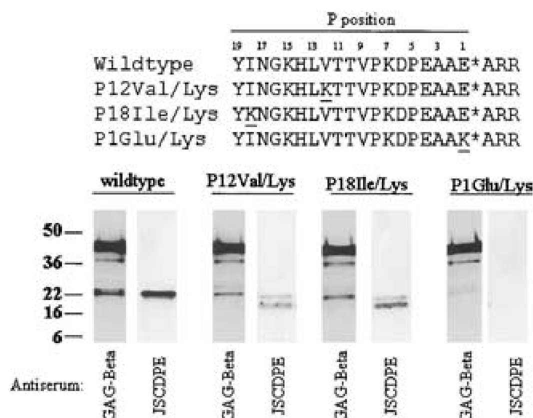

Figure 4 Effects of residues N-terminal to the scissile bond on cleavage of versican V2 by ADAMTS4

Recombinant versican V2 GAG-β (wild type and mutants at 28 μM) containing lysine substitutions at P1, P12 or P18 (as shown in the top alignment) were incubated with ADAMTS4 (60 nM) for 1 h. Digestion products were run on SDS/4–12 %-PAGE and analysed by Western analysis with antiserum JSCDPE (1:1000) or with anti-GAG-β (1:1000).

The migration behaviour and immunoreactivity profiles for species 2–7 suggested that they all represent versican V2 species which are not substituted with CS, and which are composed of an intact N-terminal globular domain attached to variable lengths of the C-terminally truncated GAG-α domain. By comparison with the migration behaviour of the G1-bearing aggrecan species characterized in cartilage extracts [19], these versican V2 species appear to represent peptides in the size range of approx. 700 residues (species 2) down to approx. 450 residues (species 7). Generation of these species *in vivo* would therefore require cleavages at multiple sites within the 250-residue region at the N-terminal end of the GAG-α domain (see Figure 9). Following this reasoning, the abundant LF99/GAG-α-positive species at 64 kDa (species 7) appeared to represent the N-terminal globular domain of versican V2 with a C-terminus located between residue 450 and the boundary (at residue 348) of the N-terminal globular domain and the GAG-α domain.

Effect of PMI on versican V2 core protein structures in brain extracts

The presence of many fragments of versican V2 in human brain extracts (Figures 1 and 2) and the apparent relative paucity of full-length versican (see Discussion) suggested that excessive proteolysis might occur in brain samples during the PMI before autopsy. To investigate this, we examined the effect of PMI before dissection on versican V2 core protein structure in proteoglycan preparations from mature rat brains. For this purpose, three mature rats were killed, stored at 4 °C, and brain tissue was removed into ice-cold PBS containing proteinase inhibitors (see the Experimental section) at PMIs of 0, 2 and 30 h. After dissection of hippocampus, the tissue was finely sliced and proteoglycans were extracted and purified in the presence of inhibitors, as described for the human hippocampus samples. For control purposes, a normal human hippocampus (PMI unknown) was analysed at the same time.

Western analysis of these samples with antibody LF99 (Figure 3) suggested that increasing PMI did not markedly affect the profile of intermediate-size rat versican species (90–130 kDa), which appeared to be equivalent to human species 3–5. Rat products equivalent to human species 6 and 7 were present in relatively low abundance. As found for the human products, treatment of the rat samples with chondroitinase ABC did not alter the relative proportions or electrophoretic migration of the different species. Therefore it appears that the PMI did not markedly affect the profile of versican species, suggesting that the compositions obtained with human brain extracts are a reasonable representation of the state of versican V2 *in vivo*.

Confirmation of the role of versican residues upstream of the ADAMTS4 cleavage site by point mutation further predicts the versican V2 scissile bond

The reactivity of the abundant human 64 kDa fragment (species 7) with LF99 and anti-GAG-α antibodies showed that this species contains the native N-terminal sequence of human versican V2. Alignment [13] of the sequences of human aggrecan, brevican and versican (V1 or V2) shows the expected high degree of identity within the disulphide-bonded N-terminal globular domain of each. Immediately C-terminal to the globular domain is a relatively non-conserved region that encompasses the established sites for ADAMTS-dependent cleavage of aggrecan (NITEGE^{373} ↓ ARGSV), brevican (EATESE^{399} ↓ SRGAI) and versican V1 (DPEAAE^{441} ↓ ARRGQ). Since the N-terminal globular domain products of these cleavages migrate at approx. 60, 50 and 70 kDa respectively, it seemed likely that the equivalent predicted cleavage site for versican V2 in this region at NIVSFE^{405} ↓ N^{406}QKATV (see Table 1 in [13]) would be responsible for generation of the 64 kDa fragment (species 7, Figures 1 and 2).

Prediction of this cleavage site [13] was based on its location relative to the N-terminal globular domain, and more importantly on the presence of apparently important recognition residues at P1 (Glu), P12 (Glu/Val) and P18 (Ile/Leu/Val). To confirm experimentally the importance of these residues in recognition of versican substrates by ADAMTS4, we prepared mutants based on the 230-residue GAG-β domain substrate [13], in which the Glu at P1, the Val at P12 and the Ile at P18 were each individually converted into Lys (see Figure 4). When these substrates were partially digested with 60 nM ADAMTS4, the wild-type substrate was approx. 20 % digested (as shown by reactivity of the 22 kDa product with both anti-GAG-β and JSCDPE); however, this rate of cleavage was markedly reduced by the mutations at either P12 or P18 individually and, as expected, essentially eliminated by the mutation at P1. The low-abundance JSCDPE-reactive bands

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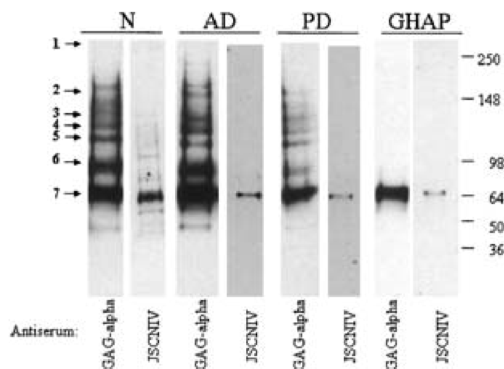

Figure 5 Characterization of the 64 kDa fragment in human hippocampus extracts

The 6 M urea/0.25 M NaCl purification fractions from normal (N), AD and PD hippocampus extracts (3.5 mg wet weight) and purified GHAP (0.5 μg) were run on 4–12 % (w/v) polyacrylamide gels and analysed by Western analysis with anti-GAG-α (1:1000) or JSCNIV (1:500). Arrows labelled 1–7 indicate multiple species of variable abundance.

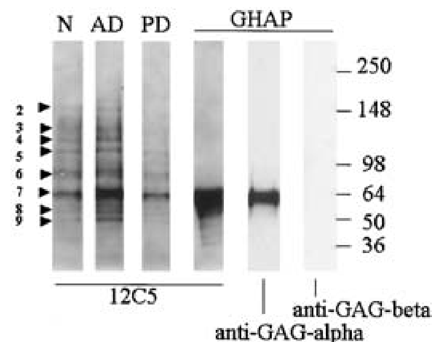

Figure 6 GHAP is major versican fragment in human hippocampus

The 0.25 M NaCl fractions from normal (N), AD and PD hippocampus (corresponding to 3.5 mg wet wt of tissue) and purified GHAP (0.5 μg) were run on 4–12 % (w/v) polyacrylamide gels and analysed by Western analysis with an antiserum to purified GHAP (12C5; 1:10). Western analysis of purified GHAP (0.2 μg) with anti-GAG-α (1:1000) and anti-GAG-β (1:1000) is shown on the right. Arrows labelled 1–9 indicate multiple species of variable abundance.

at approx. 20 kDa in the P12 and P18 mutants have not been identified.

Immunodetection of the C-terminus of the 64 kDa fragment of versican V2 in human brain, and the relationship of this fragment with purified GHAP

The mutational study (Figure 4) confirmed the importance of the P1, P12 and P18 residues in substrate recognition for ADAMTS4, and increased the likelihood that the NIVSFE^{405} ↓ N^{406}QKATV site in versican V2 is sensitive to ADAMTS4 cleavage. Therefore we generated an anti-neoepitope antiserum (JSCNIV) to the ovalbumin-linked peptide CGGNIVSFE and used affinity-purified antibodies in Western analysis of the human brain samples (0.25 M NaCl eluates of normal, AD and PD samples) shown in Figures 1 and 2. As predicted, the 64 kDa product (species 7) alone was reactive with this antiserum (Figure 5), showing that some, or all, of the versican V2 species migrating at 64 kDa were represented by the N-terminal globular domain of versican V2 with a C-terminus at NIVSFE^{405}.

To investigate the relationship of this protein to GHAP purified from human brain, the native purified glycoprotein [5] was also examined by Western analysis for its reactivity with anti-GAG-α and JSCNIV. The result (Figure 5, right panel) clearly indicated that the abundant 64 kDa species (species 7) present in normal, AD and PD human brain extracts (Figures 1, 2 and 5) was identical to GHAP. In addition, the specificity of the reactivity of JSCNIV for the 64 kDa species was confirmed by showing that preadsorption of the antibodies with the immunizing peptide totally eliminated the JSCNIV signal on Western analysis (results not shown).

Further confirmation that GHAP is the N-terminal globular domain of versican V2 with Glu^{405} as its C-terminal residue

In previous studies on GHAP, a monoclonal antibody (12C5) was raised to the purified protein and used in immunohistochemical and Western analyses [14,20]. When purified proteoglycans from normal, AD and PD human brains were analysed for 12C5 reactivity (Figure 6), the results further supported the conclusion that the 64 kDa (species 7) in human brain was the major 12C5-reactive species present and therefore represents GHAP. In these samples, the 12C5 probe also detected low-abundance signals of the species detected previously by the anti-GAG-α antiserum and described as species 2, 3, 4, 5, 6, 8 and 9 (small arrowheads at approx. 150, 130, 120, 110, 85, 55 and 50 kDa respectively in Figure 6). In addition, the reactivity of native human GHAP with 12C5 and anti-GAG-α, and its total lack of reactivity with anti-GAG-β (Figure 6, right-hand lanes), provides further strong evidence that GHAP is indeed generated by proteolysis of versican V2 and not versican V1.

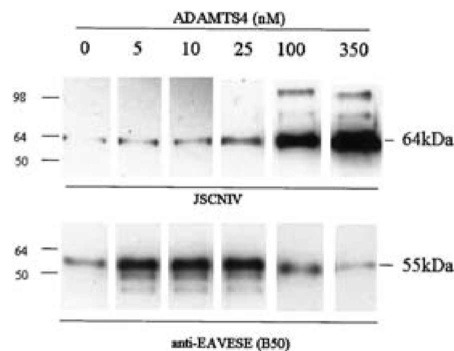

Figure 7 Versicanase and brevicanase activities of recombinant ADAMTS4

DE52-purified human cerebellum proteoglycan was incubated with various concentrations of recombinant ADAMTS4 at 37 °C in digestion buffer (20 mM Tris, pH 7.5, 100 mM NaCl and 10 mM $CaCl_2$) overnight. After treatment with chondroitinase ABC, the samples were run on SDS/4–12 %-PAGE and analysed by Western blot for ADAMTS4-generated products of brevican degradation (anti-EAVESE, B50, 1:1000) and versican V2 degradation (JSCNIV, 1:500).

Digestion of human brain versican V2 with ADAMTS4 generates GHAP

To confirm that the 64 kDa/GHAP product is indeed the product of the ADAMTS4-catalysed cleavage of brain versican V2, we digested DE52-purified proteoglycans from human cerebellum (eluted with buffer containing 1 M NaCl and corresponding to 3.7 mg wet wt of tissue) with different amounts (0–350 nM) of human recombinant ADAMTS4, and examined the products by Western analysis with antibody JSCNIV (Figure 7, upper panel).

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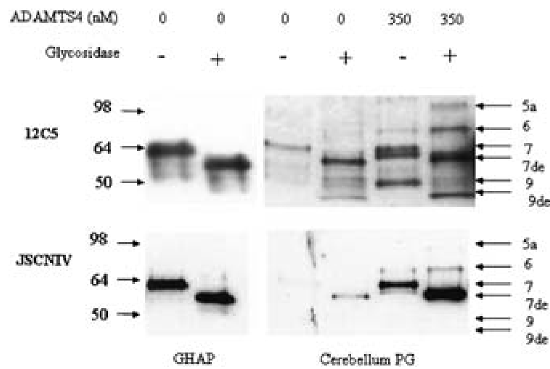

Figure 8 Effects of glycosidase treatment on GHAP and 64 kDa glycoprotein

The left panels show Western analysis of GHAP (5 μg) without (−) and with (+) deglycosylation by sialidase II, O-Glycanase and N-Glycanase. The antibodies used were 12C5 (upper) and JSCNIV (lower). The right panels show Western analysis of human cerebellum proteoglycan (PG) without (−) and with (+) deglycosylation by sialidase II, O-Glycanase and N-Glycanase. de denotes deglycosylated.

As expected, the no-enzyme control contained a low but detectable level of endogenous 64 kDa protein; however, the abundance of this product was increased markedly by incubation with 100 or 350 nM ADAMTS4. Western analyses of these same digested samples using anti-GAG-α and 12C5 antibodies confirmed that the production of species 7 by ADAMTS4 coincided with a decrease in full-length versican V2 (species 1) as well as a loss of species 2–5 (results not shown). Although we have been unable to detect full-length versican V0 using the anti-GAG-β antibody, it is possible that a small amount of GHAP is generated by cleavage of versican V0 at Glu^{405}–Gln^{406}, as this would also generate the 64 kDa fragment.

Since similar experiments were used previously to establish the ADAMTS4 cleavage site in rat brevican at EAVESE^{395} ↓ SRGAI [12], we decided to confirm this work with human brain proteoglycans. When the same digests (Figure 7) were probed with anti-EAVESE (B50), we detected the expected ADAMTS4-generated N-terminal globular domain product of human brevican at 55 kDa with a C-terminus of EATESE^{399}.

Characterization of GHAP and the human 64 kDa product by deglycosylation

To examine further the relationship between the 64 kDa species and purified human brain GHAP [5], we examined the effect of deglycosylation (with combined sialidase II, O-glycosidase and N-Glycanase) on the electrophoretic mobility and immunoreactivity of both species (Figure 8). Native GHAP (Figure 8, left panels) migrated at 64 kDa before deglycosylation and at approx. 55 kDa after deglycosylation, and in both forms it was equally reactive with both 12C5 and JSCNIV. As expected, the banding pattern was more complex with the less highly purified cerebellum proteoglycan preparation (Figure 8, right panels). Untreated material showed a single 12C5-positive band at 64 kDa that was not detected by JSCNIV (species 7). On deglycosylation, this species, in common with GHAP, also moved to approx. 55 kDa (called 7de for species 7 deglycosylated), and in this form it was readily detected with both antibodies, consistent with an unmasking of epitopes by removal of carbohydrate. On digestion (without deglycosylation) of the cerebellum proteoglycan with ADAMTS4, as expected more species 7 was generated (along with some species 9, which has not been identified). On deglycosylation of this species 7, a product equivalent to 7de and deglycosylated GHAP was generated. In addition, a deglycosylated form of species 9 was formed (9de), which was 12C5-reactive but apparently JSCNIV-negative.

We conclude that ADAMTS4 cleaves versican V2 in human cerebellum proteoglycan preparations to generate a product that corresponds to GHAP on the basis of the electrophoretic mobilities and immunoreactivity profiles (anti-GAG-α, 12C5 and JSCNIV) of the major native form (species 7) and also the product of deglycosylation (species 7de; Figure 8). To clarify discussion of the versican V2 species (1–9) described above, we have provided a schematic diagram of the structures of these core protein forms (Figure 9), which includes identification of species 7 as GHAP.

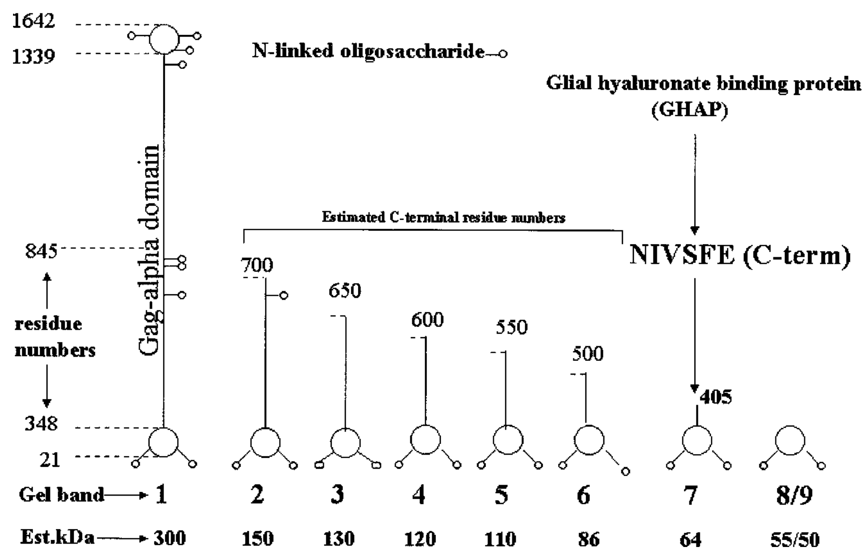

Figure 9 Model of versican V2 species present in human brain

Shown is a schematic diagram of the versican core glycoprotein species described in the text. Note that the C-terminal sequence is known only for species 7, and those of the others are estimated from their electrophoretic mobility and immunoreactivity profiles.

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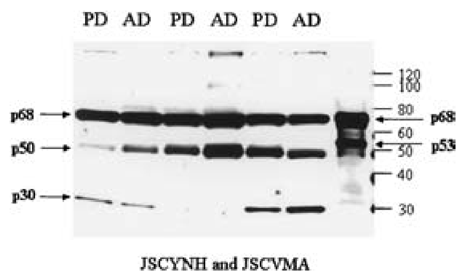

Figure 10 ADAMTS4 protein is present in human hippocampus

Crude human hippocampus samples (from three AD and three PD subjects with PMI < 10 h) extracted with Triton X-100 (see the Experimental section) were used for Western analysis with anti-ADAMTS4 (JSCYNH and JSCVMA, each at 1:2000). A 100 ng sample of recombinant ADAMTS4 (p68 containing the truncated p53 species [21]) is shown in the right lane.

ADAMTS4 protein is abundant in human brain extracts

Since it appears that human brain is abundant in the ADAMTS4-generated product of versican V2 (GHAP), we next examined crude human brain extracts for evidence of ADAMTS4 and ADAMTS5 proteins by Western analysis (Figure 10). Hippocampus from AD and PD brains contained three immunoreactive ADAMTS4 species, which are labelled p68, p50 and p30. By comparison with the major species in autoproteolysed human recombinant ADAMTS4 (Figure 10, right lane), it appears that these samples are abundant in the furin-cleaved p68 form [21], and also contain variable amounts of a p50 form, which presumably represents a highly active C-terminally truncated species related to the previously described p53 form [22,23]. The p30 band has not been studied further. We have been unable to detect ADAMTS5 in these extracts with an antibody (JSCKNG) that readily detects recombinant ADAMTS5 and native ADAMTS5 in cartilage extracts.

DISCUSSION

In the present work, we have attempted to describe the spectrum of versican core protein species in adult human brain extracts. For this purpose we have extracted proteoglycans from human hippocampus and cerebellum and used standard ion-exchange purification methods to generate products for Western analysis with antibodies to all versican species (LF99 and 12C5) and others specific for versican V0 and V1 (anti-GAG-β) or V0 and V2 (anti-GAG-α and JSCNIV). The data (Figures 1–3, 5, 6 and 8) confirm that brain versican is almost exclusively the V2 variant. However, somewhat surprisingly, the results suggest that, in the hippocampus and cerebellum at least, versican V2 is present as multiple C-terminally truncated forms (see Figure 9 for schematic diagram), with the full-length species apparently representing a quantitatively minor proportion of the total. While it is clear that full-length versican V2 can be purified from whole mature bovine brain [7], the ion-exchange conditions used in that study (0.3–2.0 M NaCl gradient) may have discarded the quantitatively major species that we have detected in our 0.25 M NaCl eluates from human hippocampus and cerebellum. On the other hand, the apparent low abundance of full-length core protein in our samples may be due to poor deglycosylation and/or electrotransfer/immunodetection of this ‘bulky’ protein in our system. In this regard, we have found that the abundance of apparent full-length versican V2 core protein(s) in human and rat samples can be increased by treatment of the 0.25 and 1.5 M NaCl eluates with keratan sulphate-degrading enzymes, suggesting that some versican V2 species are substituted with keratan sulphate (results not shown). The low yield of full-length core protein does not, however, appear to be due to post-mortem proteolysis *in vivo*, since similar profiles were obtained with rat hippocampus proteoglycans at PMIs of 0, 2 and 30 h (Figure 3). In terms of human versican V2 core species, it was also surprising to find (Figure 2) that the abundant core species 2–6 (see Figure 9) were apparently not substituted with CS, despite the presence of three Ser-Gly and three Gly-Ser motifs in this region of the GAG-α domain. This raises the possibility that CS substitution on human V2 versican is restricted to the C-terminal region of the GAG-α domain (residues 845–1339), which contains four Ser-Gly and two Gly-Ser motifs.

The data provided cannot be used to prove that 100 % of the versican V2 core present in native species 7/GHAP preparations was generated by ADAMTS-mediated cleavage. Thus, while species 7 in human hippocampus preparations has the same electrophoretic mobility and immunoreactivity profile as purified GHAP (Figures 5, 6 and 8), and species 7 and GHAP show similar relative immunoreactivity with JSCNIV and the other general antibodies, GAG-α and 12C5, minor variation in the C-terminus as a result of alternative ADAMTS- or MMP-mediated cleavage would not markedly alter the electrophoretic migration of GHAP or species 7. In this regard, there are two possible major C-termini on aggrecan and brevican G1 domains, which have been termed the ‘aggrecanase’ site and the ‘MMP’ site; however, these cleavage sites are approx. 30 residues apart in each case, and they generate species that are readily separated electrophoretically [24,25]. An alignment of this region of human versican V0, V1 and V2 suggests that there is no obvious equivalent MMP cleavage site in these proteins, suggesting therefore that most, or all, of species 7/GHAP terminates at Glu^{405}.

Studies on ADAMTS4 activity against the interglobular domain of aggrecan [26] have suggested a requirement for at least 16 residues on the upstream or ‘P’ side of the scissile bond. The present work on cleavage of recombinant versican GAG-β by ADAMTS4 (Figure 4) supports and extends this idea, since individual substitution of the hydrophobic residues at P12 or P18 with lysine markedly reduced cleavage activity. Interestingly, this apparent requirement for specific sequence information on the upstream side of the Glu–Xaa cleavage site appears to extend for at least 32 residues in aggrecan, and includes a sequence at the aforementioned MMP site [27]. The present work on human versican V2 also compliments and extends that published on the digestion of rat brevican with ADAMTS4 [12]. We have shown here that the B50 antibody to the rat epitope (EAVESE) reacts well with the slightly different human sequence (EATESE); further, it was interesting to note that the epitope was generated at a markedly lower ADAMTS4 concentration than the versican V2 product (5 relative to 100 nM for the C-terminus of NIVSFE), although the abundance of each substrate (brevican and versican) in the 1 M NaCl eluate from the DE52 column is not known. Additionally, at high enzyme concentrations (100 nM and above) the brevican neoepitope was eliminated, whereas the JSCNIV epitope is stable. Since the B50 epitope appears to be destroyed by high levels of ADAMTS4 activity, it may be difficult to strictly correlate B50 epitope abundance with the level of proteinase activity *in situ* [12]. By the same token, since the JSCNIV epitope is generated only at high ADAMTS activities and appears stable, it may be a better measure of the ADAMTS-dependent proteolysis of matrix proteoglycans in the CNS.

Since ADAMTS4 cleaves the Glu^{405}–Gln^{406} bond of brain versican V2, it is possible that this enzyme, as well as other described ‘aggrecanases’ (ADAMTS 1, 5 or 9), may play important

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roles in the processing of this molecule and other lecticans, such as aggrecan and brevican, in the CNS *in vivo* [11,25,28]. The most well documented role for lecticans in the adult CNS is their capacity to inhibit neurite growth, which is thought to limit regenerative capacity after injury [29]. Increased expression and extracellular deposition of CS-bearing proteoglycans is common in so-called 'glial scars' that form at the site of injury and inhibit axonal regrowth across it. Much of the inhibitory influence is thought to be due to the CS side chains, since enzymic removal of these chains improves axonal growth and plasticity *in vivo* [30,31]. However, even the core protein of versican V2 exerts inhibitory actions toward neurite growth *in vitro* [32], and the CS-depleted core glycoprotein of aggrecan has been shown to block neurite growth into Matrigel in a spinal cord injury model [33]. Moreover, these CS-bearing proteoglycans do not exist alone in their extracellular environment, but probably form aggregates that include hyaluronate and tenascin C [34]. It is therefore tempting to speculate that ADAMTS-mediated cleavage of lecticans *in situ* would 'solubilize' these aggregate structures and render them more permissive to the regeneration of axons.

It is also possible that the accumulation of GHAP and other truncated versican V2 species in brain is a consequence of neurodegenerative changes during normal aging. Clarification of this idea will require analysis of human brain samples from individuals covering both the developmental and aging years, and perhaps the neoepitope antiserum to ADAMTS-cleaved versican V2 (JSCNIV) may be particularly useful in this area of study. If fragmentation of versican V2 in the mature CNS is a marker of degenerative change, this might distinguish it from versican V0 and V1, which appear to have a rapid turnover during embryonic development of the peripheral nervous system and the CNS, and therefore may be involved in the formation of transient barriers to axonal growth and the migration of neuronal progenitor cells [35].

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