Versican $V_2$ Is a Major Extracellular Matrix Component of the Mature Bovine Brain*

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We have isolated and characterized the proteoglycan isoforms of versican from bovine brain extracts. Our approach included (i) cDNA cloning and sequencing of the entire open reading frame encoding the bovine versican splice variants; (ii) preparation of antibodies against bovine versican using recombinant core protein fragments and synthetic peptides; (iii) isolation of versican isoforms by ammonium sulfate precipitation followed by anion exchange and hyaluronan affinity chromatography; and (iv) characterization by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining or immunoblotting. Our results demonstrate that versican $V_2$ is, together with brevican, a major component of the mature brain extracellular matrix. Versicans $V_0$ and $V_1$ are only present in relatively small amounts. Versican $V_2$ migrates after chondroitinase ABC digestion with an apparent molecular mass of about 400 kDa, whereas it barely enters a 4–15% polyacrylamide gel without the enzyme treatment. The 400-kDa product is recognized by antibodies against the glycosaminoglycan-$\alpha$ domain and against synthetic NH$_2$- and COOH-terminal peptides. Our preparations contain no major proteolytic products of versican, e.g. hyaluronectin or glial hyaluronate-binding protein. Having biochemical quantities of versican $V_2$ available will allow us to test its putative modulatory role in neuronal cell adhesion and axonal growth.

The term *hyalectans* (or *lecticans*) defines a family of large hyaluronan-binding proteoglycans (1, 2) whose members currently include versican (3), aggrecan (4), neurocan (5), and brevican (6). Hyalectans share highly similar domain structures at either end of the core protein, whereas the chondroitin sulfate-carrying middle portions are clearly distinct. The homologous NH$_2$-terminal regions consist of an Ig loop and a tandem repeat element that interacts with hyaluronan (7–9). The COOH-terminal globular structure includes epidermal growth factor-like repeats, a C-type lectin domain, and a sushi (or complement regulatory protein) element. Recombinantly expressed C-type lectin domains of hyalectans interact specifically with tenascin-R (10) and tenascin-C (11). The binding is mediated by a calcium-dependent protein-protein interaction.

Alternative splicing and transcription termination add greatly to the structural diversity of hyalectans. Brevican, for instance, exists either as a secreted or glycosylphosphatidylinositol-anchored molecule as a result of alternative transcription termination (12, 13). Variable usage of exons encoding the epidermal growth factor-like elements and the sushi domain lead to several aggrecan isoforms (14, 15), and alternative splicing of human (16) and mouse (17, 18) versican transcripts may generate up to four versican core protein variants. The differentially spliced exons encode the central glycosaminoglycan-carrying GAG$_1$-$\alpha$ and GAG-$\beta$ domains (19).

All known hyalectans are temporally expressed in the central nervous system (2, 20, 21). Neurocan and aggrecan appear transiently during early developmental stages (22, 23) and are replaced later by brevican and versican (24–26), the major postnatal hyalectans in brain tissues. Neurocan and brevican are restricted to the central nervous system, whereas aggrecan is expressed predominantly in cartilage. The longest versican splice variants ($V_0$ and $V_1$) display an even wider distribution and are present in a number of mesenchymal (27) and epithelial (28) tissues and in cultures of endothelial cells (18).

In vitro experiments suggest that hyalectans play a modulatory role in cell-cell and cell-matrix interactions and hence may participate in the nervous system in the control of axon growth and guidance. Inhibitory activity on neuronal cell adhesion and neurite outgrowth has been described for neurocan (29) and for brevican (24). In addition, aggrecan from nasal cartilage is a potent inhibitor of neurite outgrowth (30). Because brain and cartilage aggrecan differ largely in the extent of post-translational modifications (31), this finding still needs to be confirmed with the more relevant brain-derived aggrecan. Evidence that hyalectans may also be involved in vivo in the control of axonal growth and guidance is provided by our recent observation of the transient versican $V_0/V_1$ expression in tissues that act as barriers during the development of the peripheral nervous system (32).

Although versicans $V_0$ and $V_1$ are also present in the central nervous system (27), there are indications that a third splice variant, versican $V_2$, is the major isoform in adult brain. The assumption is based on results from reverse transcriptase-PCR (16) and Northern blot experiments (18) as well as on comparative immunohistochemical studies (26). In contrast to the other versican splice variants, versican $V_2$ transcripts are only detectable in the central nervous system (16). On the protein level, a versican-like proteoglycan (25) and several small mo-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF060456, AF060457, AF060458, and AF060459 for versican $V_0$, $V_1$, $V_2$, and $V_3$, respectively.

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‡The abbreviations used are: GAG, glycosaminoglycan; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s).
lecular size hyaluronan-binding proteins (33, 34) have been identified in brain extracts. Partial peptide sequences suggest that these components are derived from versican V1 by a proteolytic process (35). No biochemical data are currently available to confirm the presence of the versican V2 proteoglycan in the central nervous system.

To close this gap, we have now determined the complete cDNA sequence of bovine versican, we have produced several domain-specific antibodies via immunization with recombinant cDNA sequence of bovine versican, we have prepared several the central nervous system.

### EXPERIMENTAL PROCEDURES

#### Tissues—Bovine tissues were obtained from the local abattoir. The tissues were immediately placed on ice and processed within 1 h postmortem.

#### PCR Cloning and Sequencing—Total RNA was isolated from bovine forebrain using a RNeasy total RNA isolation kit (Qiagen, Hilden, Germany). 1 μg of total RNA was reverse transcribed with a first-strand cDNA synthesis kit (Boehringer Mannheim, Germany) and random hexamer primers (total volume 20 μl). Either 1 μl of this reverse transcription reaction or 100 ng of bovine genomic DNA was used as the template in different PCRs. Primer sequences and cycling conditions are listed in Table I. Initial cDNA fragments were obtained by PCR with guessmer primers that were based on comparisons of chick (36), mouse (18), and human versican (3, 16) cDNA sequences. The PCR products were either sequenced directly or cloned into a pGEM-T vector using an *E. coli* cloning kit (Promega, Madison, WI). All sequences were determined (18), and human versican (3, 16) cDNA sequences. The PCR products were either sequenced directly or cloned into a pGEM-T vector using an *E. coli* cloning kit (Promega, Madison, WI). All sequences were determined

| Primer pair | Template | Cycling conditions | Cycles |
|-------------|----------|--------------------|--------|
| a*: GATGGTATTTGTTAATG | cDNA | 94 °C 1 min | 25 |
| b*: AACCGATAGGCACAA | cDNA | 94 °C 1 min | 25 |
| c*: AACCGGAGGACCGTATG | cDNA | 94 °C 1 min | 25 |
| d*: GGGCAAAATTTGGA | cDNA | 94 °C 1 min | 25 |
| e*: GAGGCACCAGTCCCAACCCGTGACTG | cDNA | 94 °C 10 s | 35 |
| f: ACCGATACACAGTCACTCTGCAGCA | cDNA | 94 °C 10 s | 35 |
| g*: CACCCAGTTCTCCTATG | cDNA | 94 °C 10 s | 35 |
| h: GGGGTTCCTGGTTTTAC | cDNA | 94 °C 10 s | 35 |
| i: CTATGGCTTGCCACAAA | cDNA | 94 °C 10 s | 35 |
| j*: CGAGTGATAGGA | cDNA | 94 °C 10 s | 35 |
| k*: GGGTCACACGATGCAGCA | cDNA | 94 °C 10 s | 35 |
| l*: CTAATGCAAGATTTCTGTAG | cDNA | 94 °C 10 s | 35 |
| m: ACTTGGTATGTCGACAGTGAAGGA | cDNA | 94 °C 10 s | 35 |
| n: GACTGCTGTCCTCCACTGACTC | gen. DNA | 94 °C 10 s | 50 |

| Cycling conditions | Cycles |
|--------------------|--------|
| Initial denaturation 94 °C 2 min; final extension 72 °C (68 °C, respectively) 7 min. | |
| After 15 cycles, 20 s step extension/cycle. | |
| T: Standard PCR with Taq polymerase (PCR Core Kit, Boehringer Mannheim). | |
| E: Long PCR (Expand Long Template PCR Kit, Boehringer Mannheim). | |
| F: PCR with proofreading (Expand High Fidelity PCR Kit, Boehringer Mannheim). | |

#### Northern Blotting—Northern blots using 1.5 μg of poly(A)+ RNA from bovine forebrain were prepared as described previously (28). Digoxigenin-labeled riboprobes covering bases 1–916 (numbers refer to bovine versican V0 cDNA), 2842–4092, and 9552–10291, respectively, were used for hybridization.

#### Preparation of Polyclonal Antibodies—All polyclonal antibodies were prepared by immunizing New Zealand White rabbits with either histidine-tagged recombinant core protein fragments (GAG-α1): amino acids 366–769, GAG-α1: 991–1335, and GAG-β: 1340–1613, all relative to versican V0) or with NH2- and COOH-terminal peptides (LQVKNEK-SPPVKGS and KHDDHRWRWQESRR, respectively) linked to keyhole limpet hemocyanin (Pierce). Both peptides were supplemented with a COOH-terminal cysteine residue to allow efficient coupling via *m*-malimidomethyl benzoyl-3-hydroxysulfosuccinimide ester (Pierce). Fusion proteins were prepared as described elsewhere (28). All antisera were purified by affinity chromatography. Antisera against GAG-α1, GAG-α1, and GAG-β were preabsorbed on bacterial control extract columns followed by purification on corresponding fusion protein columns (NHS-activated HiTrap matrix, Amersham Pharmacia Biotech). Antibodies against the NH2- and COOH-terminal ends of versican were affinity purified by binding to the corresponding proteins immobilized on UltraLink iodoacetetyl columns (Pierce).

#### Isolation and Characterization of Versican from Bovine Brain—300 g of bovine forebrain was homogenized in a Waring blender using 4 volumes of extraction buffer containing 0.5 M NaCl, 50 mM Tris, pH 7.5, 25 mM EDTA, 0.5% Nonidet P-40, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (Pefabloc SC, Pentapharm, Basel, Switzerland), 1 μg/ml pepstatin, and 1 μg/ml leupeptin (buffer modified from Ref. 37). The homogenate was stirred for 5 h at 4 °C and subsequently centrifuged at 100,000 × g for 1 h. Proteins were differentially precipitated from the supernatant by the addition of solid ammonium sulfate. After an initial step at 30% saturation, versican-containing fractions were precipitated by increasing the ammonium sulfate concentration in the supernatant to 60%. All precipitates were allowed to form overnight for 45 min. The 60% precipitate was resuspended in buffer A (6 M urea, 50 mM Tris, 10 mM EDTA, pH 6) and batch absorbed to Q-Sepharose FF (Amersham Pharmacia Biotech) at 4 °C overnight. After column packing, the resin was washed with buffer A containing 0.3 M NaCl, and elution was achieved by applying a NaCl gradient from 0.3 to 2 M. Versican-containing fractions, identified on a slot blot with anti-GAG-α1 polyclonal antibodies, were pooled and dialyzed extensively against 0.5 M NaCl, 20 mM Tris, 10 mM EDTA, pH 8 (buffer B). Subsequently, hyaluronan-EAH-Sepharose, prepared by coupling hyaluronan (Sigma) to EAH-Sepharose (Amersham Pharmacia Biotech) through cross-linking with N-(3-dimethylamino propyl)-N’-ethylcarbodiimide hydrochloride (Fluka, Buchs, Switzerland) (38), was added to the dialyzed fractions. The batch absorption was performed at 4 °C for 20 h. After packing the resin into a column, a washing gradient from 0.5 to 2 M NaCl in buffer...
RESULTS

cDNA Cloning of Bovine Versican—The starting points for the cloning of the entire bovine versican cDNA sequence were short cDNA stretches amplified from total bovine brain RNA with reverse transcriptase-PCR and guessmer primers. These guessmer primers were derived from versican cDNA sequences with reverse transcriptase-PCR and guessmer primers. These guessmer primers were derived from versican cDNA sequences described above and lay close to the alternative splice junctions (Fig. 1). Primer pairs a*//b* and c*/d*). In this way we obtained bovine cDNA fragments encoding parts of the hyaluronic binding region and the COOH-terminal domains. Based on these sequences, we generated primers (e//f) for a long reverse transcriptase-PCR, which yielded a 3.2-kb amplification product extending over the entire GAG-α-encoding portion of versican V2 and a short PCR fragment, which originated from the versican V3 transcript. No amplification product derived from the V1 splice variant was obtained in this reaction (expected size 5.5 kb). We therefore amplified two small versican V1 cDNA fragments that crossed over the splice junctions into the GAG-β-encoding sequence. For each of these two reverse transcriptase-PCRs we used one perfect match and one guessmer primer (primer pairs: e//k* and l*/f, respectively). Because the GAG-β domain is encoded by a single exon in the human and the mouse genome, we then tried to amplify the remaining bovine GAG-β sequence in a long PCR using genomic DNA as template. The 5.1-kb fragment we obtained corresponded to the expected size. Finally, the portions including the translation initiation or translation termination sequences were each amplified with a perfect match and a guessmer primer (g* or j*), coined from species-conserved 5’- and 3’-untranslated regions, respectively.

Using this strategy, we PCR cloned overlapping cDNA fragments that covered the entire coding region of bovine versican corresponding to a 3,361-amino acid core protein in bovine versican V0, 2,380 amino acids in V1, 1,623 amino acids in V2, and 642 amino acids in V3 (all exclusive of the 20-amino acid secretory signal sequence). The cDNA and deduced amino acid sequences of the four splice variants of bovine versican are not shown in this paper but are available from the GenBank data base.

Expression of Versican in Adult Bovine Brain—From our PCR results we could conclude that mRNAs of all versican splice variants (NH2- and COOH-terminal globular domains or the GAG-α encoding region).

FIG. 1. PCR primers and products. The localization of the primers used for the PCR cloning approach of the different bovine versican splice forms is depicted in the upper panel. Primers marked with an asterisk are guessmer primers based on versican sequence comparisons of human, mouse, and chicken. Resulting PCR products were analyzed by electrophoresis on 1% agarose gels. HABR, hyaluronan binding region. E/L/S, epidermal growth factor-like domains/c-type lectin domains/sushi elements.

Fig. 2. Northern blot analysis of poly(A)+ RNA from bovine brain. Versican mRNA was detected with antisense riboprobes that included the coding sequence of the NH2- or COOH-terminal globular domains or the GAG-α encoding region.
variants V₀ and V₂ (fragments GAG-α₁ and GAG-α₂) or in V₀ and V₁ (fragment GAG-β), respectively. In immunoblotting experiments with bovine brain extracts, the polyclonal antibodies specific for the GAG-α domain detected a predominant core protein band of approximately 400 kDa after chondroitinase ABC digestion (Fig. 4). This band was also weakly visible before the enzyme treatment, probably reflecting unprocessed versican core protein from the intracellular pool. An almost identical picture was obtained with antibodies directed against the NH₂-terminal versican peptide, whereas antibodies specific for the GAG-β domain did not recognize the 400-kDa component. In contrast, GAG-β-specific antibodies detected two larger core proteins, which migrated well above the 400-kDa marker protein. Because these two bands were barely visible on blots developed with the polyclonal antibodies against the NH₂-terminal peptide we concluded that these two core proteins must be present in minute amounts and are only detectable as a result of the high immunoreactivity of the anti-GAG-β antibodies. Finally, a minor band migrating at around 80 kDa was recognized by the NH₂-terminal versican peptide, whereas antibodies specific for the GAG-β domain did not recognize the 400-kDa component. In contrast, GAG-β-specific antibodies detected two larger core proteins, which migrated well above the 400-kDa marker protein. Because these two bands were barely visible on blots developed with the polyclonal antibodies against the NH₂-terminal peptide we concluded that these two core proteins must be present in minute amounts and are only detectable as a result of the high immunoreactivity of the anti-GAG-β antibodies. Finally, a minor band migrating at around 80 kDa was recognized by the NH₂-terminal versican peptide antibodies. The mobility of this band was unaffected by chondroitinase ABC treatment.

Isolation of GAG-containing Isoforms of Versican from Brain Extracts—Versicans were solubilized efficiently from brain tissues with a detergent-containing high salt buffer, confirming previous experiments by Yamagata and co-workers (37). Subsequent extraction of the insoluble fraction with 4 M guanidine-HCl yielded less than 5% additional material recognized by GAG-α- and GAG-β-specific antibodies (data not shown). More than 90% of the soluble immunoreactive material could be precipitated with ammonium sulfate at saturation levels between 30 and 60% (data not shown). Further purification of the versican proteoglycan isoforms could be achieved by anion exchange chromatography on Q-Sepharose FF column (upper). Fractions that were positive on a slot blot using the anti-GAG-α₁ antibody were pooled (solid bar) and subjected to affinity chromatography on a hyaluronan column. During washing only every second fraction was monitored (white gaps indicate fractions that were not tested). Anti-GAG-α₂-positive fractions were pooled (solid bar) and processed for SDS-PAGE analysis.

To monitor the purification process, aliquots of versican-containing fractions were analyzed with SDS-PAGE (Fig. 6). The 400-kDa core protein component observed by immunoblotting the crude brain extract became clearly visible in the Coomassie Blue-stained gels after anion exchange chromatography and was highly enriched after affinity purification on the hyaluronan column. Without chondroitinase ABC treatment, this component barely entered the gel, indicative of its proteoglycan character. At least three other GAG-carrying components were present after the Q-Sepharose chromatography step. Their core proteins either migrated on SDS-PAGE as a double band around 150 kDa or as single band slightly above the 84-kDa protein marker. None of these smaller size bands displayed...
Characterization of the Isolated Versican Core Proteins—The proteoglycan fraction obtained by hyaluronan affinity chromatography was characterized further by immunoblot analysis using the entire panel of domain-specific polyclonal antibodies (Fig. 7). Compared with the immunoblotting analysis of the crude brain extracts (Fig. 4), no major changes in the relative amounts of the large molecular mass core proteins were observed. Only the quantitatively minor 80-kDa component of the crude brain extract had been lost during our purification procedure. Again, the two large core proteins migrating around 500 kDa were recognized by the GAG-β-specific polyclonal antibodies and displayed very weak immunoreactivity with antibodies against the NH2- and COOH-terminal ends of versican. Because the upper band was also apparent on both ABC before loading. Gels were stained with colloidal Coomassie Blue.

whether or not samples were digested with chondroitinase (Ch'ase) 1 polyacrylamide gels under reducing conditions. (Samples of the different isolation steps were separated on 4–15% SDS-

immunoreactivity with anti-versican antibodies (data not shown), and they mostly disappeared after hyaluronan affinity chromatography. The final proteoglycan preparation usually contained 50–100 μg of protein/100 g of brain tissue (wt weight).

DISCUSSION

In our earlier immunohistochemical study of human brain tissues we observed a more abundant and widely spread staining with GAG-α-specific antibodies than with antibodies recognizing the GAG-β domain. Yet these antibodies did not allow a distinction between the versican isoforms V0 and V1, respectively. We therefore could only assume on the basis of the differential staining patterns that the V2 isof orm might be the predominant versican splice variant in mature brain tissues. To prove this hypothesis, we initiated experiments isolating hyalectans from brain extracts. Because autopsy samples of human brain displayed various degrees of autolysis, we used bovine brain as the tissue source. Unfortunately, our previously prepared polyclonal antibodies against human (16, 28) and chick versican (32) showed only weak or no cross-reactivity with the bovine homolog. In consequence, a more elaborate approach including cDNA cloning and preparation of recombinant core protein fragments had to be used to prepare highly reactive antibodies specific for bovine versican.

By comparing the cDNA-deduced primary structures of different species homologs of versican (Table II), we observed a very high amino acid sequence conservation in the NH2-terminal and particularly in the COOH-terminal portion of the core protein, whereas a significant drop in sequence similarity was noted in the chondroitin sulfate-carrying GAG-α and GAG-β domains. This is not surprising because both highly conserved globular ends of the core protein are involved in protein-mediated interactions with other extracellular ligands. In vitro, the NH2-terminal region of versican binds to hyaluronan (8) and possibly also to link protein (39). The recombinantly expressed C-type lectin domain, which is localized in the COOH-terminal globule of intact versican, interacts with the fibronectin type III repeats of tenasin-R (10). In the chondroitin sulfate-carrying middle portion of the versican core proteins, we observed high sequence conservation in the vicinity of serine-glycine pairs, which are likely to be recognized by β-xylosyltransferase (40). Furthermore, the highly acidic amino acid sequence present in a potential cysteine loop at the NH2-terminal end of the human GAG-β domain could also be identified in bovine versican. The function of this core protein stretch is still unknown.

All versican isoforms are expressed in bovine brain. However, the majority of the versican splice variants, namely the mRNAs encoding versican V0, V1, or V2, are only picked up by the very sensitive reverse transcriptase-PCR analysis. It seems therefore likely that these splice forms are only present at very low levels in mature brain tissues. The sole versican mRNA detected in the Northern blot experiment corresponds to the V2 isoform. Human versican splice variants appear usually as double bands on the Northern blot (16) because of multiple polyadenylation signals in the human versican gene (19). That the bovine versican V2 transcript appears as a single band may indicate either that the bovine gene only contains one polyadenylation signal or that they are localized only a few bases apart. From our reverse transcriptase-PCR and Northern blot experiments we did not obtain evidence for the occurrence of an additional “plus” domain that is present in chick versican (41),
nor was there any indication for the existence of a membrane-associated versican splice variant similar to the glycosylphosphatidylinositol-anchored form of brevican (12).

Because the globular domains of hyalectans are highly similar, the distinct sequences of the GAG-carrying middle portions are often used to generate specific antibodies (8, 16, 28). Unfortunately, these antibodies recognize only some of the splice variants, but none is able to detect versican V₃. We therefore looked for highly variable sequence portions at either end of the versican core protein and prepared anti-peptide antibodies. These antibodies recognize all four versican isoforms and allow us to test whether the core proteins are intact.

A number of reports have described the isolation of small versican fragments named glial hyaluronate-binding protein (34) or hyaluronectin (33), the issue of proteolytic degradation has gained particular interest. Aware of this fact, we used for the extraction a detergent containing high salt buffer at neutral pH (37) supplemented with a mixture of protease inhibitors including the water-stable serine protease inhibitor. This observation may indicate that brevican binds to hyaluronan with a significantly lower affinity than versican.

The functional role that versican V₂ and brevican play in mature brain tissues is currently unknown. Hockfield et al. (44) hypothesized that changes in the extracellular matrix composition during brain maturation lead to a stabilization of the synaptic structures. This could in part be achieved by the strengthening of adhesive mechanisms and by inhibiting further neurite growth. Although the loss of synaptic plasticity will certainly not only depend on changes in hyalactan expression, it seems conceivable that matrices rich in versican V₂ and/or brevican act as inhibitors of cell interactions and therefore reduce the formation of new synapses in the mature central nervous system. Such an inhibitory activity of brevican has been demonstrated recently by Yamada et al. (24) in a neurite outgrowth assay, whereas functional studies with versican V₂ are just beginning. Now having biochemical quantities of versican V₂ available will allow us to explore potential roles of this brain-specific splice variant in in vitro and in vivo model systems.

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TABLE II
Sequence identities of bovine, human, mouse, and chick versican domains

| Bovine to | Ig B B | GAG-α | GAG-β | EGF1 | EGF2 | Lectin Sushi |
|----------|-------|-------|-------|------|------|----------------|
| Human    | 91    | 89    | 90    | 72   | 69   | 97            |
| Mouse    | 87    | 91    | 90    | 58   | 52   | 89            |
| Chick    | 75    | 81    | 68    | 25   | 29   | 65            |
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