Distinct Interaction of Versican/PG-M with Hyaluronan and Link Protein*

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The proteoglycan aggregate is the major structural component of the cartilage matrix, comprising hyaluronan (HA), link protein (LP), and a large chondroitin sulfate (CS) proteoglycan, aggrecan. Here, we found that another member of aggrecan family, versican, biochemically binds to both HA and LP. Functional analyses of recombinant looped domains (subdomains) A, B, and B’ of the N-terminal G1 domain revealed that the B-B’ segment of versican is adequate for binding to HA and LP, whereas A and B-B’ of aggrecan bound to LP and HA, respectively. BIAcore™ analyses showed that the A subdomain of versican G1 enhances HA binding but has a negligible effect on LP binding. Overlay sensorgrams demonstrated that versican G1, or its B-B’ segment forms a complex with both HA and LP. We generated a molecular model of the B-B’ segment, in which a deletion and an insertion of B’ and B are critical for stable structure and HA binding. These results provide important insights into the mechanisms of formation of the proteoglycan aggregate and HA binding of molecules containing the link module.

The proteoglycan aggregate is the major structural component of the extracellular matrix of the cartilage (1). It consists of hyaluronan (HA), link protein (LP), and a large chondroitin sulfate (CS) proteoglycan, aggrecan. In this form, HA and CS chains attain stable deposition and exert their functions in the extracellular matrix. The aggregate provides cartilage with a unique gel-like property and resistance to deformation through water absorption, and regulates chondrocyte differentiation by storing extracellular signaling molecules involved in differentiation and distributing them to target cells (2). Thus, the proteoglycan aggregate plays important roles in the development and homeostasis of the cartilage.

Formation of the proteoglycan aggregate in the cartilage requires specific interactions of aggrecan, HA, and LP. Both LP and the N-terminal G1 domain of aggrecan consist of three looped subdomains; A, B, and B’ (3–5). Each of the B and B’ subdomains contains a structure, termed a link module, which is believed to bind to HA (6, 7). Functional analyses using recombinant aggrecan domains and subdomains have revealed that a stretch of B-B’ is the minimal segment for HA binding and a single link module does not bind to HA (8), contrasting with tumor necrosis factor-stimulated gene-6 (TSG-6), which can bind to HA with a single link module (9). The A subdomains of both aggrecan G1 and LP, having an immunoglobulin (Ig)-fold structure (5), are thought to interact with each other (4, 10). Both the G1 and LP are associated side by side (11), and each binds to the other and to HA, accomplishing the stable structure of the aggregate.

The proteoglycans of the aggrecan family such as versican (12, 13), neurocan (14), and brevican (15) comprise similar domains to aggrecan. Because the G1 domain is highly conserved among the members, they may form aggregates with HA and LP. Whereas both neurocan and brevican are exclusively expressed in the brain, versican is expressed in various tissues including the central and peripheral nervous systems, the luminal surface of glandular epithelia, blood vessels in normal and tumor tissues, dermis, and the proliferative zone of the epidermis, and embryonic tissue (16). In these tissues, versican plays important roles in adhesion (17), migration (18), proliferation, and differentiation (19) of cells by interacting with cell surfaces and extracellular matrix molecules. Although versican is transiently expressed during development, it is constitutively expressed in some adult tissues such as the heart, blood vessels, and the brain. As both HA and LP are ubiquitously expressed (20), versican may exert its functions on them in the aggregate form. However, a proteoglycan aggregate composed of any other member of the aggrecan family has not been identified.

In the present study, we demonstrate that versican biochemically binds to both HA and LP at a segment of B-B’ of the G1 domain, contrasting with aggrecan which binds to LP at the A subdomain. Using the link module structure of TSG-6 and CD44, we performed molecular modeling of the B-B’ segment, which may explain the requirement of tandemly repeated link modules. These results may provide a clue to the mechanism of the specific HA-interactions of molecules with link modules.
**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors**—A 325-amino acid cDNA fragment of the versican G1 domain was generated by polymerase chain reaction (PCR), using human versican cDNA (gift from Isogai), Pfu DNA polymerase (Stratagene), and a primer set (Table I) that are attached by XhoI and BamHI linker sites, respectively. The reaction program was 25 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 2 min. The amplified DNA fragment was inserted at XhoI and BamHI sites of pBFX (8). The resulting plasmid designated as VerABB/pBFX was used as the basic vector for further versican gene constructions. A cDNA fragment encoding the G1 subdomain was subcloned into pBluescript II SK− (−) as a template and a primer set as described in Table I was used for construction of expression plasmids of versican subdomains. The PCR products encoding subdomains were similarly inserted at XhoI and BamHI sites of pBFX. The DNA sequence of all constructs was confirmed using an automated DNA sequencer (ABI, PRISM 310). Expression constructs of human LP and B-B' of LP were similarly prepared by PCR using pBFX. PCR was performed to generate a full-length cDNA and a segment encoding B-B'. Using ExTaq polymerase (Takara) with the same reaction program. The PCR-amplified DNA fragment encoding LP was inserted at XhoI and BamHI sites of pBFX. The resulting plasmids were designated as LP/pBFX. An expression plasmid encoding a fusion protein of the B-B' of LP and laminin γ chain (LPBB-pBFX) was constructed by PCR followed by insertion of the fragment into pBFX.

**Expression and Purification of Recombinant Proteins**—293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and penicillin-streptomycin. The cells at ~70% confluence in six-well plates were transfected with 2 μg of expression vector DNA and 6 μl of FuGENE 6TM (Roche Molecular Biochemicals) per well of expression vector DNA and 6 μl of FuGENE 6TM (Roche Molecular Biochemicals) per well. Transfection efficiency was measured using 4 volumes of extraction buffer containing 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. The homogenate was stirred for 5 h at 4 °C and subsequently centrifuged at 100,000 g for 1 h. The supernatant was further grown to confluence. For preparation of recombinant protein constructs: F1 and R3 for VerABB; F1 and R2 for VerAB; F2 and R3 for VerBB; F1 and R1 for VerA; F2 and R2 for VerB; F3 and R3 for VerB'; F4 and R4 for LP, F5 and R5 for LPBB; and a segment encoding B-B' of LP were similarly prepared by PCR using pBFX. PCR was performed to generate a full-length cDNA of LP and laminin γ chain (LPBB-pBFX) was constructed by PCR followed by insertion of the fragment into pBFX.

**TABLE I**

| Primer | Sequence | Subdomain |
|--------|----------|-----------|
| F1 | 5′-ATGCCACCTCGAGGTCAAGTGGAAAGACCC-3′ | A loop |
| F2 | 5′-ATGCCACCTCGAGGGGGGTGTGACTTACACAGGCGC-3′ | B loop |
| F3 | 5′-ATGCCACCTCGAGGTGATCTTACCCCTGACCTGT-3′ | A loop |
| F4 | 5′-ATGCCACCTCGAGGGTGTTTCTTTCAGCAACAT-3′ | A loop |
| F5 | 5′-ATGCCACCTCGAATGATCTTACCCCTGACCTGT-3′ | B loop |
| R1 | 5′-ATGCCACCTCGAGGGGGGTGTGACTTACACAGGCGC-3′ | A loop |
| R2 | 5′-TAAGAATACCCACTGAAATGCCTGAGACCTACCCCTGACCTGT-3′ | A loop |
| R3 | 5′-CTAATTCAAGGAGAAATTCTGACAGGGTCGCTGAG-3′ | B loop |
| R4 | 5′-ACGAACTCGTGGTGTGAGACCTACCCCTGACCTGT-3′ | B loop |
| R5 | 5′-CAGATGCAAGGCTGTCAGGTCGTCGACCTACCCCTGACCTGT-3′ | B loop |

**Preparation of Native Versican**—Native versican was prepared as described (22). Native versican and biotinylated LP were mixed and incubated in a microtube at 4 °C overnight. After the sample was precleared with protein A-Sepharose for 30 min at 4 °C, 2 μl of an anti-CS-α antibody was added. After incubation at 4 °C for 2 h, immunoprecipitation was performed by incubation with 10 μl of protein A-Sepharose gel (50/50 v/v slurry) for 2 h at 4 °C. The gels were washed 10 times with ice-cold buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonylphenylethoxylate, 1 mM PMSF), electrophoresed on a 7.5% SDS-polyacrylamide gel under a reducing condition, and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h in 20 ml Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20 (TBS-T) containing 5% skim milk, and incubated with 2 μg/ml peroxidase-conjugated avidin for 1 h at room temperature. The reaction was visualized by ECL. The sample was separated by SDS-PAGE under either a reducing or a non-reducing condition and electrotransferred onto a PVDF membrane. The membrane was blocked for 1 h at room temperature in TBS-T containing 5% skim milk. For immunoblot analysis, the membrane was incubated with either an anti-CS-α (×2,000) or an anti-LP (8A4, × 1,000). After washing three times with TBS-T, the membrane was treated with peroxidase-conjugated goat anti-mouse IgG (×4,000, Pierce). After washing three times with TBS-T, the reaction was visualized by ECL (Amersham Biosciences). For transblot assay, the membrane was incubated with was either 25 μg/ml biotinylated LP (b-LP) or 25 μg/ml biotinylated HA (b-HA) for 2 h at room temperature after blocking (8). The membrane was reacted with 2 μg/ml peroxidase-conjugated avidin (Pierce) for 1 h, rinsed three times with TBS-T, and treated with ECL to detect proteins that interacted with b-LP or b-HA.

**Surface Plasmon Resonance Binding Studies**—Binding analyses were performed using a BIACoreTM 1000 instrument at 25 °C. For determination of recombinant LP and VerBB' to CMS sensor chips, and b-HA to a SA sensor chip, solutions of LP, VerBB' and b-HA at 10 μg/ml in 10 mM HEPES, pH 7.4, containing 150 mM NaCl were injected into the flow cell at a flow rate of 10 and 5 μl/min, respectively. The amount immobilized in each flow cell was controlled by varying the injection volume of the sample solution. In the kinetic studies, the binding assays were performed with a constant flow rate of 30–50 μl/min in both association and dissociation phases. In brief, a series of protein concentrations ranging from 0.5 to 3.0 mg/ml in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, and 0.005% surfactant P-20) was injected into the flow cell, and the change in resonance unit was recorded. After each run, regeneration of the sensor chip surface was accomplished by two successive injections of 15 μl of 5 mM HCl. The values for rate constants were determined by nonlinear regression analyses using BIAevaluation 2.1 software provided by the manufacturer and as described in detail previously (8). Association rate constants (k_a) were calculated from the linear portion of the sensorgram during the early association phase. Dissociation rate constants (k_d) were calculated from the linear portion of the sensorgram during the late dissociation phase.
were calculated from the early portion of the dissociation phase—20 s after the completion of the sample injection during the wash-out period. The apparent equilibrium dissociation constant ($K_{D}$) was calculated as the ratio of $k_d/k_a$. The kinetic constants were determined by 6–10 independent experiments.

For analysis of interaction of three molecules, we used the HA chip and the co-inject command. In brief, a series of protein concentrations ranging from 0.5 to 3.0 mM in running buffer was injected into the flow cell, and the change in response unit was recorded. After each run, regeneration of the sensor chip surface was performed by two injections of 15 μl of 5 mM HCl, 10 mM glycine HCl, pH 3.0, 0.5 mM guanidinium hydrochloride, and 4 mM guanidinium hydrochloride, respectively.

Sequence Alignment, Homology Modeling, and Docking of HA—Regions of the link module were selected from amino acid sequences of versican, aggrecan, LP, CD44, and TSG-6 of both mouse and human, and neurocan and brevican of mouse, and multiple alignment was performed using an algorithm of Needleman and Wunsch (23). Then, refinement of the pairwise alignment of human versican B and B′ with human TSG-6 was carried out using an ALAX program, which enables precise alignment of deletion and insertion when amino acid identity is low, and therefore critical for precise molecular modeling.

With data of the sequence alignment of the link modules of human versican B and B′ domains and human TSG-6, homology modeling of B and B′ was performed using FAMS (24) with the structure of TSG-6 as a template. The link module contains two disulfide bonds. As the FAMS program does not take disulfide bonds into consideration, the side chains of Cys may be incorrectly oriented. In this case, we bound two sulfite atoms of Cys residues fixing their distance in 2.0 Å, and performed energy minimization using MOE (CCG). For the force field of the minimization, CHARMM22 (25) was used.

Both B and B′ domains were oriented so that the insertion of B and the deletion of B′ are involved in the contact of both domains and the HA-binding surfaces predicted are aligned in a line. The model of HA14 was manually prepared, where both torsion angles of β1–3 and β1–4 bonds are ($\phi$, $\psi$) = (60°, 0°), which is one of the most stable states (26).

RESULTS

Versican Binds to Both LP and HA at the B-B′ Segment—Whereas versican has already been reported to interact with HA (27), the interaction between versican and LP has not been shown yet. We first examined whether versican binds to LP by immunoprecipitation. Versican purified from mouse brain was mixed with biotinylated LP (b-LP), and immunoprecipitated with anti-CS-α. The presence of versican in the precipitate was confirmed by immunoblot using anti-CS-α (Fig. 1A, right).

When the precipitate was applied to trans blot using horseradish peroxidase (HRP)-conjugated avidin, b-LP was detected, indicating that versican bound to LP in solution (Fig. 1A, left). We confirmed their interaction by trans blot assay. Chondroitinase ABC-treated and nontreated versican were applied to a SDS-PAGE gel under a non-reducing condition and transferred to a membrane. When immunoblot was performed using anti-CS-α, chondroitinase ABC-treated and nontreated versican were detected as a sharp band and a smear, respectively (Fig. 1B, lanes 1 and 2). When the membrane was treated with b-LP followed by treatment with HRP-avidin, a band and a smear identical to areas of versican were observed (Fig. 1B, lanes 3 and 4). Specificity of the trans blot assay was confirmed using a recombinant fusion protein of FLAG-aggrecan G1 (ABB′)-laminin γ, AgcAβ′, as a positive control (Fig. 1B, lanes 5 and 6). These results indicate that LP actually interacted with versican on the membrane.

Then, we determined the subdomains responsible for the interaction with LP by trans blot assay, using recombinant domains and subdomains of LP, and the G1 domain of versican and aggrecan as fusion proteins with a FLAG tag at the N terminus and laminin γ chain at the C terminus except for the full-length LP, which was expressed without laminin γ as a soluble protein (Fig. 2A). All recombinant proteins showed a single monomeric band except for VerB, which had an additional band that appeared to be a dimer (Fig. 2B). Biotinylated LP bound to VerABB′ and VerBB′, both of which contain B and B′ subdomains of the versican G1 domain, whereas VerAB, VerA, VerB, VerB′, and laminin γ did not (Fig. 2C). These results indicate that the B-B′ segment of the versican G1 domain is essential for interaction with LP. In contrast, AgcAβ′ and AgcA bound to LP, but AgcBβ′ did not, indicating that the A subdomain of the aggrecan G1 domain interacts with LP. Interestingly, LP and LPBB′γ did not bind to LP.

Next, we determined the subdomains that bind to HA, using a HA-trans blot assay. VerABB′, VerBB′, LP, and LPBB′γ
bound to HA (Fig. 2D), in addition to AgcBB' and AgcABB' (data not shown) as previously reported (8). These results indicate that B-B\(_2\) segments of versican, aggrecan, and LP, are responsible for interaction with HA.

**Analysis of LP or HA Binding Function Using BIAcore\(^\text{TM}\) System**—We studied the kinetics of interaction of recombinant proteins with LP and HA, using BIAcore\(^\text{TM}\) biosensor systems. VerABB', VerBB', AgcABB', and AgcA bound to recombinant LP on the chip, and all the others including VerAB, VerA, VerB, VerB', and LP did not, confirming results obtained by LP-transblot (Table II). The association and dissociation with LP were similar between VerABB' and VerBB', suggesting that the A subdomain of versican G\(_1\) has a negligible effect on the interaction with LP. Although both AgcABB' and AgcA interacted with LP, AgcA associated slower and dissociated faster than AgcABB', resulting in a 3.1-fold higher dissociation equilibrium constant (\(K_{d}\)) of AgcA, indicating that the B-B\(_2\) segment enhances interaction of the A subdomain with LP. When two G\(_1\) domains of versican (VerABB') and aggrecan (AgcABB') were compared, aggrecan G\(_1\) showed a 2.8-fold lower \(K_{d}\). VerBB' immobilized to a CM sensor chip interacted with LPBB'\(\gamma\) at a \(K_{d}\) of \(2.17 \times 10^{-7}\) M, indicating that the B-B\(_2\) segments of both versican G\(_1\) and LP interact with each other.

Next, we studied the kinetics of HA interaction of these recombinant proteins. VerABB', VerBB', LP, and LPBB'\(\gamma\) interacted with HA, but VerAB, VerA, VerB, and VerB' did not (Table III), consistent with the HA-transblot results. Although three molecules interacted with HA at their B-B\(_2\) segment, LP bound strongest to HA, followed by versican G\(_1\), and then aggrecan G\(_1\). VerBB', lacking the A subdomain, associated slowly and dissociated rapidly, compared with VerABB', resulting in a 17-fold weaker interaction as compared by \(K_{d}\). LPBB'\(\gamma\) lacking the A subdomain showed slightly weaker interaction with HA than full-length LP. AgcABB' and AgcBB' interacted with HA at a \(K_{d}\) of \(1.41 \times 10^{-7}\) and \(3.15 \times 10^{-7}\) M, respectively, consistent with our previous data (8). These results indicate that the A subdomain, without HA binding activity, enhances interaction of the B-B\(_2\) segment with HA, although its enhancing levels are different among versican G\(_1\), aggrecan G\(_1\), and LP.

| Interactions of various recombinant domains with LP | \(k_a\) | \(k_d\) | \(K_d\) |
|-----------------------------------------------|---------|---------|--------|
| VerABB'                                      | 2.47    | 3.23    | 1.31   |
| VerAB                                        | NB      | NB      |        |
| VerBB'                                      | 2.93    | 5.06    | 1.73   |
| VerA                                         | NB      | NB      |        |
| VerB                                         | NB      | NB      |        |
| VerB'                                       | NB      | NB      |        |
| Lay                                          | NB      | NB      |        |
| LP                                           | NB      | NB      |        |
| AgcABB'                                      | 2.05    | 0.95    | 0.47   |
| LPBB'\(\gamma\)                             | NB      | NB      |        |
| AgcA                                         | 0.92    | 1.34    | 1.46   |
| AgcBB'                                      |        |         |        |
Versican, LP, and HA Form a Stable Complex—Using the BIAcore™ system, we determined whether versican G1 interacts with both LP and HA to form a stable complex, or competes with LP for HA. Although quantitative analysis of three molecules could not be measured using the BIAcore™ system, the levels of interaction could be studied by the patterns of overlay sensograms. We first injected VerABB' to a flow cell of immobilized HA chip, and then injected LP. VerABB' associated with HA, and LP further did, indicating that LP interacted with the HA–VerABB' complex or a portion of the HA unoccupied by VerABB'. The interaction of three molecules was not dissociable by two successive injections of any solution except for 4 M guanidinium hydrochloride, which also dissociates streptavidin-biotin interaction. When VerBB' was used in place of VerABB', similar patterns were observed (Fig. 3B). In contrast, when AgcBB' was used in place of VerABB', they did not form a stable complex (data not shown). When we first injected VerBB' and then injected LPBB'γ, their interaction was not dissociable (Fig. 3C). These results indicate that HA and the B-B' segments of both versican and LP bind to each other to form a stable complex. When both VerABB' and LP were premixed and passed over the HA chip, the mixture tightly bound to HA, and their binding was not dissociable by 5 mM HCl (data not shown). These results suggest that sequence of their interactions may not be critical for formation of the stable complex.

Molecular Modeling of Versican B-B'—Like aggrecan, both versican G1 and LP require both B and B' domains for HA interaction, whereas TSG-6 requires a single link module. Both B and B' domains exhibit ~40 and 30% sequence identity of amino acids to the link module of TSG-6, respectively, suggesting that both B and B' were duplicated from an ancestral domain of the link module. We aligned sequences of the link module of versican, aggrecan, LP, TSG-6, and CD44 of both mouse and human, and neurocan and brevican of mouse (Fig. 4). In the sequence of the human versican B domain, the amino acid residues Arg-160, Tyr-161, Tyr-208, Tyr-219, and Tyr-230 corresponded, respectively, to Lys-46, Tyr-47, Tyr-94, Phe-105, and Tyr-113, the HA-binding residues in TSG-6 (28), and the amino acid residues Arg-160, Tyr-161, Asp-197, and Ala-198 corresponded, respectively, to Arg-41, Tyr-42, Arg-78, and Tyr-79, the essential residues for HA interaction in CD44 (29) (Fig. 5A, a). In the sequence of the B' domain, His-306, Gly-317, and Leu-325 corresponded, respectively, to Tyr-94, Phe-105, and Tyr-113 of the HA-binding residues of TSG-6, and Asp-295 and Tyr-296 corresponded, respectively, to Arg-78 and Tyr-79, essential for HA interaction in CD44 (Fig. 5A, b). The amino acid residues Arg-160, Tyr-161, Asp-197, Ala-198, Tyr-208, and Tyr-230, of the B domain and Asp-295, Tyr-296, His-306, and Leu-325 of the B' were localized on a surface, suggesting that these amino acid residues form a HA-binding surface of B-B' (Fig. 5A). In contrast, the amino acid residue Phe-105 of TSG-6 showed a solvent accessibility of 0.06, and therefore corresponding residues of B or B' were predicted to be buried inside. Thus, we excluded Gly-219 of B and Gly-317 of B', corresponding to Phe-105, as the amino acid residues forming the HA-binding surface. The sequence alignment predicted a deletion of three amino acid residues in the B' domain (Fig. 4, frame 1), and an insertion of two or three amino acid residues in the B domain (Fig. 4, frame 2). The deletion in B' corresponded to the position of amino acid residues essential for HA binding in both TSG-6 and CD44, which may explain the inability of B' to interact with HA. As the deletion and insertion were not found in TSG-6 and CD44 containing a single link module, they appeared to be associated with tandem duplication of a single link module to B and B' domains. Based on these observations, we performed molecular modeling of the B-B' domains. Because there was no linker stretch between B and B', and both were simultaneously involved in HA binding, they were thought to be oriented side by side. In this model, the insertion and deletion were likely generated to increase the contact of both B and B' domains (Fig. 5B, a

| TABLE III |
| --- |
| Interactions of various recombinant domains with HA |

Biotinylated hyaluronan was immobilized onto a flow cell of a SA sensor chip, and various proteins were injected over the chip surface. NB indicates no binding.

|  | $k_0$ | $k_1$ | $k_2$ |
| --- | --- | --- | --- |
| VerABB' | 3.62 | 6.38 | 1.76 |
| VerAB | NB | NB | 29.1 |
| VerBB' | 0.95 | 27.6 | 29.1 |
| VerA | NB | NB | NB |
| VerB | NB | NB | NB |
| VerB' | NB | NB | NB |
| Lnγ | NB | NB | NB |
| LP | 23.1 | 8.50 | 0.37 |
| LPBB'γ | 6.74 | 4.45 | 0.66 |
| AgcABB' | 0.14 | 1.94 | 14.2 |

Fig. 3. Analysis of interactions of three molecules using BIAcore™ system. Biotinylated HA was immobilized onto a flow cell of an SA sensor chip, and VerABB' and LP (A) or VerBB' and LP (B) were sequentially injected over the chip surface. The interactions of three molecules appear strong, as they are not dissociated by two successive injections of any solution except for 4 M guanidinium hydrochloride, which also dissociates streptavidin-biotin interaction. When VerBB' and LPBB'γ were successively injected (C), their interaction appeared strong. The patterns injected with 15 μl of 5 mM HCl are shown.
FIG. 4. The sequence alignment of the link module. The amino acid sequences of link modules from versican, aggrecan, brevican, neurocan, LP, TSG-6, and CD44 (m, mouse; h, human) are aligned. Amino acids are divided into five groups as follows: Asp, Glu, Gln, and Asn as group 1; His, Lys, and Arg as group 2; Phe, Trp, and Tyr as group 3; Cys, Ile, Leu, Met, and Val as group 4; Ala, Gly, Pro, Ser, and Thr as group 5. The sites where at least 80% amino acids are within a group are colored red, blue, orange, yellow, and green for the groups 1, 2, 3, 4, and 5, respectively. A deletion in B and an insertion in B′ are framed in orange (region 1) and light blue (region 2), respectively. The arrowheads below the aligned sequences represent sites involved in HA binding: red, for both TSG-6 and CD44; green, for CD44 but not for TSG-6; purple, for TSG-6 but not for CD44.

FIG. 5. Molecular modeling of the B and B′ domains. A, the molecular model of human versican B (a) and B′ (b) domains with predicted HA-binding surfaces. Predicted HA-binding surfaces are indicated in circles. Amino acid residues that correspond to ones involved in HA binding in TSG-6 and/or CD44 are indicated in the same colors as arrowheads in Fig. 4. B, the tube (a) and Connolly (b and c) models are presented. The B and B′ domain are colored in pink and white, respectively. Light blue and orange regions represent an insertion of B and a deletion of B′, respectively. Without the deletion in the B′, the contact surface would decrease, which might abrogate stable energy for the contact of B and B′. Without the insertion in the B, the contact surface would decrease. C, the complex model of the B-B′ segment (Connolly model) and HA14 (stick model). In this model, the length of decasaccharide (HA10) contacts with the HA-binding surface, as predicted.
and b). When a chain of HA14 was docked to the model, it was localized onto the predicted surface, where it recognized a length of HA10 (Fig. 5B, c).

**DISCUSSION**

We have investigated the molecular interactions in the formation of proteoglycan aggregates and its in vivo function. As has long been believed, versican forms proteoglycan aggregates with LP and HA. The versican G1 domain, like aggrecan G1, binds to HA at a minimal segment of B-B’, which is enhanced by the A subdomain. Whereas aggrecan G1 binds to LP at the A subdomain, versican G1 does at the B-B’ segment, indicating that the B-B’ is adequate for stable aggregate formation (Fig. 6). Comparison of binding affinity between domains/subdomains and HA by BIAcore™ analyses revealed important roles of each domain/subdomain in the aggregate formation. To address the requirement of both B and B’, we further performed molecular modeling of the tandemly repeated link modules of B and B’ of versican G1. With a deletion of the B’ and an insertion of the B, they are oriented side by side, which increases the contact with HA. These results provide important insights into the mechanisms of HA binding of molecules with the link module.

For detailed functional analysis of HA binding, recombinant proteins should ideally be expressed and measured in the same systems. We used the BIAcore™ instrument, which allows real-time interaction analysis without any labeling, such that association and dissociation rate constants can be measured directly. We have found that HA-transblot and HA-polyethylenamine solid phase assays give results essentially similar to BIAcore™, but that HA solid phase and cetylpyridinium chloride co-precipitation assays may pick up ionic interactions between HA and basic amino acid residues of the ligand (8). The HA binding affinity of versican G1 (VerABB’, $K_D = 1.76 \times 10^{-8}$ M) was stronger than that of aggrecan G1 (AgcABB’, $K_D = 14.2 \times 10^{-8}$ M). Slightly lower levels of HA binding than those previously reported ($4 \times 10^{-9}$ M and $\sim 1 \times 10^{-8}$ M for versican (Ref. 27) and aggrecan (Refs. 30 and 31)) may be the result of different assay systems and different levels of glycosylation, which enhances HA binding (8).

We present here for the first time the molecular model of the B-B’ segment, which is in agreement with the facts that a single-link module of versican does not bind to HA and that the minimal length for HA-interaction is HA10. Sequence alignment reveals a deletion in the B’ and an insertion in the B common to all the members of the aggrecan family and LP, suggesting that they took place when an ancestral link module was tandemly duplicated in evolution. It remains unclear why two tandemly repeated link modules are required for HA binding. As versican G1 and LP bind to HA tighter ($K_D$ of $1.76 \times 10^{-8}$ and $0.38 \times 10^{-8}$ M, respectively) than the link module of TSG-6 (with $K_D$ values ranging from $2-5 \times 10^{-7}$ M) (9), these large proteoglycans might have required tighter HA binding by tandem duplication of the link module in evolution.

Versican G1 and LP interact at their B-B’ segments, and their interaction does not interfere with their HA binding. These facts indicate that the area of the versican B-B’ segment for LP binding is independent of the surface for HA binding. Whereas the B-B’ segment of LP is positively charged (pI = 8.43), versican B-B’ is negatively charged (pI = 4.94), suggesting their ionic interaction. However, their binding was unaltered when the kinetics analysis was performed with 1.0 M NaCl (data not shown). Thus, their binding would require specific structures. The amino acid sequence of the versican B-B’ segment shows extremely high homology to that of aggrecan G1. The critical amino acid residues responsible for LP binding of versican B-B’ remain to be determined.

We have shown at least four novel aspects of functions and properties of the A domain. First, the A domains of versican and LP enhance HA-interaction of the B-B’ segment, as previously reported for aggrecan G1 (8), although the intensity of enhancement is different among them. Second, full-length LP recombinant protein is expressed as a monomer as assessed by SDS-PAGE under a non-reducing condition (data not shown), and it neither binds to LP itself nor to its B-B’ segment. These results contrast with the previous report that LP forms a hexamer in the absence of divalent cations and converts to a dimer when bound to HA (32). Third, the A subdomain of aggrecan G1 alone can bind to LP, which we demonstrated here for the first time. This provides direct evidence supporting previous observations obtained from partial tryptic digestion of aggrecan-LP-HA complex (4), and functional studies using recombinants with domain-specific deletions (10). Fourth, the A subdomain of versican fails to bind to LP. The A subdomains of versican G1, aggrecan G1, and LP contain a well conserved Ig fold. Our results imply that a slight difference in the amino acid sequence may account for distinct functional differences. Although we have clearly demonstrated the requirement of an appropriate partner for interactions, the mechanism of selection of the partner remains to be elucidated.

We have described the structure essential for the formation of proteoglycan aggregate and its in vivo function. Our results of functional analysis may lead to elucidation of the mechanism of HA interaction and to tissue engineering of the extracellular matrix to support the resistance to mechanical load. Recently, other members of the LP family have been identified such as the brain link protein1 (Bral1) (34), resulting in four members, which are designated as HA and proteoglycan-binding link protein (HAPLN) 1–4 (35). The four members of the aggrecan family may select appropriate partners from LP family members, and other types of proteoglycan aggregate with different functions may be present. As the A subdomains of both aggrecan G1 and LP interact with each other, the A subdomain is supposed to play a key role in selection of the partner. However, the fact that the B-B’ of both versican and LP interact with each other suggests more complicated aspects of the selection. Versican is constitutively expressed in some tissues such as the brain and the aorta. HAPLNs other than the cartilage LP (HAPLN-1) may participate in the versican aggregate formation in these tissues. In the cartilage, versican is present in the interterritorial zone of the growth plate and the articular surface, whose expression is up-regulated in osteoarthritis (33). There, versican may form aggregates with LP and HA, and the versican aggregate may have distinct function from the aggrecan aggregate. Further studies on the structure and in vivo function of these aggregates remain to be performed.

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3 H. Watanabe, unpublished observation.
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