Aggrecan, a large cartilage proteoglycan, interacts with hyaluronan (HA), to form aggregates which function to resist compression in joints. The N-terminal region of aggrecan contains two structurally related globular domains, G1 and G2 separated by IGD domain. The G1 domain consists of three subdomains, A, B, and B9, structural features characteristic to many other HA-binding proteoglycans. Here, we studied the interaction of aggrecan domains with HA using recombinant proteins expressed in 293 cells, an embryonal kidney cell line. Deglycosylation of the recombinant aggrecan fragment reduced the HA binding activity. We found that both the B and B9 subdomains were required for HA binding and that a single module of A, B, or B9 was unable to bind HA. The A subdomain increased the HA binding activity of the B-B9 region. The G2 domain had no HA binding activity confirming previous reports. Studies of HA-binding properties using a BIAcore biocomass sensor system revealed that the Kd of recombinant aggrecan fragment (AgW) consisting of G1, IGD, and G2 was 0.226 μM, whereas the Kd of another HA-binding protein, native bovine link protein, is 0.089 μM. In contrast, AgMut11 which lacked subdomain A showed little HA binding activity. AgMut12 consisting of only B-B9 had a 3.4-fold lower affinity and AgMut13 containing A-B-B9 was 1.5-fold lower than AgW. These results suggest that carbohydrates are essential for high level aggrecan binding to HA and that the A subdomain of aggrecan functions in a cooperative manner with subdomains B and B9.

Hyaluronan (HA) is an ubiquitous repeating disaccharide chain. HA has various functions, including tissue morphogenesis, wound repair, cell migration, tumor invasion, and immune recognition (for review, see Refs. 1 and 2). These functions of HA are mediated through specific interactions with HA-binding molecules. A number of these extracellular matrix and cell-surface molecules have been identified and possible mechanisms of interaction with HA have been proposed (for review, see Refs. 2–4). Several studies have suggested that a proteoglycan tandem repeat (PTR), acts as a functional site of interaction with HA (5, 6). Most of the HA-binding molecules, including link protein and aggrecan, contain PTRs. For example, link protein contains three looped domains: A, B, and B9, in which B and B9 contain PTR modules. Aggrecan (7) is a member of a family of large extracellular matrix proteoglycans, which includes versican/proteoglycan-mesenchyme (8, 9), neurecan (10), and brevican (11). These proteoglycans have N-terminal globular domains, G1, whose structure is homologous to link protein. Like link protein, G1 consists of an A subdomain and PTR-containing B and B9 subdomains. Aggrecan is the only member of this family containing an additional globular domain, G9, which has a structure similar to G1 and is separated from it by the interglobular domain, IGD, at the N terminus. The G2 domain also contains B and B9 subdomains but without the A subdomain and lacks HA binding activity. The HA receptor CD44 (12) and the arthritis-associated protein tumor necrosis factor-stimulated gene-6 (TSG-6) (13) have been reported as HA-binding molecules. These molecules contain one PTR motif, suggesting that a single PTR can interact with HA.

Although various HA-binding molecules with PTR have been reported, the HA-binding mechanism of PTR has not yet been well elucidated. For example, it is not clear which one of the PTRs of link protein or of the proteoglycans interacts with HA. The role of the A subdomain for HA binding is also unknown. Various expression systems have been employed to produce recombinant PTR molecules. However, studies of the HA-binding function using these systems have been hampered by insolubility of the PTRs, ternary structure formed by disulfide bonds, and possible effects of glycosylation on HA-binding function. Link protein (14) and TSG-6 (15) have been expressed in Escherichia coli. Since bacteria have no protein disulfide isomerases, disulfide bonds occur at random and the recombinant proteins do not form a correct structure. Therefore, refolding of the molecule with glutathiones (14) or laborious purification steps to obtain molecules with correct structure were performed (15). Link protein has also been expressed in a baculovirus system (16). Since the glycosylation machinery of insect cells is different from that of mammalian cells, the HA binding of the recombinant protein may not be properly assessed. Recent nuclear magnetic resonance (NMR) studies of the PTR structure of TSG-6 show that the ternary structure is similar to the C-type lectin domain (17). Since the TSG-6 was expressed in bacteria, these studies have not considered the role of glycosylation on structure and activity.

Various assays have previously been established such as the transblot assay using labeled HA (18–20), HA-Sepharose column chromatography (21), co-precipitation using HA-Sepharose or cetylpyridinium chloride (CPC) (13), and enzyme-linked...
immunosorbet assay (22). Since HA has a highly negative charge, it may interact with any positively charged molecules or basic amino acid residues in a protein. Also by virtue of its viscous nature, HA may trap molecules nonspecifically. In addition, previous studies that used synthetic peptides to identify an active sequence of link protein for HA-binding (23), were inconclusive (24).

In this study, we developed a novel mammalian expression system and expressed a soluble recombinant human aggrecan fragments consisting of various portions of the G1–G2 domains linked to the C-terminal portion of the laminin γ1 chain. This system allows us, for the first time, to study the HA-binding function of recombinant aggrecan domains expressed in mammalian cells. We analyzed these recombinant proteins for HA-binding by several different methods including the HA-Transblot assay and lipid-conjugated HA-binding assay (HA-PE assay). We also used a BIACoreTM biosensor instrument to evaluate the kinetics of binding of recombinant aggrecan fragments and bovine link protein to HA. By measuring the changes in surface plasmon resonance signal of aggrecan domain- and subdomain-specific mutants associating to and dissociating from the immobilized HA on the sensor chip, the binding characteristics of these molecules were monitored in real-time. This technique allows accurate and highly reproducible calculation of their kinetic rate constants. Our results suggest that both B and B' are required for HA binding and that the A subdomain and carbohydrates are important for high level interaction between HA and aggrecan.

**EXPERIMENTAL PROCEDURES**

**Construction of the Expression Vectors—**A basic expression vector pBFX was constructed from the pcDNA3 vector (Invitrogen, San Diego, CA) with several modifications. The pBFX vector contains the following sequences: cytomegalovirus promoter, a rabbit β-globin splicing site (25), a human interleukin-2 receptor signal peptide sequence (26), a FLAG epitope tag (Kodak Scientific Imaging Systems, Rochester, NY), a factor Xa cleavage site, and a segment of mouse laminin γ1 chain (27). The splicing signal was placed in the 5′-end and the reverse primer encoding two threonines are replaced with a GCG codon for alanine. The primers for AgMut21 were F6 and R5. In these primers, a TGG codon for tryptophan is replaced with a GCG codon for alanine. The primers for AgMut22 were F7 and R6. The sequence GCCGCC in the forward primer and GGCGGC in the reverse primer encoding two threonines are replaced with an Apol linker site and a translation termination codon at 5′-ends. The reaction program was 25 cycles of 94 °C for 12 s, 58 °C for 30 s, and 72 °C for 1 min (Gene Amp PCR System 9600, Perkin-Elmer, Norwalk, CT). The PCR-amplified DNA fragment that encodes factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the mouse laminin segment (27) was inserted at a BamHI and ApoI sites of pcDNA3. The interleukin-2 receptor and FLAG sequences were prepared by PCR with the plasmid cytomegalovirus-interleukin-2 receptor (28) as a template using the following set of primers: 5′-CGTACCCAAGGTCAG-GAAAGTGAGGAT-3′ with a KpnI linker site; 5′-GATTCCCCGATTGTTCTGTTGTTCTGG-3′ with an EcoRI linker site. The β-globin splicing signal was prepared by PCR with the plasmid pKCR (25) and was cloned into the HindIII site of pcDNA3. The sequence underlined represents a HI linker site. The following combinations of primers were used to prepare aggrecan constructs: F1 and R1 for AgW; F2 and R1 for AgMut11; F2 and R2 for AgMut12; F1 and R2 for AgMut13; F1 and R4 for AgMut16; F2 and R3 for AgMut17; F3 and R2 for AgMut18; F4 and R1 for AgMut19.

**TABLE I**

Primers for polymerase chain reactions

| Primer | Sequence | Subdomain/domain (residues) |
|--------|----------|-----------------------------|
| F1     | 5′-CTCAGGAAACCTCTCCAGGATGACAC-3′ | A loop/G1 (121–141) |
| F2     | 5′-CTCAGGAGGCTTCTGTCTCCCAGA-3′ | B loop/G2 (1489–1509) |
| F3     | 5′-CTCAGAGGGTCAGGTTTGGTACCAC-3′ | B′ loop/G1 (817–837) |
| F4     | 5′-CTCAGGAGGCTTCTGTCTCCCACCGC-3′ | B′ loop/G1 (1110–1160) |
| F5     | 5′-CAAGAACCTCAAGGCGCCTGCTTCAAG-3′ | A loop/G1 (271–300) |
| F6     | 5′-CTCAGGAGGCTTCTGTCTCCCACCGC-3′ | A loop/G1 (228–257) |
| R1     | 5′-GGATCCCTCGGAGCAAAGGATGTCG-3′ | B′ loop/G1 (300–271) |
| R2     | 5′-GGATCCCTCGGAGCAAAGGATGTCG-3′ | A loop/G1 (300–271) |
| R3     | 5′-GGATCCCTCGGAGCAAAGGATGTCG-3′ | A loop/G1 (257–228) |
| R4     | 5′-GGATCCCTCGGAGCAAAGGATGTCG-3′ | A loop/G1 (257–228) |
| R5     | 5′-CTTGGACAGACCGCTTGGCTTTGCTTG-3′ | A loop/G1 (257–228) |
| R6     | 5′-CTTAGAAGGCCCGCGGCACAGGGTTGACTG-3′ | A loop/G1 (257–228) |

6037 to 6007) with an Apol linker site and a translation termination codon at 5′-ends. The reaction program was 25 cycles of 94 °C for 12 s, 58 °C for 30 s, and 72 °C for 1 min (Gene Amp PCR System 9600, Perkin-Elmer, Norwalk, CT). The PCR-amplified DNA fragment that encodes factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the mouse laminin segment (27) was inserted at a BamHI and ApoI sites of pcDNA3. The interleukin-2 receptor and FLAG sequences were prepared by PCR with the plasmid cytomegalovirus-interleukin-2 receptor (28) as a template using the following set of primers: 5′-CTCAGCCAAGGTCAG-GAAAGTGAGGAT-3′ with a KpnI linker site; 5′-GATTCCCCGATTGTTCTGTTGTTCTGG-3′ with an EcoRI linker site. The β-globin splicing signal was prepared by PCR with the plasmid pKCR (25) and was cloned into the HindIII site of pcDNA3. The primers used for pBFX was used as the basic vector for further aggrecan gene constructions.

DNA segments for various regions of aggrecan were generated by PCR with pSA005 (7) as a template using a set of primers carrying a XhoI linker site for the forward primer and a BamHI linker site for the reverse primer. The amplified DNA was inserted into the BamHI site of AgMut16. The mutant proteins, AgMut21 and AgMut22, were constructed using a QuickChangeTM site-directed mutagenesis kit (Stratagene). The CDNA insert from AgMut13 was subcloned into pBluescript II and the plasmid was used as a template for mutagenesis. The primers used for AgMut21 and F6. In these primers, a TGG codon for tryptophan is replaced with a GCG codon for alanine. The primers for AgMut22 were F7 and R6. The sequence GCCGCCG in the forward primer and GCCGCCG in the reverse primer encoding two threonines are replaced by ACCAC encoding two alanines. The PCR fragments with the mutations were subcloned into the XhoI and BamHI sites of pBFX. The sequence of all expression constructs was confirmed by DNA sequencing using an automated DNA sequencer (Applied Biosystems, Foster City, CA).

**In Vitro Expression, and Purification of Recombinant Proteins—**293 cells (CRL 1573, ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, penicillin-streptomycin, and 1-glutamine (Dulbecco's modified Eagle's medium-10). The cells at ~70% confluency in 6-well plates were transfected with 2 μg of expression vector DNA and 6 μl of LipofectAMINE™ (Life Technologies, Gaithersburg, MD) per well according to the manufacturer's instructions. Forty-eight hours after transfection, the medium was replaced with Dulbecco's modified Eagle's medium-10 containing 650 μg/ml G418 (Life Technologies). The cells were cultured for 10 days in the presence of G418 and a pool of stable transfectants was further grown to confluency. For preparation of recombinant proteins, 10 dishes (15 cm in diameter) of the confluent cells were used. The cells were rinsed three times with phosphate-buffered saline (PBS) and collected in 10 ml of 20% Trit-HCl, pH 7.4, containing 0.15 m NaCl, 0.05% Brij 35, 10 ml EDTA, 2 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide. The protein fractions were extracted twice from the cell lysate by homogenization and centrifugation for 30 min at 10,000 ×
Co-precipitation with HA-Sepharose or CPC—

N-glycosidase F

Chemical deglycosylation of the protein was performed using trifluoromethane sulfonic acid (TFMSA) as described previously (31). Briefly, 100 ng of the dry sample was reconstituted in 10 μl of anisol (Fluka, Ronkonkoma, NY). Ninety μl of TFMSA was added to the reaction and it was incubated at 4 °C for 2 h. The sample was precipitated and washed five times with ice-cold diethyl ether. The precipitate was vacuum dried. For immunoblot analysis or HA-binding transblot assay, the sample was dissolved in SDS sample buffer. The GlycoShift De-N-Glycosylation kit (Oxford GlycoSystems, Bedford, MA) was used for enzymatic deglycosylation. Briefly, 200 ng of the recombinant proteins was incubated with 0.4 units of peptide N-glycosidase F for 2 h at 37 °C.

Co-precipitation with HA-Sepharose or CPC—

The co-precipitation experiment was performed (14), using HA-Sepharose (21) or CPC as described previously (13).

BIAcore™ Biosensor—

For immobilization of HA to a SA sensor chip (Biacore, Inc., Piscataway, NJ), a solution of biotinylated HA at 40 μg/ml in 20 mM Tris-HCl, pH 7.4, containing 0.3 mM NaCl, 0.005% Tween 20 was injected into the flow cell at a flow rate of 5 μl/min. The amount of biotinylated HA immobilized in each flow cell was controlled by varying the injection volume of the protein solution. In the kinetic studies, the binding assays were performed at 25 °C with a constant flow rate of 50–100 μl/min in both association and dissociation phases. In brief, a series of protein concentrations ranging from 0.5 to 5.0 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 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 by Karlsson et al. (32, 33).

RESULTS

Expression of Recombinant Aggrecan Fragments with Hyaluronan Binding Activity—

The pBFX vector was constructed for expression of recombinant aggrecan fragments (Fig. 1A). A recombinant aggrecan fragment (AgW) containing the N terminus of human aggrecan core protein spanning from G1 to G2 domains was expressed in 293 cells, a human embryonal kidney cell line. AgW is a fusion protein consisting of a segment of the mouse laminin γ1 chain at the C terminus and the FLAG tag sequence at the N terminus. The cleavage sequence for factor Xa was inserted between the aggrecan G2 domain and the segment of the laminin γ1 chain. The FLAG tag was used for identification and purification of AgW. Expression of the recombinant protein as a fusion protein was necessary because the N-terminal portion of aggrecan without the γ1 chain was not expressed in 293 cells probably due to rapid degradation of the protein (data not shown). Hence, all recombinant aggrecan molecules in this study were expressed as a fusion protein. The AgW monomer was purified to an apparent homogeneity by two-step column purification with an anti-FLAG M2 antibody and a Superdex-200 FPLC column (data not shown). Approximately 200 μg of the recombinant protein was obtained from 10 confluent tissue culture dishes (15 cm in diameter). The expression of the recombinant protein was examined by immunoblotting using an anti-FLAG M2 antibody (Fig. 1B). The HA binding activity of AgW was examined by several methods. First, the HA-Transblot assay confirmed the HA binding activity of AgW (Fig. 1B). Reduced AgW did not bind to HA (data not shown), suggesting the importance of disulfide bonds in the G1 domain for the activity as previously reported for link protein (23). To examine the effect of the laminin γ1 chain on HA-binding, the fusion protein (AgW) was cleaved by activated factor Xa and examined for its HA-binding activity by the HA-Transblot analysis (Fig. 1C). Western blotting with anti-FLAG M2 antibody revealed that the truncated protein (M, 138,000) was cleaved to a protein consisting of FLAG sequence and the G1 and G2 domains (M, 86,000) with factor Xa. HA-Transblot showed that there is no difference in HA binding ability between the fusion protein and the factor Xa-cleaved 86-kDa product (Fig. 1C).

These results indicate that the laminin γ1 chain does not affect HA binding to AgW.

The lipid-conjugated HA-binding (HA-PE) assay was also used to measure the activity of AgW and to compare its activity with bovine link protein and with the HABR, which represents the G1 domain prepared by partial proteolytic digestion of native bovine aggrecan (30). In this method, PE dipalmityl, a lipid, was covalently coupled to the reduced terminus of the HA chain (HA-PE) and coated on a 96-well dish (29). PE has a high affinity for plastic while HA does not, which leads to the preferential attachment of PE to the dish, and leaves the HA
portion of the HA-PE conjugate free in solution and available for binding. AgW had approximately 50 and 25% of HA binding activity when compared with the bovine aggrecan fragment and bovine link protein (Fig. 2A). A control recombinant protein, FLAG-laminin $\gamma_1$ chain, showed no HA binding activity. AgW did not bind to chondroitin sulfate or heparan sulfate (Fig. 2B), indicating specific interaction of AgW with HA. Increasing salt concentrations did not inhibit interaction of AgW with HA, suggesting that HA-binding is independent of ionic interactions (Fig. 2C).

Expression of Domain-specific Recombinant Proteins and Their HA-binding Ability—A series of domain-specific constructs were prepared and expressed in 293 cells (Fig. 3A). The recombinant proteins were partially purified by anti-FLAG-M2 columns. The proteins were separated by 4–20% SDS-PAGE and analyzed by Western blot using anti-FLAG antibody (Fig. 3B) and by HA-Transblot (Fig. 3C). All recombinant proteins showed a single monomeric band except AgMut14 which had an additional slow migrating band that appeared to be a dimer (Fig. 3B). HA-Transblot analysis demonstrated that AgMut11 and AgMut12, which contain both B and $B_9$ subdomains of $G_1$ but lack A subdomain had HA binding activity although Ag-
binding activity. A recombinant protein consisting of A and B (AgMut14) also showed no activity even after prolonged exposure to a x-ray film (Fig. 3C, bottom panel). Faint bands seen in the prolonged exposure in lanes 12 and 14 in Fig. 3C represent nonspecific reactions since the band did not correspond to the molecular weight of recombinant AgMut12 and AgMut14. The B-B' segment from G9 (AgM19) did not show HA binding activity. The presence of the A subdomain (AgMut20) also failed to bind to HA. Similar results were obtained from HA-PE analysis (data not shown). These results indicate that the minimal segment for HA-binding is the B-B' loops of the G9 domain.

Our results differed from previous reports on recombinant link protein expressed in insect SF9 cells (16) and in bacteria (14) in that a single loop of B or B' was sufficient to bind HA using the HA-Sepharose or CPC precipitation method. We, therefore, examined HA binding activity of the recombinant aggrecan fragments using these same methods. In the HA-Sepharose method, the recombinant proteins were incubated with HA-Sepharose beads and bound proteins were analyzed. We found that all recombinant proteins shown in Fig. 3A were bound to HA-Sepharose even after extensive washes with different conditions (data not shown). We also found that a control protein containing FLAG and the C terminus laminin y1 chain (FLAG-laminin y1) derived from the basic vector pBFX, bound to the HA-Sepharose beads although another control protein, bovine serum albumin, did not bind to HA-Sepharose (data not shown). These results suggest that the HA-Sepharose method was not appropriate to evaluate specific binding of recombinant aggrecan fragments to HA. In the CPC assay, the recombinant proteins were incubated with HA and bound proteins were precipitated with 1% CPC. All recombinant proteins except the negative controls, FLAG-y1 and bovine serum albumin, were co-precipitated with HA. However, even under reducing conditions these recombinant proteins were also co-precipitated with HA (data not shown). Thus, the CPC method is incapable of detecting conformation-dependent HA binding.

Effect of the A Subdomain on HA Binding—Although AgW, AgMut11, AgMut12, and AgMut13 were active for HA binding, their binding activity levels differed. HA binding activity of these recombinants was compared quantitatively using immunoblot and HA-Transblot. Four serially diluted samples from each recombinant aggrecan fragment were separated on a set of two SDS-PAGE gels under nonreducing conditions. One of the gels was analyzed by immunoblot to estimate the amount of the proteins and the other by HA-Transblot for HA binding activity. Fig. 4A (AgW and AgMut11) and Fig. 4B (AgW, AgMut13, and AgMut12) show representative patterns of the immunoblots and HA-Transblots. The HA-Transblot showed much less relative HA binding activity of AgMut11 than AgW, compared with the same ratio between AgW and AgMut11 in immunoblots. Similarly, AgMut12 showed less binding reactivity to HA as compared with AgMut13. The affinity to HA was quantitated by measuring the density of the bands and the ratio of the reactivity was statistically analyzed from two independent experiments (Fig. 4C). The HA binding ability of AgMut11, AgMut12, and AgMut13 were calculated as 3.1 ± 1.9%, 32.1 ± 16.3%, and 84.0 ± 22.9% of AgW, respectively. These data suggest that the A subdomain significantly enhances HA binding activity of the B-B' segment.

The A subdomain structure is characteristic of an immunoglobulin (Ig) type-fold with three and four $\beta$-sheets in parallel orientation (6). As the structure of the hypervariable region is dependent on interaction with antigens, the segment of the A loop may be important for the enhancement of HA binding activity. The tryptophan (Trp-75) in the center of the Ig-like
fold functions as a hinge of these β sheets and is critical for its ternary structure (Fig. 5A). To examine whether the conformation of the A subdomain is essential for its enhancing effect on the HA-binding function of aggrecan, a mutant protein, AgMut21, was generated with the tryptophan replaced with alanine. Using immunoblot and HA-Transblot assays (Fig. 5, B and C), HA binding activity of AgMut21 was determined to be 45 ± 9% that of AgMut13. A similar result was obtained with the HA-PE assay (Fig. 5, D). Double substitutions (AgMut22) at two threonines (Thr-61 and Thr-62) with alanines reduced HA binding activity to 31 ± 15% that of AgMut13, respectively. Relative HA binding activity of the recombinant proteins are shown. The density of the bands in Western blot and HA-Transblot was measured by densitometry and their ratios were compared. Data with 200 ng and 100 ng of each recombinant proteins in two separate experiments were averaged. HA binding activity with AgW was taken as 100%.

**Effect of Deglycosylation on HA Binding Activity**—The G1 domain has been shown to have O- and N-linked carbohydrate chains (34, 35). We examined the role of these carbohydrate chains in HA binding activity by removing them either chemically or enzymatically. TFMSA treatment which removed both O- and N-linked carbohydrates, reduced the molecular weight of AgMut13 significantly, indicating that AgMut13 is glycosylated. Since a pilot experiment revealed a significant reduction in HA binding of deglycosylated AgMut13, 5-fold more was applied on a SDS-PAGE gel and compared with unglycosylated AgMut13 in immunoblot and HA binding assays in Fig. 6A. Densitometric calculation of the immunoblot with anti-FLAG antibody showed that 97% of AgMut13 was deglycosylated and 3% remained glycosylated (Fig. 6A). HA-Transblot showed that the ratio of the intensity of the bands for HA binding is 36.5 to 63.5 for deglycosylated to glycosylated proteins. Hence, HA binding activity of deglycosylated AgMut13 was about 2% that of glycosylated AgMut13. Peptide N-glycosidase F treat-

**FIG. 4.** Semi-quantitation of HA binding activity. Various amounts of recombinant proteins were electrophoresed on two sets of 4–20% SDS-polyacrylamide gels under nonreducing conditions. One gel was analyzed by Western blot with anti-FLAG M2 antibody and the other gel by HA-Transblot. A, comparisons of AgW and AgMut11. Lanes 1–4 for 200, 100, 50, and 25 ng of AgW, respectively; lanes 5–8, for 200, 100, 50, and 25 ng of AgMut11, respectively. B, comparisons of AgW, AgMut12, and AgMut13. Lanes 1 and 2, 200 and 100 ng of AgW, respectively; lanes 3–6, 200, 100, 50, and 25 ng of AgMut13, respectively. C, relative HA binding activity of the recombinant proteins are shown. The intensity of the bands in Western blot and HA-Transblot was measured by densitometry and their ratios were compared. Data with 200 ng and 100 ng of each recombinant proteins in two separate experiments were averaged. HA binding activity with AgW was taken as 100%.

**FIG. 5.** HA binding activity of the recombinant proteins with a mutation in the A loop. A, a part of the sequence from the A subdomain of the G1 domain of aggrecan is shown. AgMut21 and AgMut22 are derivatives of AgMut13. AgMut21 contains a substitution of Trp-75 with Ala, and AgMut22 contains two substitutions of Thr-61 and Thr-62 with Ala. B, immunoblot and HA-Transblot are shown. Lanes 1–4, 200, 100, 50, and 25 ng of AgMut13, respectively; lanes 5–8, 200, 100, 50, and 25 ng of AgMut21, respectively; lanes 9–12, 200, 100, 50, and 25 ng of AgMut22, respectively. C, relative HA binding activity of the recombinant proteins are shown. The intensity of the bands in the Western blot and in HA-Transblot was measured by densitometry and their ratios were compared. Data with 200 and 100 ng of each recombinant proteins in two separate experiments were averaged. The HA binding activity obtained with AgMut13 is taken as 100%. D, using HA-PE assay, HA binding activity of AgMut21 (closed circle) and AgMut22 (open circle) were compared with that of AgMut13 (×). The graph is a representative of three identical experiments.
Hyaluronan-binding Function of Aggrecan

Summary of rate and equilibrium dissociation constants of AgW and AgW mutants interactions with HA

|         | $k_a (M^{-1} s^{-1}) \times e^3$ | $k_d (s^{-1}) \times e^{-3}$ | $K_D (M) \times e^{-7}$ |
|---------|-------------------------------|----------------------------|---------------------|
| AgW     | 17.6                          | 3.97                       | 2.26                |
| AgMut11 | 8.46                          | 6.56                       | 7.75                |
| AgMut12 | 12.70                         | 4.41                       | 3.47                |
| Bovine-LP | 14.8                          | 1.31                       | 0.89                |

HA, which is consistent with the results obtained by HA-Transblot and HA-PE assays.

**DISCUSSION**

Using a mammalian expression system, we expressed recombinant proteins containing various domains of human aggrecan and tested their activity for HA binding. A part of the mouse laminin γ1 chain was fused to the C terminus of the N-terminal segment of aggrecan. The γ1 chain fusion is required for stable expression of the recombinant proteins and the highly soluble property chain contributes to the increase in solubility of the recombinant proteins. The γ1 chain and the FLAG tag had no effect on HA binding to aggrecan globular domains.

In this study, we used several different assays for HA binding, including HA-Transblot, HA-PE, and a BIAcore™ biosensor system. Essentially similar results were obtained from these assays. We found that both the B and B′ domains in the G3 domain are inactive for HA binding. Individual subdomains, A, B, or B′ alone are inactive for HA binding. We also found that the A subdomain plays a critical role in enhancing the HA binding activity of the G1 domain of aggrecan. There is a more profound effect of the A subdomain on HA binding for a longer recombinant molecule consisting G1, IGD, and G2 (AgMut11) than for a smaller molecule containing B and B′ (AgMut12). In the BIAcore™ assay, this is more evident in that AgMut11 did not show any HA binding activity, whereas inclusion of the A subdomain in AgMut11 (AgW) resulted in HA binding activity to nearly the same level as native link protein. In the absence of the A subdomain, the IGD and G2 domains may block the B-B′ loops of the G1 domain and interfere with...
their interaction with HA. It is of interest to note that all extracellular structural macromolecules which have HA binding activity contain the A loop, while CD44 and TSG-6 do not. The A loop may play a crucial role in stabilizing the interaction with HA in the extracellular matrix to form a solid tissue structure.

Our results disagree with previous reports that a single PTR module of B or B’ loop of link protein can bind to HA (14, 16, 17). This discrepancy is probably due to different assays and expression systems. To evaluate these differences, we analyzed HA binding activity of the recombinant aggrecan molecules with the same assays used in previous studies with recombinant proteins expressed in bacteria and in baculovirus systems. These assays included the HA-Sepharose binding and CPC precipitation methods. In the HA-Sepharose binding assay, the recombinant aggrecan proteins, AgW, AgMut11–14, 16–18, and the control FLAG-laminin y1 bound to HA-Sepharose. In the CPC-precipitation assay, recombinant proteins bound to HA even under reducing conditions (data not shown). These results indicate that, under these assay conditions, recombinant aggrecan molecules interact with HA in a nonspecific manner and may not reflect an in vivo mechanism that involves the ternary structure of PTR. Since HA is highly negatively charged, basic amino acid-rich regions of the proteins have a tendency to interact nonspecifically with HA.

It is of interest to note that CD44 and TSG-6, which each contain a single PTR, bind to HA by enzyme-linked immunosorbent assays (22) and CPC coprecipitation methods (13). It has also been shown that the HA binding activity of CD44 is regulated by glycosylation levels and by its clustering on the cell surface (for review, see Ref. 2). Different glycosylation levels of CD44 are achieved by alternative splicing of mRNA. The correlation of the clustering and HA binding activity of CD44 suggests that dimerization of the molecule may be involved in its HA binding activity. The sequence of CD44 shows little homology to link protein or to the G1 domain of aggrecan except for a PTR motif. It is also possible that the flanking sequence of the PTR of CD44 may contribute to HA binding of a single PTR. It would be of interest to know whether dimer formation of CD44 is a prerequisite for HA binding. The function of TSG-6, a secreted protein found in articular joints has not yet been identified. Since its domain structure is more similar to CD44 than to link protein, TSG-6 may bind to HA through a mechanism similar to that of CD44.

The BIAcore instrument allows real-time interaction analysis without any labeling such that association and dissociation rate constants can be measured directly. We used a fast flow rate and a low concentration of HA on the sensor chip surface for kinetic measurements. These measures minimize the effect of size and diffusion rate differences among these proteins that would affect the rate constant calculations. Our data indicated that binding of AgW and native link protein to HA have a similar relatively fast on-rate despite the fact that AgW is three times larger than link protein. This suggests that diffusion rates do not play a role in the kinetic rates. AgW has a moderately fast off-rate while link protein has the slowest off-rate of all the recombinant proteins examined in this study. Thus, the affinity of AgW to HA (K_D = 0.226 μM), which is consistent with previous report (36), is about three times lower than link protein (K_D = 0.089 μM). The half-life (t_1/2) for dissociation of AgW from the complex is given by the equation t_1/2 = ln2/k_off. The half-life of the AgW-HA complex and link protein are calculated as 175 s and 529 s, respectively. We tested the HA binding property of all the other recombinant proteins. Only AgMut12 and AgMut13 bound to HA, although their affinity to HA is significantly lower than that of either AgW or link protein.

Proteins that bind to HA without a PTR have been identified such as the receptor for HA-mediated motility (37), the intercellular adhesion molecule-1 (35), and the cumulus extracellular matrix stabilizing factor (39). Receptor for HA-mediated motility binds to HA with high affinity (K_D = 10^{-8} M) via clusters of basic amino acids in the molecule. Denatured and reduced receptor for HA-mediated motility can bind to HA in the transblot assay, suggesting that ionic interactions between the two molecules appear to be more important (28). Application of the BIAcore biosensor system to HA binding analysis for these molecules may lead to a better understanding of the binding properties.

We found that the A subdomain significantly enhanced the HA binding activity of the aggrecan G1 domain. The A subdomain forms an Ig-fold which consists of 7 β-strands. Tryptophan in the strand C and a disulfide bond are important for its conformation (40). Replacement of the tryptophan with alanine indicates that the ternary structure of the A loop is necessary for its enhancing effect on the HA binding function of the G1 domain. The Ig-fold contains loops that correspond to hypervariable regions of immunoglobulin. A Thr-Thr-Ala-Pro sequence in the A loop located in the loop corresponding to L1 of immunoglobulin has an O-linked carbohydrate side chain(s) (35, 41) (6). Substitution of these threonine residues with alanines to remove possible O-linked carbohydrates decreased aggrecan HA binding activity, suggesting that carbohydrate side chain(s) in the A loop may fortify the HA binding affinity of the G1 domain. Our results demonstrate, for the first time, the significant contribution of the N-linked and O-linked carbohydrate side chains of the G1 domain for its HA binding function. It has been reported that the carbohydrate side chains become elongated in preference to keratan sulfate chains with age (34, 41). Carbohydrate side chains, by regulating charge interactions in the microenvironment, may enhance interactions with HA and thereby strengthen the cartilage matrix structure. Studies with mutations in glycosylation sites of CD44 have also suggested that glycosylation regulates its lectin activity (42).

It is intriguing that the aggrecan G2 domain did not bind to HA. Our results confirmed the previous finding that the G2 domain of pig aggrecan failed to bind to HA (43). Attachment of the A subdomain at the N terminus of the G2 domain (AgMut20) did not enhance HA binding function. The G2 domain shows a high homology of amino acid sequence to B-B’ of the G1 domain (44) and contains the same disulfide bond patterns as those of the G1 domain (45). It has been suggested that an extra N-linked oligosaccharide may exist in the B’ subdomain of G2r, 4 amino acid residues after the first cysteine residue, and may disrupt the folding of the domain (46). Creation of chimeric proteins and substitution mutations would give us clues for the identification of critical differences between G1 and G2 for HA binding.

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