The function of link protein in stabilizing the interaction between aggrecan and hyaluronan to form aggregan aggregates, via the binding of link protein to the aggrecan G1 domain and hyaluronan, is well established. However, it is not known whether link protein can function with similar avidity with versican, another member of the large hyaluronan-binding proteoglycan family that also binds to hyaluronan via its G1 domain. To address this issue, we have compared the interaction of the versican and aggrecan G1 domains with link protein and hyaluronan using recombinant proteins expressed in insect cells and BIAcore analysis. The results showed that link protein could significantly improve the binding of both G1 domains to hyaluronan and that its interaction with VG1 is of a higher affinity than that with AG1. These observations suggest that link protein may function as a stabilizer of the interaction, not only between aggrecan and hyaluronan in cartilage, but also between versican and hyaluronan in many tissues.

The family of large aggregating proteoglycans (hyalectans) includes aggrecan, versican, neurocan, and brevican (1). Although aggrecan is most abundant in cartilage, neurocan and brevican are largely restricted to nervous tissue, and versican has a rather wide tissue distribution. Versican has been identified in loose connective tissues, in fibrous, articular, and elastic cartilages, in the central and peripheral nervous system, and in the epidermis, and in all three wall layers of veins and elastic arteries (2). These large aggregating proteoglycans have common structural features. Their core proteins are composed of terminal globular (G) domains and a central chondroitin sulfate attachment domain. The N-terminal G1 domain contains an Ig-like motif, which mediates protein-protein interaction, and two link protein-type modules that are responsible for binding to hyaluronan (HA). The C-terminal G3 domain consists of one or two EGF repeats, a C-type lectin-like motif, and a complement regulatory protein-like repeat (3).

Link protein (LP) is a major structural component of cartilage and, like versican, is also present in many other tissues (4). Its amino acid sequence exhibits considerable homology with the G1 domains of the large aggregating proteoglycans, including conservation of five disulfide bonds. LP binds to the G1 domain of aggrecan and to HA, and its major function is thought to be the stabilization of the interaction between aggrecan and HA in the aggrecan/HALP ternary complex in cartilage (5). However, the function of LP in non-cartilaginous tissues devoid of aggrecan is less clear. The high degree of homology shared by the G1 domains of the different aggregating proteoglycans suggests that LP may also bind to their G1 domains and to HA to produce stable proteoglycan aggregates.

In this study, we compare the interaction affinities of versican G1 (VG1), aggrecan G1 (AG1) and LP with each other and with HA using recombinant proteins expressed in insect cells via a baculovirus expression system. Our results demonstrate that LP has a higher affinity for VG1 than for AG1. We also demonstrate that LP stabilizes versican aggregates in addition to stabilizing aggrecan aggregates, its traditionally accepted role.

EXPERIMENTAL PROCEDURES

Construction of DNA Fragments and Recombinant Donor Plasmids—A DNA fragment encoding residues 1-333 (LHK... to ... DSE) of mature human versican (Vs splice variant of the versican gene (6)) plus its signal peptide (7) and a C-terminal His tag was generated from a human keratinocyte RNA preparation by reverse transcription and PCR using the primers VF and VR (Table I) as described previously (8). A DNA fragment encoding residues 1-333 (VET... to ... TGE) of mature human aggrecan plus its signal peptide (9) and a C-terminal His tag was generated from a human chondrocyte RNA preparation by reverse transcription-PCR using the primers AF and AR. Similarly, a DNA fragment encoding human LP plus its signal peptide (10) and a C-terminal His tag was also generated from the human chondrocyte RNA preparation by reverse transcription-PCR using the primers LPF and LPR. Human primary culture keratinocytes were purchased from Colnetics Co. (San Diego). Human chondrocytes were isolated from human articular cartilage obtained at autopsy. Total RNA was extracted using TriZol® reagent (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was synthesized using the SuperScript first strand synthesis system (Invitrogen) and used as a template for PCR. The amplified PCR products were cloned into the pCiR2.1 vector using a TA cloning kit (Invitrogen), and their authenticity was confirmed by DNA sequencing. To express recombinant VG1, AG1, and LP in insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen), the DNA fragments were subcloned into the pFastBac donor vector between the BamHI and EcoRI sites to obtain the recombinant donor plasmids pFastBac-VG1, pFastBac-AG1, and pFastBac-LP. Construction of a DNA fragment encoding for honeybee melitin signal peptide (11), mature LP, and a C-terminal His tag, was accomplished in two steps. First, a DNA fragment encoding for mature LP and a C-terminal His tag was PCR amplified from pFastBac-LP

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‡ The abbreviations used are: G, globular; AG1, aggrecan G1 globular domain; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; HA, hyaluronan; LP, link protein; PBS, phosphate-buffered saline; RU, response unit; SPR, surface plasmon resonance; VG1, versican G1 globular domain.

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using primers LPFas and LPR and cloned into a TA vector. This fragment was then subcloned into pMebBac (B) (Invitrogen) between the BamHI and EcoRI sites to obtain pMebBac (B)-LP. Second, the DNA fragment encoding honeybee melittin signal peptide, mature LP, and a C-terminal His tag was PCR amplified from pMebBac (B)-LP using primers HMSFP and LPR. The resulting PCR product was also cloned into a TA vector and then subcloned into pFastBac vector at the EcoRI site.

Expression and Purification of Recombinant Proteins—Recombinant bacmid and baculoviruses were prepared, as described previously (8), using the Bac-to-Bac Baculovirus Expression System, and the recombinant baculoviruses were used to infect High Five insect cells (Invitrogen) at a multiplicity of infection of 10. Recombinant proteins were purified from the culture supernatant by Sephadex G-25 and nickel-nitrotriacetic acid-agarose (Qiagen, Mississauga, ON) column chromatography (8). The concentrations of the recombinant proteins were determined by the method of Bradford (12).

Characterization of Purified Recombinant Proteins—To assess purity, recombinant proteins were analyzed by 12% SDS-PAGE under reducing conditions and silver staining and by Western blotting using specific monoclonal antibodies. The anti-His tag antibody was purchased from Qiagen. Antibody 12C5, specific for versican, IC6 for aggrecan, and 8A4 for LP were provided by the Developmental Studies Hybridoma Bank (Department of Biological Sciences, the University of Iowa, Iowa City, IA). Antibody 12C5 recognizes an epitope located on the G1 domain of versican, and IC6 recognizes a conserved sequence present once on each of the aggrecan G1 and G2 domains (13). To confirm further the authenticity of the recombinant proteins they were analyzed by 12% SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane for N-terminal amino acid sequencing.

Biotinylation of HA and Recombinant Proteins—Biotinylated HA was prepared by a modification of the method described by Melrose et al. (14), in which a reduced amount of biotinylation reagent was employed. In brief, 100 mg of HA (Sigma) was dissolved overnight at 4 °C in 20 ml of water. 0.8 g of adipic acid dihydrazide (Sigma) was added and the pH adjusted to 4.75 with 0.1 N HCl. 0.1 g of EDAC HCl (Sigma) was then added and the pH maintained at 4.75 by the addition of small aliquots of 0.1 N HCl. After incubation for 2 h at room temperature, the solution was adjusted to pH 7.0 with 0.1 N NaOH, dialyzed against water, and lyophilized to obtain hydrazido-HA. 100 mg of hydrazido-HA was redissolved overnight in 20 ml of 0.2 M NaHCO3 at 4 °C. 10 mg of biotin-3-sulfosuccinimidyl-N-hydroxysuccinimide (Sigma) was added to the solution and incubation continued for 18 h at room temperature. Finally, 0.25 ml of 1 N Tris-HCl, pH 7.6, was added, and the product was dialyzed against PBS. In the original method of Melrose et al. (14), an approximately equimolar amount of biotinylation reagent, relative to HA disaccharide content, resulted in about 35% substitution of HA carboxyl groups. In the present study the biotinylation reagent was reduced to one-tenth of that described by Melrose et al. so that the expected glucuronic acid substitution would be correspondingly reduced to about 3.5% but certainly not to exceed 10%. Recombinant proteins were biotin labeled using NHS-LC-Biotin (Pierce Chemical Co.) according to the manufacturer’s instructions.

ELISA Analysis of Protein Binding—AG1, VG1, LP, ovalbumin, or HA in carbonate buffer, pH 9.6, was added to 96-well flat bottomed microtiter plates (20 μg/ml, 50 μl/well) and kept overnight at 4 °C. The wells were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with PBST containing 3% bovine serum albumin and 0.1% NaN3 (blocking buffer) for 1 h at room temperature. After blocking, 50 μl of biotinylated protein at various concentrations ranging from 0.4 ng/ml to 4 μg/ml diluted in the above blocking buffer was added to each well and incubated for 2 h at room temperature. After washing the wells with PBST, alkaline phosphatase-conjugated streptavidin, diluted 1:1,000 in the blocking buffer, was added and incubated for 1 h. To quantify binding, the alkaline phosphatase substrate p-nitrophenyl phosphate was added, and the absorbance was read at 405 nm in a microplate reader.

Slot Blot Analysis—2-fold serial dilutions of recombinant proteins (AG1 or VG1) in PBS and 1% β-mercaptoethanol, or PBS alone, were incubated at 55 °C for 15 min, then spotted onto nitrocellulose membrane using a 48-well Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Ovalbumin was used as a control. The membrane was blocked for 1 h at room temperature in 20 mM Tris-HCl, pH 7.6, containing 0.15 μM NaCl, 0.05% Tween 20 (TBST), and 0.1% bovine serum albumin and washed three times with TBST. Subsequently, the membrane was incubated with 1 μg/ml biotinylated LP diluted in TBST and 0.1% bovine serum albumin for 2 h at room temperature. After washing, horseradish peroxidase-conjugated streptavidin was applied, and bound LP was detected using an ECL substrate (Amersham Biosciences). Surface Plasmon Resonance (SPR)—All SPR experiments were carried out at 25 °C using a BiAcore 1000 system (BIACore, Uppsala, Sweden) with a same running buffer (20 mM Hepes, pH 7.4, containing 0.15 M NaCl, 3.4 mM EDTA, and 0.05% Polysorbate 20) at a flow rate of 100 μl/min. However, 5 μl/min was used for immobilization. Amine coupling kits and B1 and SA sensor chips were from BiAcore. Recombinant LP was immobilized on a B1 pioneer sensor chip using the amine coupling procedure. 80 μl of 0.05 M N-hydroxysuccinimide and 0.2 M EDAC HCl mixture was injected over a flow cell for activation. 5 μg/ml recombinant LP diluted in 10 mM formic acid, pH 3.5, was immobilized onto a B1 sensor flow cell until the desired amount of the protein was captured. Finally, 80 μl of 0.1 M ethanolamine HCl, pH 8.5, was injected over the flow cell to block the remaining activated carboxyl groups.

Control surfaces were generated using the same procedure by replacing the protein with running buffer. For immobilization of HA to an SA sensor chip, 1 μg/ml biotinylated HA in running buffer was injected into a flow cell of an SA sensor chip at a flow rate of 5 μl/min until the desired amount of the biotinylated HA was captured. The control surface was also generated using the same procedure by replacing the biotinylated HA with running buffer. Different concentrations of recombinant proteins, diluted in the BiAcore running buffer, were injected over protein or HA-captured surface for 90 s for association, followed by a 300-s-long running buffer injection for dissociation. Parallel injections of analytes over a control surface were always performed for background measurements. The protein-captured surface was regenerated for subsequent injections by two successive injections of 100 μl of 10 mM HCl. The HA-captured surface was regenerated for subsequent injections by two successive injections of 100 μl of 25 mM HCl.

SPR Studies on the Interactions of VG1 and AG1 as LP Complexes with HA—A solution of 500 nM VG1 or AG1 was mixed with a solution of 500 nM HA in PBS. Serial 2-fold dilutions of the starting solution were made, and each solution was then injected over biotinylated HA captured on a flow cell or over a blank control flow cell. The preincubation time of VG1 or AG1 with LP prior to their injection on the HA surface was at least 1 h. VG1, AG1, and LP at different concentrations (250, 125, and 62.5 nM) were injected over a pair of flow cells for comparison.

BiAcore Data Analysis—Data were prepared using the SPRevolution software package (www.irb.bri.cnrc-nrc.gc.ca/dh/health/receptors/equipment_e.html). Sensorgrams generated using a control surface were subtracted from the corresponding experimental sensorgrams.
and the resulting curves were transformed to concentration units. All curves were reduced to 500 evenly spaced sampling points. For each set of individual curves, corresponding to injections of various concentrations of protein over the same surface, integration was carried out by fitting the data to the simple binding model. Association ($k_{on}$) and dissociation ($k_{off}$) rate constants were determined directly from the integration. The apparent dissociation equilibrium constant ($K_d$) was calculated as the $k_{off}/k_{on}$ ratio.

The schematic representations of the protein binding were prepared using the BIAevaluation software (version 3.1). The calculation of the association equilibrium constant ($K_a$) by Scatchard analysis was done by determining the slope of the maximum arbitrary resonance units ($RU_{max}$) reached at each concentration divided by the concentration, as a function of $RU_{max}$-$K_a$ is equal to $1/K_a$.

**RESULTS**

**Expression and Characterization of Recombinant Proteins**

The G1 domains of aggrecan (AG1) and versican (VG1) and LP were expressed as His-tagged recombinant proteins with their own signal peptide. The recombinant proteins were purified. Generally, 4 mg of recombinant VG1 and 1.5 mg of AG1 were obtained per liter of the medium; however, the yield of LP was so low that it was hard to obtain enough material for further studies. To improve expression and secretion efficiency, the LP signal sequence was replaced with the honeybee melittin signal sequence. This approach gave up to 500 µg of protein/liter of supernatant. The purity of the recombinant proteins was demonstrated by SDS-PAGE and silver staining. The recombinant proteins were also characterized by Western blotting using a panel of antibodies (Fig. 1). N-terminal sequencing analysis showed that recombinant VG1 has a single N-terminal sequence (LHKVKVGKSPP—) identical to that reported for the mature core protein, that the recombinant AG1 preparation contained two N-terminal sequences: VTVETSDHDNSLSV— and VETSDHDNSLSV—, representing the mature core protein and cleavage two residues downstream. For the two LP species, the slower migrating component gave an N-terminal sequence beginning DPDHLSDNYTL—, which corresponds to the native protein plus two additional residues (DP). These two extra residues were introduced by the replacement of the LP signal peptide with the honeybee melittin signal peptide. The faster migrating component contained an N-terminal sequence (IQANGH—) beginning with residue 17 of the mature LP. The molecular sizes of the three recombinant proteins on SDS-PAGE are larger than predicted for the proteins alone and are

![Fig. 1. Characterization of recombinant proteins by SDS-PAGE.](image)

![Fig. 2. Demonstration of VG1, AG1, LP, and HA interactions by ELISA.](image)
Analysis of G1, LP, and HA Interactions by ELISA and Slot Blotting

To determine whether VG1 binds to LP and HA in a manner similar to AG1, an ELISA was performed, where LP or HA was immobilized, and the ability to bind soluble biotinylated VG1 or AG1 was tested. Both LP and HA bound biotinylated VG1 (Fig. 2A) and AG1 (Fig. 2B) in a dose-dependent manner. In contrast, no binding of biotinylated VG1 or AG1 to ovalbumin was detected. The inverse assay was performed where VG1 or AG1 or HA was immobilized, and the binding of soluble biotinylated LP was tested (Fig. 2C). Immobilized VG1 and AG1 both bound soluble biotinylated LP in a dose-dependent manner. The ability of LP to bind VG1 appeared to be stronger than for AG1 (EC_{50} = 60 ng/ml for VG1 versus >200 ng/ml for AG1), and the binding affinity of VG1 for HA was greater than that of AG1 (EC_{50} = 250 ng/ml for VG1 versus >1,500 ng/ml for AG1).

The slot blot assay indicated that the incubation of VG1 and AG1 with β-mercaptoethanol completely abolished the binding activity to immobilized LP (Fig. 3). A reverse experiment indicated that the incubation of LP with β-mercaptoethanol also completely abolished its binding activity to either immobilized VG1 or AG1 (data not shown). These results demonstrate that disulfide bonds in VG1 and AG1 and LP are critical for maintaining intermolecular interactions.

BIAcore Analysis of Binding Affinities

G1-LP Interaction—To investigate further the binding of VG1 and AG1 to LP, real time interactions of AG1 and VG1 with LP were analyzed by SPR. In a classical BIAcore experiment, one of the binding partners is immobilized in the matrix of a sensor chip surface, and the other interactant is injected over that sensor chip surface. As the injection is proceeding, a mass accumulation of the analyte (the injected species in BIAcore terminology), as it binds to the ligand (the immobilized species), causes an increase in the refractive index of the interfacing medium at the surface, and this is recorded in RU. The signal is proportional to the mass accumulation of the analyte. This corresponds to the wash-on phase of the experiment. The analyte solution is then replaced by buffer, and dissociation of the surface complexes is recorded (the wash-off phase). These steps generate a sensorgram, and from a set of sensorgrams at different analyte concentrations it is possible to derive kinetic parameters. In our experiment, the recombinant VG1 or AG1 (analyte) were flowed over immobilized recombinant LP (ligand). Experiments were performed at a flow rate of 100 μl/min to avoid any mass transport limitation. Parallel injections of VG1 or AG1 over a control surface on which no protein was immobilized were always performed as a reference. Fig. 4 shows representative overlays of sensorgrams of the binding of LP to VG1 (Fig. 4A) and to AG1 (Fig. 4B). Each set of curves represents injections of different concentrations of VG1 or AG1 over the same pair of flow cells (immobilized LP or control). The kinetic data (Table II) indicated that the association of VG1...
with LP is 4.8-fold faster than AG1 with LP, and the dissociation rates are similar for both VG1 and AG1. This resulted in the apparent dissociation equilibrium constant for VG1 being 4.6-fold lower than for AG1.

**HA Interactions**—Biotinylated HA was captured onto an SA sensor chip surface. A control surface was generated by carrying out the same procedure but replacing biotinylated HA with running buffer. Fig. 5 shows representative overlay of sensorgrams of the binding of VG1 to HA (Fig. 5A), AG1 to HA (Fig. 5B), and LP to HA (Fig. 5C). The kinetic data (Table II) indicate that the association of VG1 with HA was 12-fold faster than AG1 and 5.4-fold faster than LP, and dissociation of LP from HA was 12.5-fold slower than VG1 and 5.3-fold slower than AG1. This resulted in the apparent dissociation constant for LP being the lowest and for AG1 being the highest. The results obtained for the interaction of VG1 binding to HA were also analyzed by Scatchard plot (data not shown). The calculations estimated the dissociation equilibrium constant $K_d$ to be 40 nM, which is in good agreement with the $K_d$ determined by global analysis using a simple model (Table II).

**Aggregate Interactions**—To study the ability of LP to stabilize the interaction of versican and aggrecan with HA, VG1 or AG1 that had been preincubated with LP was injected over biotinylated HA. Sensorgrams were compared with those from the injection of VG1, AG1, and LP alone. In Fig. 6 A and B, curve 1 represents the injection of 250 nM VG1 (Fig. 6A) or AG1 (Fig. 6B). Curve 2 represents the injection of 250 nM LP. Curve 3 represents the injection of VG1 preincubated with LP (250 nM each, Fig. 6A) or AG1 preincubated with LP (250 nM each, Fig. 6B). Curve 4 represents the sum of curve 1 plus curve 2. The difference between curve 3 and curve 4 reflects the effect of interactions between VG1 (or AG1) and LP on the binding of both to HA. For VG1, the dissociation phases differed between curves 3 and 4 (Fig. 6A), indicating that LP primarily stabilizes

| Table II |
| Association ($k_{on}$) and dissociation ($k_{off}$) rate constants and equilibrium dissociation constants ($K_d$) for the interactions of VG1 and AG1 with link protein and VG1, AG1, and link protein with hyaluronan. |
| The values are the averages ± S.D. of six independent experiments done on two different link protein surfaces and four determinations done on two different hyaluronan surfaces. |
| $k_{on}$ | $k_{off}$ | $K_d$ |
| $\text{mM}^{-1} \text{s}^{-1}$ | $\text{s}^{-1} \times 10^{-2}$ | nM |
| Link protein | VG1 | 81.3 ± 5.1 | 1.30 ± 0.06 | 16.0 ± 1.2 |
| | AG1 | 17.0 ± 0.8 | 1.24 ± 0.03 | 72.7 ± 3.9 |
| Hyaluronan | VG1 | 351 ± 7 | 5.87 ± 0.10 | 16.7 ± 0.3 |
| | AG1 | 29.2 ± 0.7 | 2.51 ± 0.06 | 85.7 ± 2.0 |
| | Link protein | 65.4 ± 1.6 | 0.47 ± 0.02 | 7.2 ± 0.2 |

**Fig. 5.** Evaluation of VG1, AG1, and LP interaction with HA by BIAcore analysis. Biotinylated HA (122 RU) was captured by streptavidin onto an SA sensor flow cell. Various concentrations of VG1, AG1, or LP were injected over the HA-immobilized flow cell. Results from parallel injections of each concentration over a control flow cell were subtracted from the experimental data. Representative sensorgrams are depicted for VG1 (A), AG1 (B), and LP (C) injections.
the interaction of VG1 and HA. For AG1, the difference between curves 3 and 4 was evident throughout both the association and dissociation phases (Fig. 6B), suggesting that LP both promotes the association and reduces the dissociation of AG1-HA complexes. It is apparent that under the conditions used in this study, LP does not irreversibly stabilize the association of either VG1 or AG1 with HA; rather, a slow dissociation is observed.

DISCUSSION

The baculovirus expression system used in this study gave yields of VG1 and AG1 in the range of 1–4 mg/liter, whereas LP was recovered in much lower amounts when the constructs contained their endogenous signal peptides. A low yield of LP in this expression system has been observed previously (15). Considerable improvement of the LP yield was obtained when an insect signal peptide was used to replace the endogenous mammalian sequence. The beneficial use of the insect signal peptide has been reported previously for other proteins (11). The recombinant VG1 and AG1 were recovered as intact proteins by N-terminal sequence analysis and size evaluation on SDS-PAGE, whereas the LP showed evidence of partial proteolytic processing at its N terminus. The cleavage site observed is the same as that generated by mammalian matrix metalloproteinases (16), which have not been reported to modify the G1 domain of aggrecan or versican. It is not clear in the insect cells which protease is responsible for this cleavage. However, there is no evidence that N-terminal truncation of LP alters its ability to interact with G1 domains or HA. It was also apparent that the insect cells generated G1 and LP possessing disulfide bonds that are essential for their functions, as occurs in the native proteins (17), and that they are glycosylated.

All recombinant proteins used for analysis were analyzed at concentrations of less than 3.2 μM (about 160 μg/ml), to minimize the possibility of self-association interfering with the analyses. This is of particular concern in the case of LP where oligomerization in solution, which ultimately results in insolubility, has been reported (18). In our case the recombinant proteins were all fully soluble at the concentrations used. Furthermore when BIAcore analysis was used to assess the interaction of soluble LP with LP immobilized on a sensor chip, there was no evidence for any interaction (data not shown).

It has been shown previously that the aggrecan G1 domain and LP can interact with each other and independently with HA (19, 20). Furthermore, it was demonstrated that the presence of cooperative interactions results in the formation of a stable ternary complex (21, 22). The present study demonstrates that the interaction of VG1 with either LP or HA is higher affinity than that of the AG1. In both cases this appears to be caused by a higher rate of association. It was also apparent that of all three proteins studied, LP has the highest affinity binding for HA, which is the result of its much lower rate of dissociation. Previous BIAcore studies had also indicated that the equilibrium dissociation constant for LP binding to HA was lower than that for AG1 binding to HA (23). These results are in accord with studies demonstrating the relative insensitivity of the LP-HA interaction to changing pH from 7 to 5, in contrast to the decreased binding observed for aggrecan-HA interaction over the same pH range (24). In addition, earlier studies using equilibrium dialysis and frontal gel chromatography had determined equilibrium dissociation constants for LP-HA and aggrecan-HA complexes to be in the 10–100 nM range, respectively (25, 26), which agrees well with the present study.

Very recently a BIAcore-based study has also compared the interaction of recombinant VG1 and AG1 with LP and HA (27). Their values for the equilibrium dissociation constants for the interaction of VG1, AG1, and LP with HA are all in the same range and order as those shown in Table II, i.e. the equilibrium dissociation constants of the interaction with HA increase in the order, LP < VG1 < AG1. However, their results for the interaction of VG1 and AG1 with LP are different from those reported in the present study. Although both studies determined a similar equilibrium dissociation constant for the AG1 interaction with LP, in our study we found that the affinity of
VG1 for LP was higher than that of AG1, whereas Matsumoto et al. (27) reported that the affinity of VG1 for LP is less than that for AG1. There are several potential reasons for this discrepancy, including the use of different sensor chips for BIAcore analysis and expression of recombinant proteins from different cell types. The B1 sensor chip used to study LP binding in the present work eliminated most of the nonspecific binding observed with the use of the CM5 chip and was therefore found to be a more reliable system. Variation could also arise from differences in recombinant protein structure, particularly glycosylation, resulting from the use of alternate expression systems. In this regard one might expect that the glycosylation pattern most resembling the native structure would result in the greatest binding affinity. It is therefore interesting to note that the equilibrium dissociation constant for the VG1-LP interaction obtained with the insect cell system in the present work was lower than that obtained with the human embryonic kidney 293 cell system. It is impossible to say whether either system generates the native glycosylation pattern as this may vary with age, site etc.

It is apparent from the present studies that the complexes formed upon LP stabilization of VG1 or AG1 interaction with HA are not irreversible under the conditions of BIAcore analysis. This is not too surprising for any noncovalent interaction under the conditions of infinite dilution present during the wash-off phase of BIAcore analysis, when all noncovalent interactions should show some degree of dissociation, however small. The level of dissociation apparent in our work is consistent with that depicted in the paper by Matsumoto et al. (27). At first sight this would appear to contradict classical studies of proteoglycan aggregate stabilization by LP toward HA oligosaccharide-mediated dissociation (5, 28, 29). This apparent discrepancy could be caused by the high sensitivity of BIAcore analysis for monitoring low levels of dissociation.

The differences in the ability of LP to interact with the versican and aggrecan G1 domains and stabilize their complexes raise the question of whether LP was indeed designed to function with both proteoglycans. This issue is of particular relevance at the present time because analysis of the human genome sequence has revealed the existence of four members of the LP family, with each LP gene residing adjacent to one of the members of the aggrecan family of proteoglycans (aggrecan, versican, brevican, and neurocan) (30). Perhaps surprisingly, the gene coding the cartilage LP used in this study resides adjacent to the versican gene (rather than that of aggrecan), even though aggrecan is by far the most abundant aggregating proteoglycan in cartilage. It is therefore possible that the cartilage LP is in fact designed to interact preferentially with versican because this proteoglycan and LP are present at low abundance in most tissues. This may explain the higher affinity of LP for this proteoglycan. At present there is no evidence for the presence of the LP that partners the aggrecan gene in cartilage, and there is no information on its ability to interact with the different proteoglycans. Interestingly, the LP gene that partners the brevican gene on chromosome 1 is expressed in brain (31), where brevican is preferentially located (32).

The present study shows that there are differences in the ability of the cartilage LP to interact with the versican and aggrecan G1 domains and stabilize the G1-HA interactions. For this in vitro observation to be of physiological significance, it is necessary that the proteoglycan-bound G1 domains behave in a manner similar to their free counterparts and that other components of the extracellular matrix do not influence these interactions. In addition, tissue concentrations of the proteoglycans can be much higher than those used in this in vitro work, particularly in the case of aggrecan which is present in the 10–20 μM range in human articular cartilage. Again, it is not clear whether the differences between G1 interactions will persist at the higher physiological concentrations. Although further studies will be required to validate the physiological relevance of these observations, it is clear that the versican and aggrecan G1 domains were not created to be functionally equivalent under all conditions.

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