G3 Domains of Aggrecan and PG-M/Versican Form Intermolecular Disulfide Bonds that Stabilize Cell−Matrix Interaction†

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ABSTRACT: The extracellular matrix plays a critical role in maintaining tissue integrity. Among the matrix molecules, the large aggregating chondroitin sulfate proteoglycans are the major structural molecules and are the primary contributors to the stability for some tissues such as cartilage. The notable exceptions are nanomelic cartilage and arthritic cartilage: the former contains a point mutation leading to a stop codon before translating to the C-terminal G3 domain; the latter contains a large proportion of aggrecan from which the G3 domain has been cleaved. These phenomena suggest that the G3 domain may be important in cartilage stability. Here, we demonstrated for the first time that the G3 domains of aggrecan and another proteoglycan, PG-M/versican, formed intermolecular disulfide bonds, and all subdomains were involved. Further studies indicated that each of the 10 cysteine residues of the aggrecan G3 domain could potentially form intermolecular disulfide bonds in vitro. The disulfide bonds were disrupted in the presence of reducing reagent β-mercaptoethanol and dithiothreitol. As a result, normal chondrocyte−matrix interaction was disrupted, and the structure of the extracellular matrix was altered. Furthermore, disruption of disulfide bonds also reduced the role of PG-M/versican G3 domain in mediating cell adhesion. Our study provides strong evidence of the importance of proteoglycan interactions through intermolecular disulfide bonds in cartilage firmness and cell−matrix stability.

Proteoglycans and hyaluronan are important components in the extracellular matrix (ECM)[1] of cartilage. Proteoglycans are a family of glycoconjugates with a central core protein to which one or more glycosaminoglycan side chains are covalently linked posttranslationally. Most of the proteoglycans exist as aggregates formed by the noncovalent association of proteoglycan with hyaluronan and link protein (*1*, *2*). The major proteoglycan in cartilage is aggrecan, which is a member of the large aggregating chondroitin sulfate proteoglycan family that also includes PG-M/versican (*3*−*5*), neurocan, and brevican (*2*). A common feature of these large aggregating chondroitin sulfate proteoglycans is their structural similarity. Their core proteins are composed of an N-terminal G1 domain, a sequence for chondroitin sulfate (CS) modification, and a C-terminal G3 domain (*2*, *4*−*6*). G1 follows the signal peptide and is composed of three subdomains: one IgG-like and two tandem repeats. CS is a large extended region (situated in the middle) for glycosaminoglycan chain attachment (*4*, *6*). G3 domain makes up the carboxyl-terminal end. It is composed of folded modules including alternatively spliced epidermal growth factor-like motifs, a carbohydrate recognition domain (CRD), a complement binding protein (CBP)-like domain, and a short carboxyl terminal tail (*5*−*7*).

Aggrecan provides much of the resilience and load-bearing strength of cartilage. Loss of aggrecan is a major feature of cartilage degradation associated with arthritis (*8*−*10*), which is characterized by age-related slow progressive deterioration of cartilage (*11*). There is evidence that aggrecan G3 domain may be important in maintaining functional cartilage. The G3 domain is known to be lost from aggrecan during metabolism, perhaps because of proteolytic activities (*12*, *13*). Using rotary shadowing electron microscopy, it was shown that this domain is present in the majority of aggrecan isolated from young tissues; however, G3 was often missing in aggrecan prepared from adult tissues (*14*). It was later confirmed that a decrease in G3 content in human articular cartilage is age-related (*15*). As arthritic diseases are also age-related, it seems likely that a decrease in G3 content may be involved in the development of arthritis. In particular, we speculated that the G3 domain may play a role in determining the stability of cartilage structures. This study aimed to investigate the effect of the G3 domain on chondrocyte−matrix interaction. We demonstrated that the G3 domain forms intermolecular disulfide bonds, which are important in maintaining the stability of ECM−cell interactions.

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[1] Abbreviations: ECM, extracellular matrix; CS, chondroitin sulfate (chain attachment sequence); G3, the globular domain in the carboxyl terminus of aggrecan or selectin-like domain; CRD, carbohydrate recognition domain; CBP, complement binding protein; IgG, immunoglobular domain; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DTT, dithiothreitol.

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EXPERIMENTAL PROCEDURES

Materials. Lipofectin, Dulbecco's modified Eagle's medium (DMEM) culture medium, fetal bovine serum (FBS), Hank's balanced salt solution, Fungizone, trypsin/EDTA (Cat. No. 15305-014), and penicillin/streptomycin were purchased from Invitrogen. The ECL Western blot detection kit was from Amersham. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Sigma. The DNA Midi-prep kit and Ni-NTA agarose were from Qiagen Inc. Tissue culture plates (60 mm, 6-well and 24-well) were from Nunc Inc. All chemicals were from Sigma. COS-7 cells and astrocytoma cell line U87 were purchased from American Type Culture Collection. Chicken chondrocytes were isolated from the sterna of 18-day chicken embryos. Briefly, chicken sterna from 18-day embryos (Shaver Poultry Breeding Farms Ltd., Cambridge, Canada) were removed and rinsed with PBS. Each sternum was cut into pieces and incubated in dissociation medium (0.3% collagenase in HBSS) at 37 °C for 30 min. The dissociation medium was changed, and the incubation continued for a further 1.5 h. An equal volume of medium (DMEM containing 10% FBS) was added to stop the enzymatic reaction. Cells were collected by passing through cell strainers (40 μm) and centrifuged at 1100 rpm for 10 min. The newly isolated chondrocytes were grown as suspension cultures in bacterial Petri dishes.

Recombinant Construct Generation and Expression. In this study, a total of 29 recombinant constructs were used. Most have been described in our previous studies. The mini-aggrecan (G1CSG3), originally described in ref *30*, contains the leading peptide, which is composed of a link protein signal peptide and an epitope recognized by the monoclonal antibody 4B6 (*31*), and the aggrecan G3 domain with the carboxyl tail in pcDNA3. The construct aG3 comprises the CRD motif, the CBP motif, and a short tail (*30*). The constructs CRD, CRDC2C3C4C5, CRDC1C2C3, CRDC4C5C6, CRDC3C4, CRDC1C3, CRD4C6, CRDC4C5, CRDC4, CBPtail, CBP, CBPC1C2C4, CBPC2C3C4, CBPC1C2, CBPC2C4, CBPC2C3, CBPC2, and G1CSCRD are described in ref *16*. The structures of vG3, vG3DEGF, vCRD, and vCBP have also been published previously (*28*, *32*−*34*).

The final four constructs used were LPCSG3, LPG3, LPIgG, and HisG3. To generate LPCSG3, the sequence for the leading peptide and G1 domain of the mini-aggrecan construct was replaced with link protein cDNA. Link protein cDNA was synthesized with two primers (5′aaagaattcgccgccaccatggcaagt, named LPNKozak*Eco*RI, and 5′atacgggatccgttgtaagctctgaaacagta), while the CS sequence was amplified with the primers 5′ ccatccgggatcccgtatttcagcgga and 5′ aaactcgagagtggcagcagtgtcagtgct. The G3 domain was isolated from the G1CSG3 construct by digestion with *Xho*I and *Xba*I. LPG3 was generated by connecting link protein cDNA and the G3 domain, in which the link protein cDNA was amplified by the primers LPNKozak*Eco*RI and 5′atactcgaggttgtaagctctgaaacagta. LPIgGG3 was produced by replacing the link protein cDNA with link protein IgG-like motif, which was synthesized with the primers LPNKozak-*Eco*RI and 5′ aaaggatccttccaaattcagagctac.

The HisG3 construct was generated by ligating two fragments, the leading peptide containing a His-tag and the G3 domain, into pcDNA3. The leading peptide containing a His-tag was produced using two primers, 5′aaactcgaggtgatggtgatggtgatgagatcctctcattaggcggggtccattttc named LP40Chis-*Xho*I and LPNKozak*Eco*RI, and a link protein as a template. The PCR product was doubly digested with *Eco*RI and *Xho*I, purified, and ligated with the G3 fragment, which was derived by digesting the G1CSG3 construct with *Xho*I and *Xba*I, into pcDNA3.

COS-7 cells were cultured in 6-well plates at a cell density of 2×10^5 cells/well. The cells were allowed to attach and grow overnight in DMEM supplemented with 5% FBS. The following day, cells were subjected to gene expression as described (*35*−*37*).

Western Blot. Samples containing recombinant products were subjected to SDS−PAGE electrophoresis. The stacking gel contained 4% polyacrylamide and the separating gel, 5%. In some cases, higher concentration gels were used as described elsewhere. The buffer system is 1x TG (Amresco) containing 1% SDS. In reducing conditions, protein samples were loaded directly to the gels for electrophoresis. Reducing loading dye (1x) contained 50 mM Tris-Cl, pH 7.2, 2% SDS, 10% glycerol, and 0.02% bromophenol blue. In nonreducing conditions, protein samples were prepared in nonreducing loading dye (1x loading dye containing 125 mM Tris-Cl, pH 6.8, 2 M deionized urea, 4% SDS, and 0.024% bromophenol blue), followed by electrophoresis as above. To obliterate any trace amount of reducing agent that might be present in the electrophoresis system, we occasionally added H_2O_2 to the electrophoresis buffer to a final concentration of 0.3%, followed by pre-electrophoresis at 100 V for 30 min. We expected that after the pre-electrophoresis, the oxidative capacity of H_2O_2 was ruined. With this treatment, the patterns of protein bands were improved somewhat. After the pre-electrophoresis, protein samples were loaded. Proteins separated on SDS−PAGE were transblotted onto a nitrocellulose membrane (Bio-Rad) in 1x TG buffer (Amresco) containing 20% methanol. The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% nonfat dry milk powder (TBSTM) for 1 h at room temperature and then incubated at 4 °C overnight with the monoclonal antibody 4B6, which recognizes an epitope in the leading peptide of all constructs, prepared in TBSTM. The membranes were washed with TBST (3×30 min) and then incubated for 1 h in TBSTM with goat anti-mouse antibody conjugated to horseradish peroxidase. After washing as above, the bound antibody was visualized with an ECL kit according to the manufacturer's instructions.

Ni-NTA Purification. Culture medium was harvested from COS-7 cells transiently cotransfected with vG1CSG3 and HisG3, vG1CSG3 and pcDNA3 vector, LPCSG3 and HisG3, or LPCSG3 and pcDNA3 vector. The HisG3 product contains a His-tag that binds to Ni-NTA resin. Culture medium from each transfected culture was harvested and passed through the Ni-NTA column in native conditions or denaturing conditions according to the manufacturer's instructions (Qiagen Inc. purification system). The loaded columns were washed extensively. After washing, the bound products were eluted. The eluate was precipitated with TCA, boiled in 1x protein loading dye for 5 min, and subjected to Western blot analysis probed with the monoclonal antibody 4B6 that recognizes the leading peptide in all recombinant products.

Treatment with Reducing Agents. The effects of β-mercaptoethanol on the formation of intermolecular disulfide bonds were examined in cells expressing recombinant constructs. Briefly, COS-7 cells transiently transfected with aG3, aCRD, or aCBP or U87 cells stably transfected with vG3, vG3ΔEGF, vCRD, or vCBP were treated with 40 mM β-mercaptoethanol for 0, 1, 5, 10, or 20 min. Culture medium was harvested, mixed with nonreducing loading dye, and analyzed on Western blot in nonreducing conditions probed with 4B6. U87 cells stably transfected with vG3, vG3ΔEGF, vCRD, or vCBP were also treated with β-mercaptoethanol at 0, 0.5, 1, 2, 5, 10, 20, or 40 mM for 10 min. The treated culture media were analyzed on Western blot in nonreducing conditions probed with 4B6. COS-7 cells transiently transfected with G3 were also treated with dithiothreitol (DTT) at 0, 4, 8, 15, 28, or 48 mM for 10 min. The treated culture media were analyzed on Western blot in nonreducing conditions probed with 4B6.

Culture medium from G3-transfected cells was also subjected to treatment as follows: (i) The medium was treated with 28 mM DTT at room temperature for 1 h. (ii) H_2O_2 (50 mM) was added to the DTT-treated medium and allowed a further incubation of 1 h. (iii) Iodoacetic acid was added to the DTT-treated medium to a final concentration of 0.2 M and allowed a further incubation of 1 h. (iv) H_2O_2 (50 mM) was added to the DTT/iodoacetic acid-treated medium and allowed a further incubation of 1 h. The treated products in (iii) and (iv) were dialyzed against PBS in a cold room overnight. The above products were analyzed on Western blot in nonreducing conditions.

Suspension chondrocytes were isolated from chicken embryos as described previously (*18*, *38*). For culture splitting, cells along with medium were passed to new plates followed by addition of fresh medium. This method minimized the disruption of cell−matrix interaction. To test the effect of reducing agents on cell−matrix interaction, chondrocytes (2×10^5 cells) were inoculated to new plates and maintained for at least 3 days. The cultures were then incubated with or without 40 mM β-mercaptoethanol or 28 mM DTT for 10 min in 6-well plates, followed by centrifugation at 3700 rpm at room temperature for 30 min. Culture media was carefully removed by absorption, leaving the chondrocytes and the matrix network surrounding the cells attached to the plates with minimal disturbance. After air-drying, cells/matrix were fixed with 75% ethanol (pre-chilled at −20 °C) for 10 min followed by 4% paraformaldehyde for 10 min. The fixed cells/matrix were washed with 3% glacial acetic acid and then stained with 0.5% alcian blue in 3% glacial acetic acid for 1 h. The stained cells/matrix were destained with 3% glacial acetic acid until the background was clear and briefly rinsed with PBS. The staining of glycosaminoglycan chains with alcian blue was examined and photographed under an inverted light microscope.

Cell Attachment Assays. Culture medium from versican G3- and vector-transfected cells were treated with 28 mM DTT at room temperature for 1 h. Ammonium sulfate was added to the treated or untreated medium to a final concentration of 50% saturation, followed by an incubation on ice for 30 min and pelleted at 10 000*g* at 4 °C for 30 min. The pellets were resuspended in PBS in the same volume as the original medium. The solution was used to coat 15 × 60 mm Petri dishes at 4 °C overnight. The solution was removed, and the dishes were inoculated with 10 mM-EDTA-harvested U87 cells or NIH3T3 fibroblasts. The cells were incubated in the plates at 37 °C for 20 (U87 cells) or 30 (NIH3T3 cells) min. Unattached cells were removed, and the plates were washed with PBS three times. Cell attachment was examined under light microscopy and photographed. Six areas were randomly selected for cell counting.

RESULTS

Effect of G3 Domain on the Formation of Intermolecular Disulfide Bonds. We designed experiments to examine whether the aggrecan G3 domain could form intermolecular disulfide bonds. The G3 domain, or part of the domain, was linked with a fragment modified by chondroitin sulfate (CS) chains, the aggrecan G1 domain, or link protein (LP) that is structurally similar to the G1 domain, producing the recombinant constructs G1CSG3, G1CSCRD, LPCSG3, LPG3, and LPIgGG3. The structures of these constructs are shown in Figure 1, and generation of these constructs is described under Experimental Procedures. Products from COS-7 cells transfected with these constructs were analyzed on Western blot, and the experiments indicated that, in reducing conditions, all products migrated as major bands or with limited diffusion (Figure 1). A small amount of diffusion was expected since these recombinant products were expected to undergo modification by glycosylation and glycosaminoglycan chain attachment. However, in nonreducing conditions, all products migrated as multiple bands, in sizes corresponding to monomers, dimers, trimers, tetramers, and so on (Figure 1). This suggests that these products interact with each other covalently since the interaction was stable even after boiling in the presence of SDS.

Formation of intermolecular disulfide bonds was further examined in the G3 domain. COS-7 cells were transfected with four constructs, aG3, HisG3, aCRD, and aCBPtail; the former two contain full-length G3, and the latter two contain one of the G3 subdomain (Figure 2A). Product analysis confirmed that all of these products migrated as multiple bands in nonreducing conditions (Figure 2B) but produced only a single band in reducing conditions (Figure 2C). When samples were separated on a higher concentration gel (12%; Figure 2D), the sizes of the monomers are more easily compared. We noted that the multimeric forms of the products were not well-transferred to the membrane, and some of them had not even migrated into the gel. As a result, the intensities of larger oligomers were weak as compared to those separated in 5% gels. The above four constructs were also expressed in bovine chondrocytes, and product analysis revealed similar migration patterns (data not shown).

To further confirm that the disulfide bonds were formed intermolecularly, we coexpressed two constructs containing G3, HisG3, and LPCSG3 in COS-7 cells. As the HisG3 construct contained a His-tag, culture medium was passed through the Ni-NTA column to purify the HisG3 product. The purified product was analyzed on Western blot in reducing conditions. LPCSG3 was co-purified with HisG3 (Figure 3), suggesting the interaction of LPCSG3 with HisG3. As a control, no LPCSG3 product was obtained when the same procedure was carried out with a vector control. We further tested whether G3 domains from different members of the large chondroitin sulfate proteoglycan family

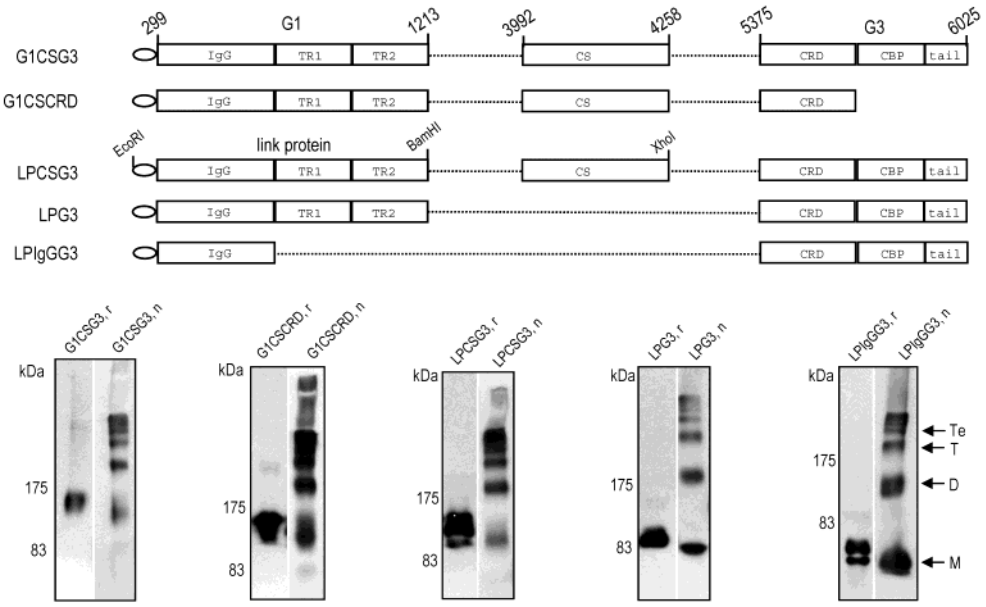

FIGURE 1: Formation of intermolecular disulfide bonds in recombinant products containing the aggrecan G3 domain. COS-7 cells were transiently transfected with the recombinant constructs G1CSG3, G1CSCRD, LPCSG3, LPG3, and LPIgGG3. Products in culture medium were analyzed on Western blot in reducing and nonreducing conditions probed with 4B6. In reducing conditions (r), all products migrated as major bands, or with some diffusion. In nonreducing conditions (n), all products migrated as multiple bands, at sizes corresponding to monomers (M), dimers (D), trimers (T), tetramers (Te), and so on.

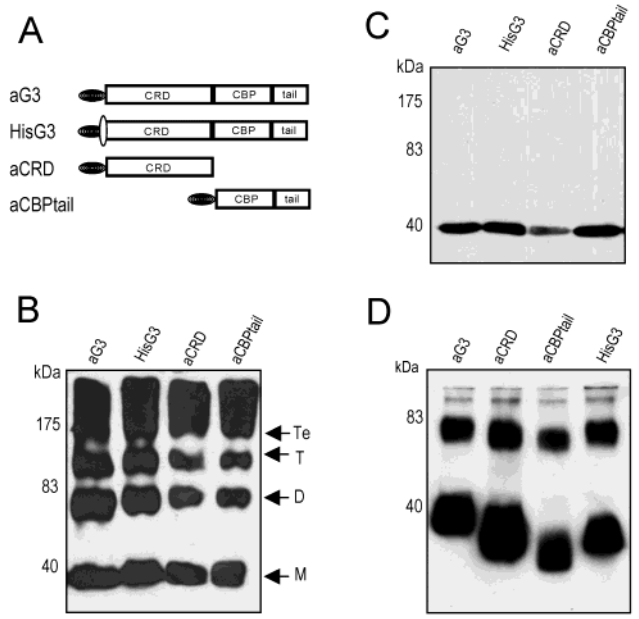

FIGURE 2: Formation of intermolecular disulfide bonds in the subdomains of G3. Four recombinant constructs, aG3, HisG3, aCRD, and aCBPtail, were transiently expressed in COS-7 cells (A). The recombinant products were subjected to 5% SDS−PAGE in nonreducing (B) or reducing (C) conditions. A 12% gel in nonreducing conditions was also used as a control (D). Proteins separated in the gels were analyzed on Western blot probed with 4B6. In reducing conditions, all products migrated as single major bands. In nonreducing conditions, all products migrated as multiple bands, at sizes corresponding to monomers (M), dimers (D), trimers (T), tetramers (Te), and so on.

could form intermolecular disulfide bonds. COS-7 cells were cotransfected with vG1CSG3 (mini-versican) and HisG3 or vG1CSG3 and a vector. vG1CSG3 did indeed co-purify with HisG3 (Figure 3), indicating the interaction between the aggrecan G3 domain and the versican construct.

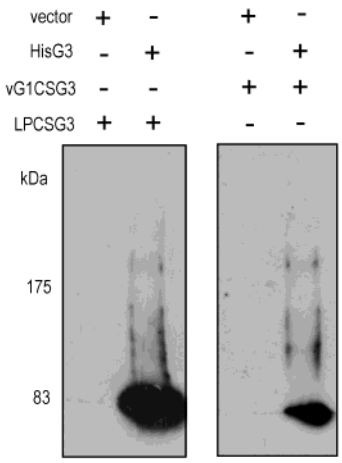

FIGURE 3: Formation of intermolecular disulfide bonds between fragments of different proteoglycans. COS-7 cells were cotransfected with vG1CSG3 and HisG3, vG1CSG3, and pcDNA3 vector, LPCSG3 and HisG3, or LPCSG3 and pcDNA3 vector. After affinity purification over a Ni-NTA column, bound products from the culture medium were eluted and analyzed on Western blot probed with 4B6. In the absence of coexpressed HisG3, neither vG1CSG3 nor LPCSG3 interacted with the Ni-NTA resin. In the presence of HisG3, both vG1CSG3 and LPCSG3 were recovered from the Ni-NTA column.

Identification of Cysteines Involved in the Formation of Intermolecular Disulfide Bonds. The aggrecan CRD motif contains six cysteine residues, and CBP contains four cysteine residues. To investigate how these cysteine residues were involved in the formation of intermolecular disulfide bonds, we expressed CRD, CRDC2C3C4C5, CRDC1C2C3, CRDC4C5C6, CRDC3C4, CRDC1C3, CRD4C6, CRDC4C5, CRDC4, CBPtail, CBP, CBPC1C2C4, CBPC2C3C4, CBPC1C2, CBPC2C4, CBPC2C3, and CBPC2 in COS-7 cells. The structures of these constructs are shown in Figure 4A, and generation of these constructs has been described previously (*16*). In reducing conditions, all products in the

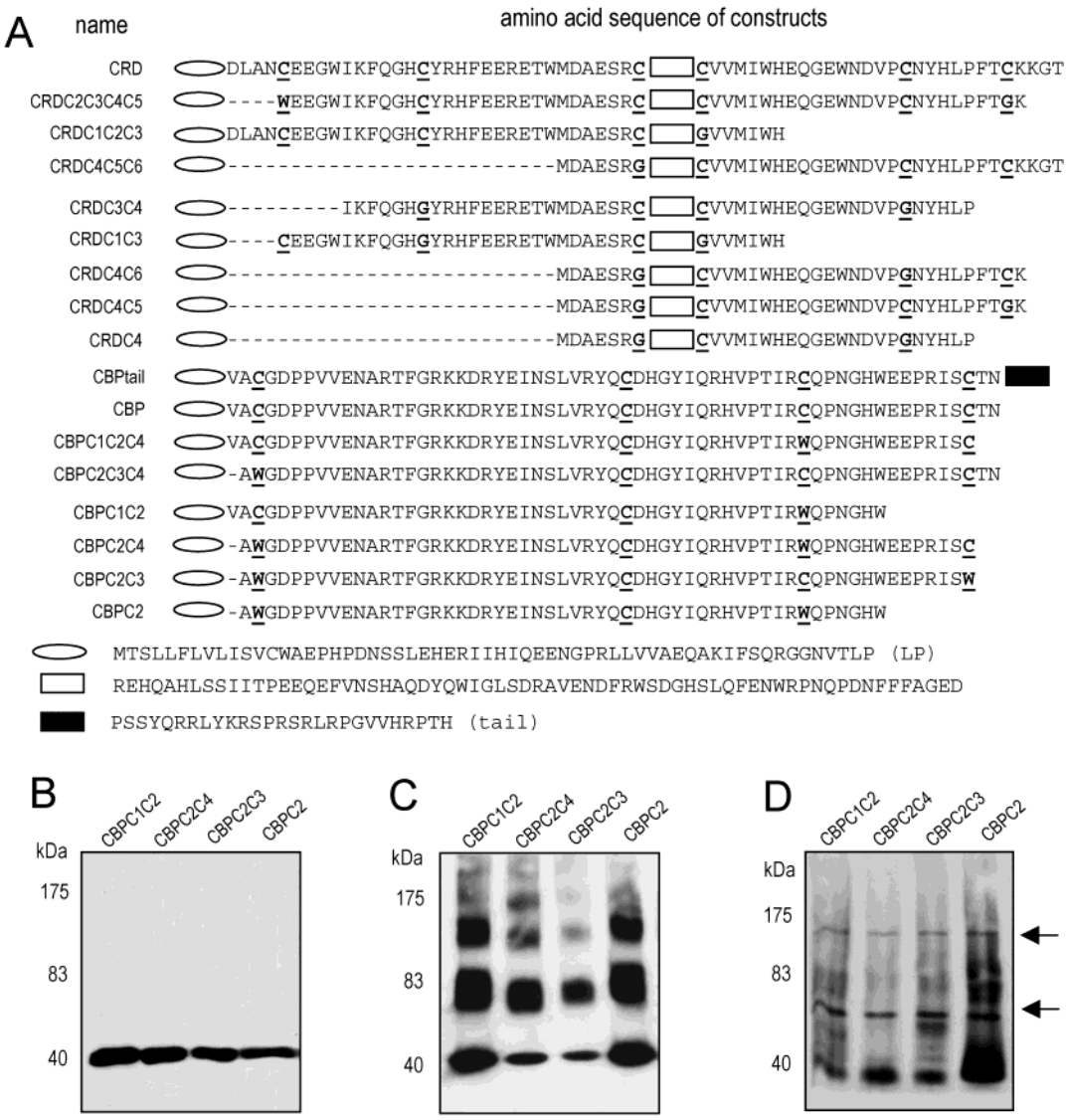

FIGURE 4: Effect of each cysteine residue on disulfide bond formation. (A) 17 constructs with or without mutations of the cysteines in the CRD or CBP motif were generated as shown. All fragments are linked to LP by the restriction endonuclease site *Xho*I, and each fragment is linked to the vector by *Xba*I. The amino acid sequences of each fragment and LP are listed. The cysteine residues are indicated in boldface, and the mutated amino acids (replacement of cysteines) are underlined. (B) Culture medium from COS-7 cells transiently transfected with the constructs indicated was analyzed on Western blot in reducing conditions probed with 4B6. All products migrated as single bands. (C) The medium was also analyzed in nonreducing conditions probed with 4B6. All products migrated as multiple bands, at sizes corresponding to monomers, dimers, trimers, tetramers, and so on. (D) Cell lysate was also prepared and analyzed on Western blot in nonreducing conditions probed with 4B6 as above. All products migrated as diffuse smears. Two distinct bands (arrows) were observed.

culture medium migrated as single bands (Figure 4B). In nonreducing conditions, the products migrated as multiple bands, at sizes corresponding to monomers and multimers (Figure 4C). This same pattern—of multiple bands on Western blot in nonreducing conditions—could not be duplicated in cell lysate. All cell-associated products migrated as diffuse smears, and two clear bands were observed (Figure 4D), which were also apparent in the control (not shown). These might represent the endogenous peroxidase. Since we did not detect any obvious difference among different mutants, only the results of some constructs are presented in Figure 4B−D. Our results suggest that all cysteine residues in the aggrecan G3 domain can form disulfide bonds, and there is no significant difference in the abilities of individual cysteines to form intermolecular disulfide bonds.

We also examined the possibility of disulfide bond formation in the G3 domain of versican. COS-7 cells were transiently transfected with constructs vG3, vG3ΔEGF, vCRD, and vCBPtail, containing different motifs of versican G3 domain as shown (Figure 5). In nonreducing conditions, all products migrated as multiple bands on Western blot, at sizes corresponding to monomers and multimers (Figure 5). In reducing conditions, all products migrated as single bands. Stable expression of these constructs in astrocytoma cells (U87) produced similar results (not shown).

Disruption of Disulfide Bonds by Reducing Reagents. To confirm that the appearance of multiple bands on Western blot was the result of the formation of disulfide bonds, we added the reducing reagent β-mercaptoethanol into the transfected cultures to disrupt the disulfide bonds. COS cells transiently transfected with aG3, aCRD, or aCBP were treated with 20 mM β-mercaptoethanol for different time intervals. Without β-mercaptoethanol treatment (0 min), all samples migrated as multiple bands as expected (Figure 6A).

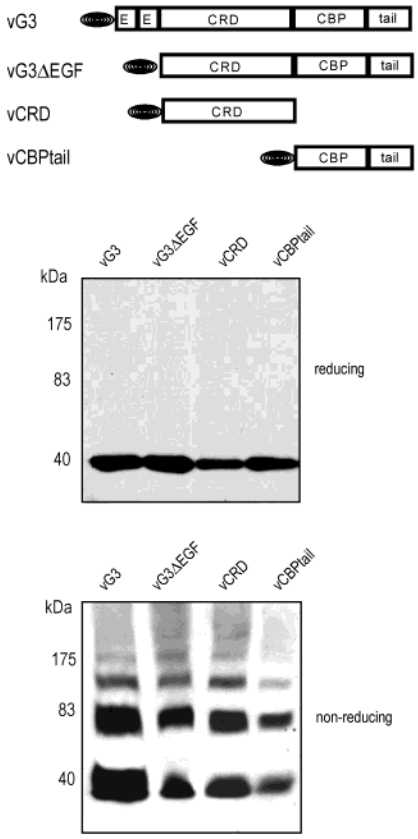

FIGURE 5: Formation of intermolecular disulfide bonds in versican G3 domains. Four recombinant constructs vG3, vG3ΔEGF, vCRD, and vCBPtail were transiently expressed in COS-7 cells, and their products were analyzed on Western blot in reducing and nonreducing conditions probed with 4B6. In reducing conditions, all products migrated as major bands. In nonreducing conditions, all products migrated as multiple bands, at sizes corresponding to monomers, dimers, trimers, tetramers, and so on.

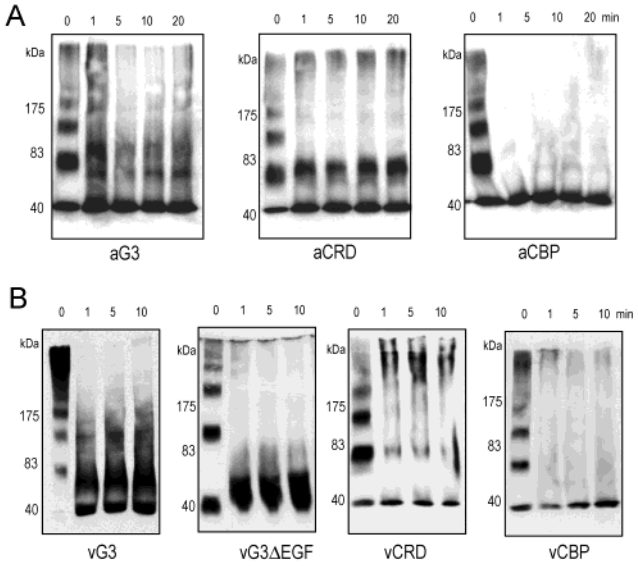

FIGURE 6: Disruption of disulfide bonds by a reducing reagent. (A) COS cells were transiently transfected with aG3, aCRD, and aCBP. Two days after transfection, the cultures were treated with 20 mM β-mercaptoethanol for 0, 1, 5, 10, and 20 min. Culture medium was analyzed on Western blot in nonreducing conditions probed with 4B6. Without β-mercaptoethanol treatment (0 min), all samples migrated as multiple bands as expected. In the presence of β-mercaptoethanol, many of the multiple bands disappeared. However, aG3 product still showed some diffuse smearing and weak bands at dimer and trimer sizes. aCRD product appeared as monomers and dimers, while aCBP product migrated mainly as a monomer single band. (B) U87 cells transfected with vG3, G3ΔEGF, vCRD, and vCBP were treated with β-mercaptoethanol for 0, 1, 5, and 10 min, and the products were analyzed on Western blot in nonreducing conditions probed with 4B6. In the absence of β-mercaptoethanol (0 min), all samples migrated as multiple bands. In the presence of β-mercaptoethanol, many of the multiple bands disappeared.

In the presence of β-mercaptoethanol, many of the multiple bands disappeared. However, the aG3 product still showed some diffuse smears and a weak dimer and trimer. The product of aCRD appeared as a monomer and dimer, while the aCBP product migrated mainly as a monomer single band (Figure 6A). Since products treated for 1 min exhibited similar results as those treated for 10−20 min, it appeared that the reagent functioned in the existing products rather than in changing cellular behavior. We then examined if intermolecular disulfide bonds formed in versican G3 domain were affected by β-mercaptoethanol. U87 cells transfected with vG3, G3ΔEGF, vCRD, or vCBP were treated with β-mercaptoethanol as above, and product analysis revealed similar results (Figure 6B).

U87 cells transfected with vG3, G3ΔEGF, vCRD, or vCBP were also treated with β-mercaptoethanol at concentrations of 0, 0.5, 1, 2, 5, 10, 20, and 40 mM. Product analysis in nonreducing conditions demonstrated that without β-mercaptoethanol treatment (0 mM), all samples migrated as multiple bands as expected (Figure 7A). In the presence of high concentrations of β-mercaptoethanol, many of the multiple bands disappeared. G3ΔEGF-transfected U87 cells were also treated with 10 and 20 mM β-mercaptoethanol for 0, 1, 5, and 10 min. Products were mainly detected as monomers and dimers when treated with 10 mM β-mercaptoethanol but were mainly detected as monomers when treated with 20 mM β-mercaptoethanol (Figure 7B). The results revealed that this reagent could affect migration behavior of the product at a concentration as low as 0.5 mM, and it is known that low concentrations of reducing agents do not affect product trafficking and cellular activities (*17*). Unexpectedly, some products seemed to aggregate in the presence of low concentrations of reducing agents.

We also examined the effect of DTT at different concentrations on the formation of intermolecular disulfide bonds and demonstrated that DTT at a concentration of 15 mM was able to disrupt disulfide bond formation (Figure 8A). To examine the responsibility of disulfide bonds for the formation of multiple bands, we added H_2O_2 to DTT-treated product. Addition of H_2O_2 reversed the effect of DTT and generated multiple bands in nonreducing conditions. While iodoacetic acid was added to the DTT-treated product, it blocked the free SH-groups, and addition of H_2O_2 could no longer produce multiple bands (Figure 8B).

Intermolecular Disulfide Bonds Are Required to Maintain a Stable ECM and Cell−Matrix Interaction. To examine the effect of a reducing reagent on the stability of matrix structure, suspension chondrocytes were treated with β-mercaptoethanol or DTT. After the treatment, chondrocytes were pelleted by centrifugation. The matrix molecules that interacted with the cells were also pelleted to the surface of the plates. Medium was removed, and the cultures were air-dried and stained with alcian blue as described (*18*, *19*). Without

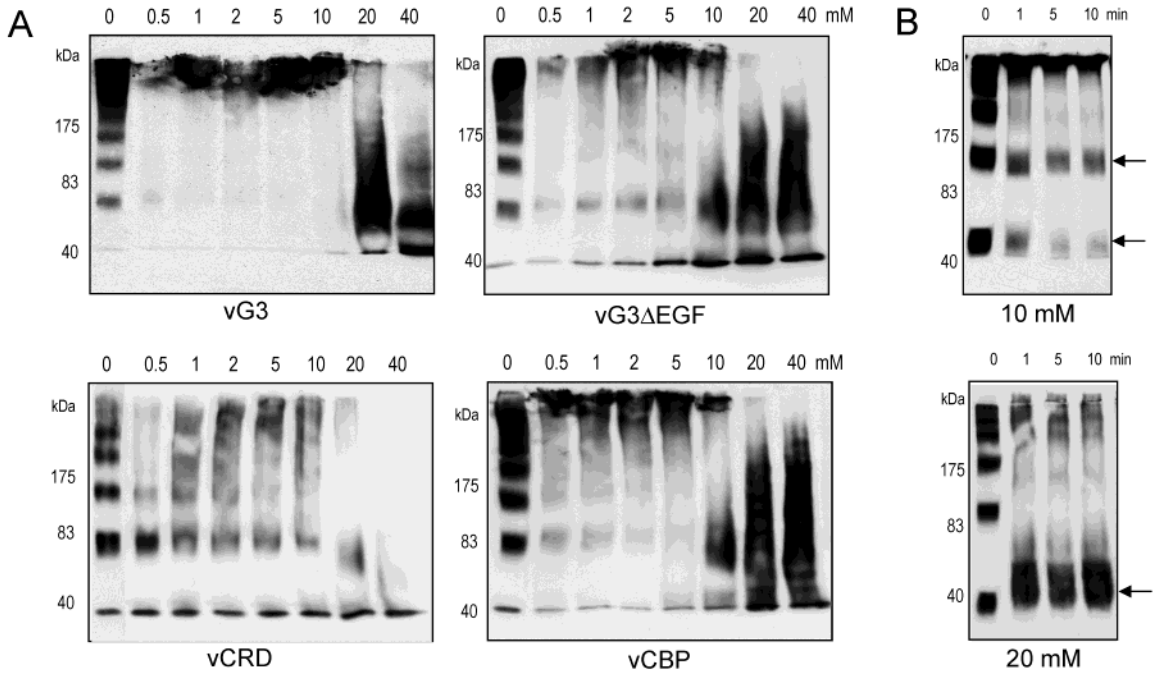

FIGURE 7: Concentration dependence of β-mercaptoethanol. (A) U87 cells transfected with vG3, G3ΔEGF, vCRD, and vCBP were treated with β-mercaptoethanol at varying concentrations (mM) as indicated at room temperature for 10 min. Culture medium was analyzed on Western blot in nonreducing conditions. Without β-mercaptoethanol treatment (0 mM), all samples migrated as multiple bands. In the presence of high concentrations of β-mercaptoethanol, many of the multiple bands disappeared. Loss of multiple bands seemed to correlate to increasing β-mercaptoethanol concentrations. (B) U87 cells transfected with G3ΔEGF were treated with 10 and 20 mM β-mercaptoethanol for 0, 1, 5, and 10 min. Culture medium was analyzed on Western blot in nonreducing conditions. Treated with 10 mM β-mercaptoethanol, products were mainly detected as monomers and dimers (arrows). Products treated with 20 mM β-mercaptoethanol were mainly detected in monomer form (arrow).

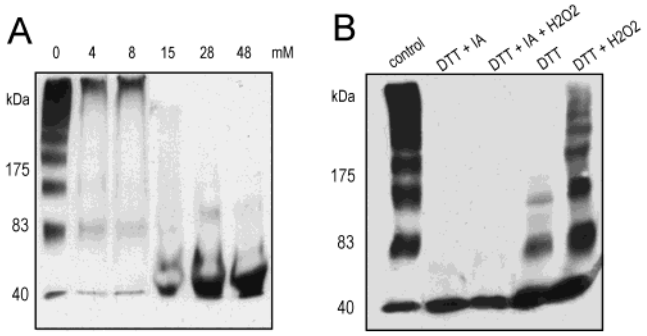

FIGURE 8: Disruption of disulfide bonds by DTT. (A) COS cells transfected with G3 were treated with DTT at different concentrations, and the medium was analyzed in nonreducing conditions. Products treated with 15, 28, and 48 mM DTT migrated as single bands. (B) Culture medium transfected with G3 were treated with (i) DTT; (ii) 28 mM DTT followed by H_2O_2 (DTT + H_2O_2); (iii) DTT followed by iodoacetic acid ((DTT + IA); and (iv) DTT followed by iodoacetic acid and H_2O_2 (DTT + IA + H_2O_2). Treated products were analyzed on Western blot in nonreducing conditions. Untreated product was used as a control.

β-mercaptoethanol treatment, the matrix formed fiberlike structures surrounding the cell complex (Figure 9A). Treatment with β-mercaptoethanol (Figure 9B) or DTT (Figure 9C) disrupted cell−matrix interaction. At higher magnification (Figure 9D−F corresponding to Figure 9A−C), the fiberlike matrix structures are more evident. These fiberlike materials might include other hyaluronan-binding molecules. The viability of the reagent-treated cultures was similar (data not shown).

To examine the biological function of the multimeric forms of versican G3, we performed cell attachment assays. Medium from G3- or vector-transfected cells was treated with or without DTT, followed by ammonium sulfate precipitation. These protein samples were used to coat Petri dishes for attachment assays using U87 cells (Figure 10A) and NIH3T3 fibroblasts (Figure 10B). Coating with G3-containing proteins enhanced cell attachment. DTT treatment reduced G3's function in cell adhesion but had little effect on vector-transfected medium. A typical photograph of cell attachment using U87 cells was shown in Figure 10C. The photographs for the attachment of NIH3T3 fibroblasts were similar (data not shown). Our results suggested that versican G3 domain enhanced cell attachment by forming multimeric forms.

DISCUSSION

In the joint, cartilage supports body weight and lubricates the joints during movement. The polymeric network of cartilage is an essential structural requirement for its load-bearing properties. In this network, the most abundant macromolecules are collagens, proteoglycans, and hyaluronan. The major proteoglycan in cartilage is aggrecan. The importance of aggrecan in cartilage has been illustrated by loss of function phenomena in the lethal genetic disease in chicken called nanomelia. Nanomelia is the result of a point mutation at the 5′ end of aggrecan G3 domain, which creates a premature stop codon (*6*, *20*, *21*). The mutated aggrecan cannot be processed normally nor secreted to the matrix. As a result, chondrocytes can continue to grow, but the extracellular space becomes smaller. This lethal condition indicates that aggrecan is essential in forming the extracellular space of cartilage. The exact mechanisms of aggrecan's role, however, are not entirely clear. Our results show that the

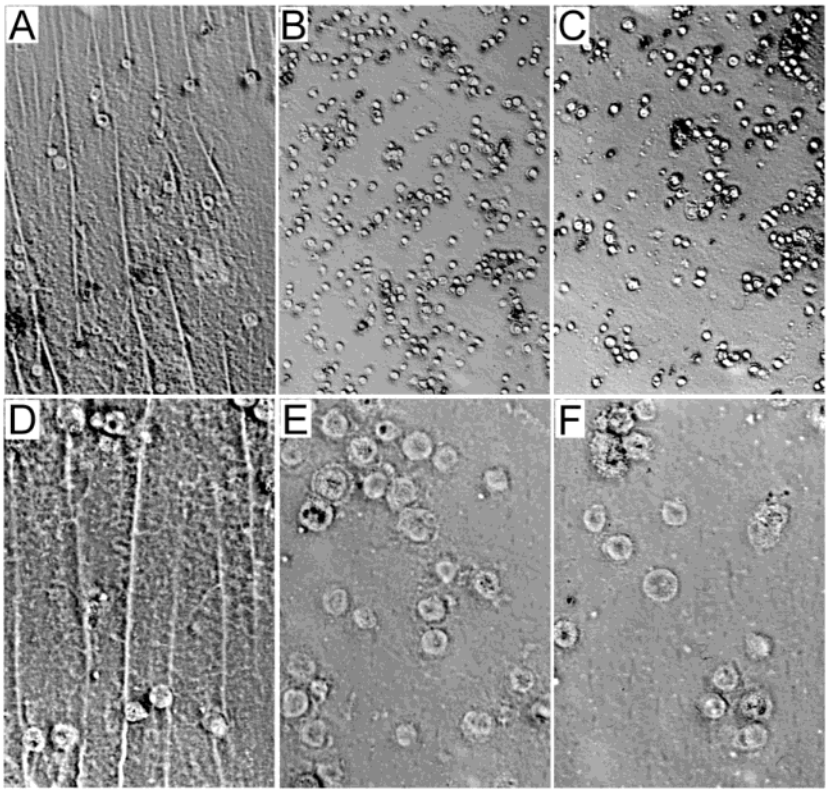

FIGURE 9: Effect of reducing agent on matrix structure. Suspension chondrocytes in 6-well tissue culture plates were incubated with or without β-mercaptoethanol (40 mM) or DTT (28 mM) at room temperature for 10 min with gentle shaking. The plates were subjected to centrifugation at room temperature at 3500 rpm for 30 min. Medium was removed, and the cultures were air-dried and stained with alcian blue to examine matrix structure. Without treatment, ECM was detected surrounding complex cells (A). Treatment with β-mercaptoethanol (B) or DTT (C) resulted in disappearance of matrix. Panels D−F, photographed at higher magnification, correspond to panels A−C.

aggrecan G3 domain forms intermolecular disulfide bonds between and among different proteoglycans providing an entirely novel mechanism in understanding matrix−matrix interaction.

In the presence of the reducing reagents, all products migrated as major single bands. Without reducing reagents, all products migrated as multiple bands, at sizes corresponding to monomers, dimers, and multimers. As the bonds forming the multimers were resistant to the cleavage in the presence of SDS and high heat, but were labile in the presence of reducing reagents, we have strong evidence that the multimers were formed via disulfide bonds, which resemble covalent bonds in their behavior. The existence of intermolecular disulfide bonds was further supported by the results of co-purification: the large molecules vG1CSG3 and LPCSG3 were co-purified with the small molecule HisG3.

It seems that every cysteine residue in the G3 domain has the potential to form intermolecular disulfide bonds. Theoretically, a polypeptide containing two cysteine residues is able to form dimers, trimers, tetramers, and so on to form a complex of unlimited size. As a G3 domain contains 10 cysteine residues, six in CRD and four in CBP, it has a potential to form infinitely large complexes. To identify the cysteine residue(s), which is crucial to the formation of the observed multimers, we generated a series of point mutations to replace the individual cysteine residues with noncysteine residues in the CRD and CBP constructs. Unexpectedly, all mutated constructs formed intermolecular disulfide bonds well. We noted that the disulfide bond in aggrecan (*22*, *23*) and link protein (*24*) have been assigned experimentally. Our results suggested that there might be some exceptions, and we propose a model of the formation of intermolecular disulfide bonds (Figure 11A). In this model, two free cysteine residues are sufficient to form intermolecular disulfide bonds. These free cysteine residues can be randomly located. Perhaps this exception would not affect the assignments of the disulfide bonds in aggrecan and link protein. Our mutation experiments also suggest that every cysteine residue in the G3 domain can potentially form intermolecular disulfide bonds. This is not to suggest that this is the case in vivo; indeed, it is more likely that a certain number of intermolecular disulfide bonds are necessary for aggrecan−aggrecan interaction and for cartilage stability. It is also likely that a single cysteine residue is involved in multiple interaction over time, some of which are intermolecular and some, intramolecular. Our results could not exclude the possibility of some nonspecific aggregation/interaction. In particular, we could not exclude the effect of glycosylation on the formation of multimers. If this was the case, there must be some novel mechanism(s) involved since the multimers were stable in the presence of SDS with high heat. Protein concentrations were not the concern as we had diluted the products and obtained similar results (data not shown).

The G3 domain of versican, another chondroitin sulfate proteoglycan, also forms intermolecular disulfide bonds. The formation of intermolecular disulfide bonds may be a property shared among many molecules in the ECM. This increases the probability that disulfide bonds are formed between different molecules in the ECM, creating multimolecular aggregates. Evidence to support this came from our

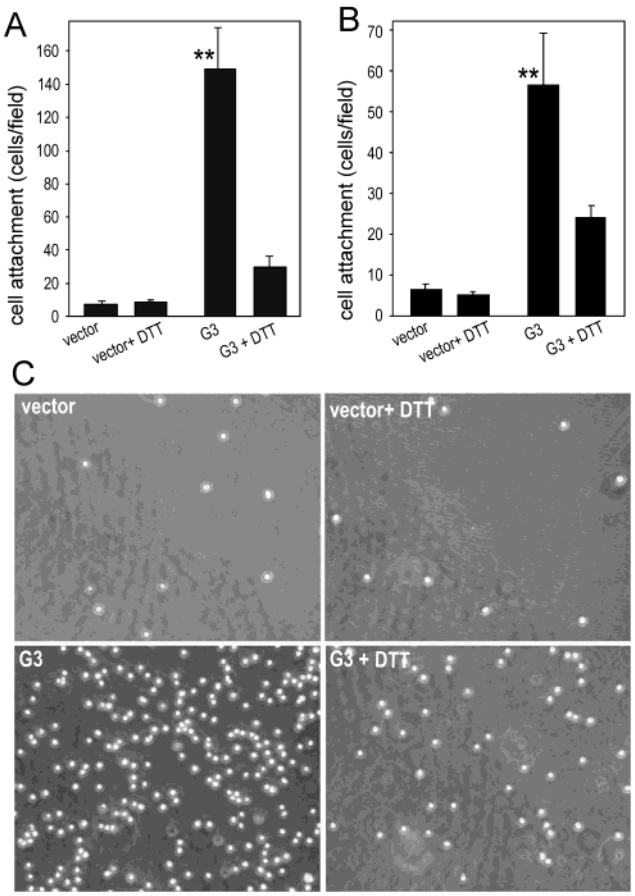

FIGURE 10: Effect of reducing agent on versican G3-mediated cell attachment. U87 cells (A) and NIH3T3 fibroblasts (B) were inoculated on Petri dishes precoated with proteins precipitated from a G3- or vector-containing medium, which had been treated with or without DTT. G3-containing proteins enhanced cell attachment, while DTT treatment reduced G3's function ($n = 3$, $p < 0.01$). A typical photograph for each treatment of U87 cells was shown (C).

finding that the aggrecan G3 domain forms intermolecular disulfide bonds with the products of a mini-versican and a chimeric construct LPCSG3.

The formation of intermolecular disulfide bonds in cell-associated preparations is more complex. Since no extra band was detected in products prepared under reducing conditions, the two bands detected appeared to be the result of the activity of an endogenous peroxidase. Since we did not detect multimers in the cell lysate, the smears might be the result of covalent interaction between the expressed proteins and the unknown cellular components. These cellular components may be involved in product processing. For example, it has been reported that heat shock protein-25 covalently interacts with aggrecan G3 domain and plays a role in G3 processing (*25*). We have also demonstrated that the G3 domain of aggrecan and versican plays important roles in product processing (*26*−*28*). Whether the intermolecular disulfide bonds have any effect on G3 processing is not known.

The effect of the reducing reagent on the formation of intermolecular disulfide bonds is concentration-dependent. At lower concentrations, multimers were still observed, but a single major band (monomer) could be detected. When cultures were treated with the lowest concentration of reducing reagents that produced the major single band, we obtained the same pattern of bands consistently over time

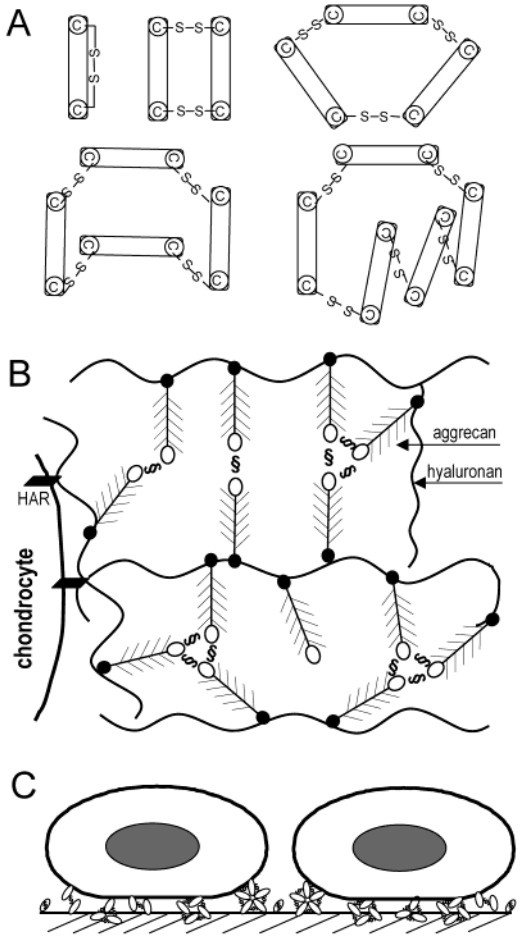

FIGURE 11: Model of matrix structure affected by disulfide bond formation. (A) Schematic intramolecular and intermolecular disulfide bond formation producing monomers, dimers, trimers, tetramers, and so on. (B) The interaction of hyaluronan and aggrecan, showing the formation of intermolecular disulfide bonds (§) via the C-terminal G3 domain. (C) A model showing multimeric forms of versican G3 product mediating cell attachment.

points. This suggests that the effect of reducing reagents on the formation of intermolecular disulfide bonds is time-independent and occurs very quickly. It has been known that reducing agents such as DTT do not affect most cellular functions including protein transport within the secretory pathway (*17*, *29*). DTT does not have serious adverse effects on translation, translocation, and glycosylation. The lack of general inhibitory effects of DTT in the secretory pathway indicates that proteins in the cytosolic environment have already adapted to the more reducing conditions (*17*). We have demonstrated that treatment with DTT for 1 min was sufficient to disrupt the disulfide bonds. It is unlikely that such a short period of treatment could disrupt the secretory pathway resulting in release of lytic enzymes that could be responsible for the cell−matrix dissociation. Rather, it appears that the reducing reagents acted directly on the existing products in the matrix.

Our results provide strong evidence that the G3 domain of aggrecan and versican form intermolecular disulfide bonds, resulting in the formation of multimeric forms observable on Western blot. These intermolecular disulfide bonds play an important role in ensuring the stability of the matrix structure, as shown in Figure 11B, where we provide a hypothetical explanation for the way in which the C-terminal

G3 domain forms intermolecular disulfide bonds and this allows aggrecan, combined with hyaluronan, to stabilize matrix structures of the ECM network in cartilage. In Figure 11C, we propose a model to explain the role of G3 multimeric forms in mediating cell attachment to the ECM.

It is not clear how the G3 domain of native aggrecan forms intermolecular disulfide bonds in cartilage. As aggrecan is a large molecule, the Western blot technique described here is not applicable to the study of the formation of aggrecan multimers. It is impossible to predict whether the cysteine residues that are responsible for the formation of multimers in small constructs behave similarly in native aggrecan. Understanding the formation of intermolecular disulfide bonds in vivo and their role in cell−matrix interactions and maintaining functional tissues awaits further investigation.

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