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Cell Adhesion and Proliferation Mediated Through the G1 Domain of Versican

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Abstract We have demonstrated previously that versican stimulated cell proliferation through the G3 domain. In these experiments, we show that versican mini-gene-transfected cell lines exhibited decreased cell-substratum interaction and increased cell proliferation. Exogenous addition of growth medium containing the versican gene product produced the same results. Because the G1 domain of versican is structurally similar to the G1 domain of aggrecan and to link protein, both of which play role in cell adhesion, we hypothesized that versican's proliferative effects may be a consequence of its ability to reduce cell adhesion, and may be mediated through the G1 domain. To investigate this, we expressed a G1 construct in NIH3T3 cells and showed that it reduced cell adhesion and enhanced cell proliferation. We then demonstrated that deletion of the G1 domain from versican greatly, but not completely, reversed the effects of versican: G1-deletion mutants of versican show slightly reduced amounts of cell adhesion and slightly increased rates of proliferation. We concluded that versican can stimulate cell proliferation via two mechanisms: through two EGF-like motifs in the G3 domain which play a role in stimulating cell growth, and through the G1 domain, which destabilizes cell adhesion and facilitates cell growth. We purified the G1 product with an affinity column and demonstrated that it reduced cell adhesion and enhanced cell proliferation. J. Cell. Biochem. 72:210–220, 1999.

Key words: cell growth; overexpression; G1 domain; hyaluronan

Versican [Shinomura et al., 1993; Zimmermann and Ruoslahti, 1989], also called PG-M, is a member of the large aggregating chondroitin sulfate proteoglycan family. Other family members include aggrecan, which is mainly expressed in cartilage, and neurocan and brevican, which are specifically expressed in the nervous system [Margolis and Margolis, 1994]. The core proteins of these large chondroitin sulfate proteoglycans range in size from 200 to 400 kDa, and a large chondroitin sulfate side chain-bearing sequence is localized in the middle region. In aggrecan, this sequence contains sites for the attachment of 100 to 150 chondroitin sulfate chains and keratin sulfate chains, but versican has only 12 to 15 chondroitin sulfate side chains covalently attached [Zimmermann and Ruoslahti, 1989; Krusius et al., 1987]. Other common features of these proteoglycans are globular domains at the amino terminus (G1) and carboxyl terminus (G3). The G1 domain is composed of an immunoglobular (IgG) domain, which binds link protein, and two tandem repeats, which bind hyaluronan. The G3 domain consists of a set of lectin- (also called carbohydrate recognition domain), epidermal growth factor-, and complement binding protein-like subdomains. These are a structural property of the selectin family [Springer and Lasky, 1991].

Like other chondroitin sulfate proteoglycans, versican has been reported to inhibit the adhesion of the cell to the substrata. Versican may repress focal contact formation and inhibit cell adhesion since it is excluded from focal contacts in fibroblast cultures [Yamagata et al., 1993a]. It has been reported that versican interferes with the attachment of cells to various extracellular matrix components such as collagen I, fibronectin, and laminin [Yamagata et al., 1989] and inhibits adhesion of normal cells [Yamagata et al., 1993a; Yamagata and Kimata, 1994].

The versican gene (called CSPG2) [Iozzo et al., 1992; Naso et al., 1995] is expressed in a variety of tissues. Versican is found in embryonic tissues [Naso et al., 1995], including

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specifically human embryonic lung fibroblasts [Zimmermann and Ruoslahti, 1989], the mesenchymal cell condensation area of limb buds [Kimata et al., 1986; Shinomura et al., 1990], the perinotochordal mesenchyme between the notochord and neural tube and basement membranes facing the neuroepithelial cells of chicks [Yamagata et al., 1993b]. Versican is also distributed in the embryonic aorta, lung, cornea, and skeletal muscle [Yamagata et al., 1993b]. In adult tissues, versican is detected in the loose connective tissue of various organs including most smooth muscles, the central and peripheral nervous system, the luminal surface of glandular epithelia [Bode-Lesniewska et al., 1996], blood vessels [Yao et al., 1994], vessels of brain tumors [Paulus et al., 1996], dermis and in the proliferative zone of the epidermis [Zimmermann et al., 1994]. Since versican is highly expressed in fast growing tissues and cells, it is likely that versican plays a role in cell proliferation. Using molecular biology approaches, we overexpressed versican in NIH3T3 fibroblasts and showed that it promotes cell proliferation. Exogenous addition of gene product had the same effect. In previous studies, we had observed that the G3 domain of versican alone had the ability to enhance cell proliferation, presumably through the activity of its two EGF-like motifs [Zhang et al., 1998]. Deletion of the G3 domain of versican greatly decreased cell growth. However, when we deleted the CS sequence from the G3-deletion mutant, we produced a G1 construct, which was able to stimulate cell growth. Furthermore, we observed that overexpression of the aggrecan G1 domain in fibroblasts and chondrocytes resulted in decreased cell-substratum interaction [Cao et al., 1998].

Since cell proliferation and cell adhesion are often related, we generated a series of constructs and performed cell adhesion and cell proliferation assays in order to study the possibility that versican may stimulate cell proliferation through its ability to decrease cell adhesion. We found that, the G1 domain of versican enhanced cell proliferation perhaps through its ability to decrease cell-substratum interaction.

MATERIALS AND METHODS

Materials

Prep-A-Gene DNA purification kit, prestained protein markers and protein assay kit were from Bio-Rad (Richmond, CA). DNA miniprep kit was from Biocan Scientific (Mississauga, Ontario, Canada). The RT-PCR kit was from Clontech (Palo Alto, CA). Taq DNA polymerase, T4 DNA ligase, and restriction endonucleases were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). The bacterial growth medium was from Difco Labotories (Detroit, MI). Lipofectin, Geneticin (G418), DMEM growth medium, fetal bovine serum, Hank's balanced salt solution, trypsin/EDTA, and IPTG were from GIBCO BRL (Gaithersburg, MD). ECL Western blot detection kit was from Amersham (Arlington Heights, IL). DNA Midi-prep kit, Ni-NTA agarose, and ^{MRGS}His antibody were from Qiagen (Chatsworth, CA). The ^{3}H thymidine was from Amersham. The six-well, 24-well, and 96-well tissue culture plates were from Nunc, Inc. (Naperville, IL). Goat anti-mouse IgG HRP, goat anti-rabbit HRP, and all chemicals were from Sigma (St. Louis, MO).

Gene Construction and Expression

A recombinant chicken versican gene was constructed using G1, CS, and G3 domains which were cloned separately by us earlier [Zhang et al., 1998]. This construct has already been shown to possess characteristic proteoglycan properties, namely, attachment of glycosaminoglycan chains (GAGs) and secretion of the gene product. For this study, a G1 construct was generated from the recombinant versican using PCR according to the standard methods described by Sambrook et al. [1989]. 5′-AAA AAG CTT GCC GCC ACC ATG GTG TTA AAC ATA AAA AGC ATC-3′ and 5′-AAA GCA TGC TTC GTA GCA GTA GGC ATC-3′ were used as primers. These primers are located in nucleotide sequence 145–165 and 1162–1182, respectively in the versican cDNA sequence published by Shinomura et al. [1993]. The resulting G1 construct was transiently transfected into COS-7 cells. Analysis of the gene product demonstrated that the G1 construct was expressed, but not secreted (data not shown). This means that the signal peptide of versican was not sufficient for G1 secretion.

To allow secretion of the products, the signal peptide of link protein was added to the G1 construct. To do this, 5′-AAA GAA TTC GCC GCC ACC ATG GCA AGT CTA CTC TTT CTG-3′ and 5′-AAA GGA TCC CTC GAG AGG CAG

TGT GAC GTT GCC-3′ were used as primers in a PCR reaction to generate a cDNA sequence containing the signal peptide of link protein [Deak et al., 1986]. The leading peptide also contains an epitope recognized by a monoclonal antibody 4B6 [Binette et al., 1994]. 5′-AAA GGA TCC ACT CTA CTA CTA GTG-3′ and 5′-AAA GCA TGC TTC GTA GCA GTA GGC ATC-3′ were used to generate the G1 domain without the signal peptide of versican. The PCR products were purified using agarose gel electrophoresis and a Prep-A-Gene DNA purification kit. The signal peptide of link protein and G1 were linked together and inserted into pcDNA1 or pcDNA3. Using the same strategy, the signal peptide of link protein was engineered into the CS and G3 sequences of versican or the mini-versican in a correct reading frame to obtain the CSG3 and versican constructs (Fig. 1A). The CS sequence represented the region of nucleotides 1183–2424 and the G3 domain located at nucleotides 9904–10830 [Shinomura et al., 1993]. The constructs obtained were confirmed by DNA sequencing.

The recombinant genes constructed in pcDNA1 were transfected transiently into COS-7 cells (American Type Culture Collection, Rockville, MD) using Lipofectin (GIBCO) as originally described by Felgner et al. [1987]. The growth medium and cells were harvested separately after 3 days of transfection and analyzed in Western blot. Stable expression was carried out once the transiently expressed products had the expected properties (i.e., glycosaminoglycan chain attachment to proteoglycan resulting in a smear of proteoglycan bands on Western blot and expected sizes of the gene products). The vector used was pcDNA3. After 3 days of transfection, 1 mg/ml Geneticin was added to the growth medium and the cells were maintained in this medium until individual colonies were large enough for cloning. Chemically selected cell lines were maintained in 0.5 mg/ml Geneticin or stored in liquid nitrogen. The PCR technique was used to confirm that the selected cell lines had incorporated the genes of interest. Briefly, genomic DNA was prepared from cell lysate. One pair of primers was used in a PCR reaction with the prepared DNA as a template. The PCR products were subjected to electrophoresis in 7% polyacrylamide gel and stained with ethidium bromide for size determination.

Purification of G1 Gene Products

To study the effect of G1 gene products on cell adhesion and proliferation, G1 was purified from the G1-transfected bacteria. Briefly, cell lysate from G1-transformed bacteria were dialyzed against equilibration buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). This buffer was also used to equilibrate a Ni-NTA column. The dialyzed samples were incubated with the Ni-NTA column and washed extensively with washing buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF). G1 peptide was eluted with elution buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole). The purified products were dialyzed against PBS, and then analyzed on SDS-PAGE and visualized with Coomassie blue dye or silver staining.

Western Blot

Cell lysate and growth medium were collected from the cultures of interest and subjected to SDS-PAGE electrophoresis, on either a 4% separating gel (for the recombinant versican gene product) or a 12% separating gel (for smaller proteins such as the G1 and G3 domains). The buffer system was 1× TG (Tris-glycine buffer, Amresco product) containing 1% SDS. The proteins separated on SDS-PAGE were transblotted onto a nitrocellulose membrane (Bio-Rad) in a 1× TG buffer 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 primary antibody in TBSTM. The membranes were washed with TBST (3 × 30 min) and then incubated for 1 h with the secondary antibody conjugated to horseradish peroxidase in TBSTM. After washing as described above, the bound antibody was visualized with an ECL kit according to the manufacturer's instructions (Amersham).

Cell-Substratum Interaction Assay

This method has been described previously by us in detail [Yang et al., 1998]. Briefly, cell were seeded in 24-well plates at a concentration of 2×10^4 cells/well in 0.5 ml DMEM containing 5% FBS and allowed to grow to 90% confluence (after 2 days). Cell adhesion was determined at different concentrations of EDTA

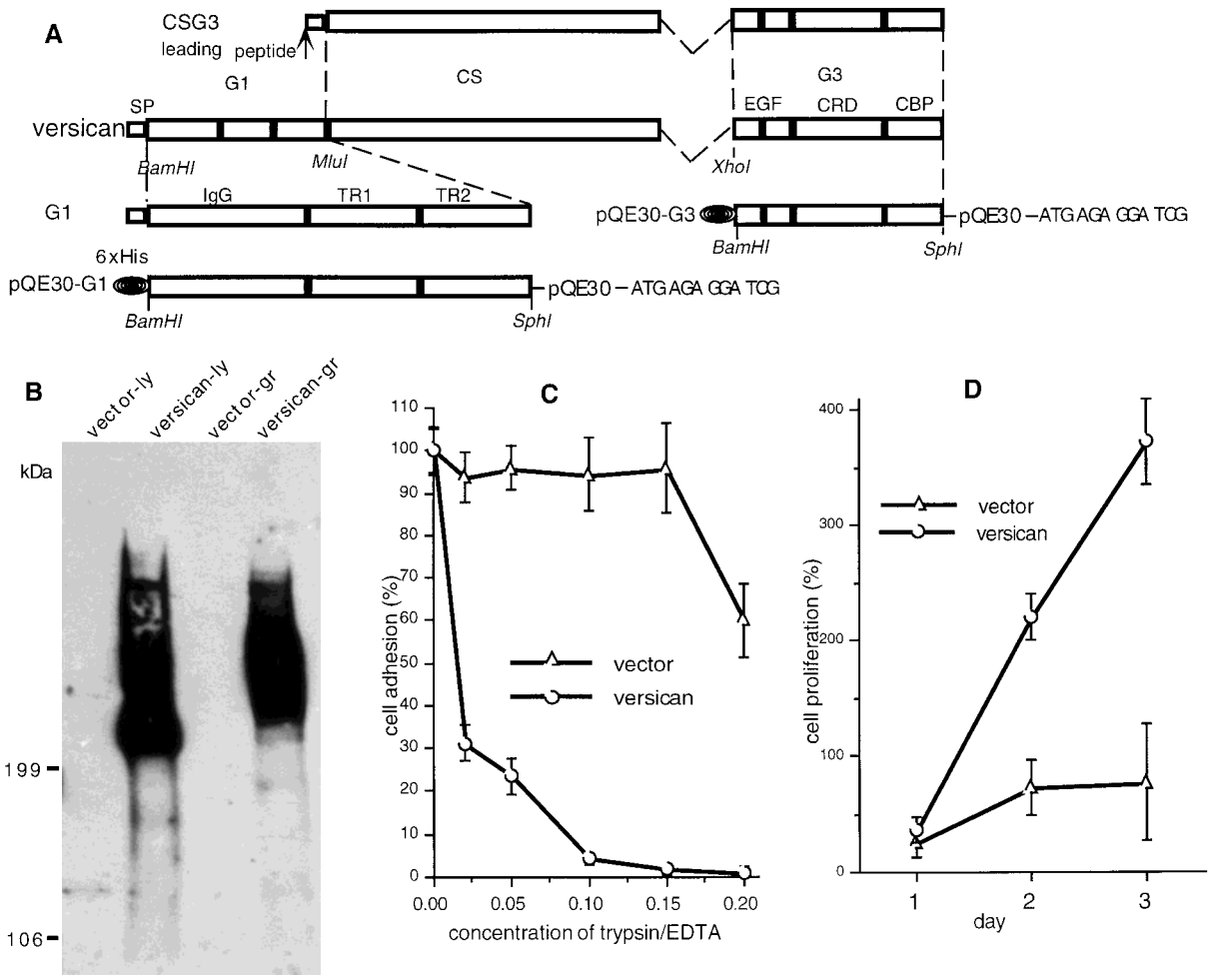

Fig. 1. Overexpression of versican destabilizes cell adhesion. **A**: To study the role of versican, recombinant cDNAs were constructed as shown in A. SP, signal peptide or leading peptide; IgG, Immunoglobulin; TR, tandem repeat; CS, chondroitin sulfate (chain attachment sequence); EGF, epithelial growth factor-like, CRD, carbohydrate recognition domain; CBP, complementary binding protein; G1, globular domain 1; 6× His, MRGSHHHHHH. The mini-versican, the CSG3 construct and the G1 construct were generated in pcDNA1 for transient expression in COS-7 cells and in pcDNA3 for stable expression in NIH3T3 fibroblasts. The pQE30-G1 and pQE30-G3 were generated in bacterial expression vector pQE30. **B**: The versican mini-gene was stably expressed in NIH3T3 fibroblasts. Growth medium (gr) and cell lysate (ly) containing the gene products were analyzed in Western blot. The mini-versican gene products had a characteristic smear of proteoglycan bands to which glycosaminoglycan chains of different sizes have been covalently attached. The size of the recombinant versican ranged from 190 to 250 kDa. **C**: A cell line stably expressing the mini-versican and a cell line transfected with a control vector was cultured in 24-well tissue culture plates for 2 days to 90% confluence. Cell adhesion was assayed by incubating the cultures with different concentrations of trypsin/EDTA (i.e., 0.20 means 0.20× dilution of the original product containing 0.05% trypsin and 0.53 mM EDTA). Overexpression of the mini-versican inhibited cell attachment compared to the vector-transfected cell line. **D**: Cell proliferation was tested in the cell line expressing the mini-versican and the cell line transfected with vector alone. The cells were seeded in 24-well plates and incubated at 37°C for 3 days. Cell number was determined every day. The cell line expressing versican had a significantly higher rate of proliferation.

prepared in Hank's balanced salt solution. Each treatment had four replicates. The EDTA concentrations used were 1, 2, 5, 10, and 20 mM (500 µl/well). Cells without the EDTA treatment were used as controls. The growth medium was removed and the cells were then incubated with 1 ml of EDTA solution at various concentrations for 30 min with shaking at a speed of 50 rpm at room temperature. The EDTA solution was removed and the cells were washed with PBS. The remaining cells were collected for cell counting. Cell adhesion was expressed as: Cell adhesion (%) = T/C, where T is the cell number in each treatment, and C is the cell number in the control. We also used trypsin/EDTA at different concentrations (0,

0.02, 0.05, 0.10, 0.15, and 0.20× of the original product that contains 0.05% trypsin and 0.53 mM EDTA) into the 24-well cell culture plate (500 µl/well) and incubated them for 30 min at room temperature, with shaking at 50 rpm. Cell adhesion was determined as described above.

Cell Proliferation

Cell proliferation was measured by ^{3}H thymidine incorporation and cell counting. For ^{3}H thymidine incorporation, 5×10^3 cells were seeded into 96-well plates and cultured for 2 days. We introduced 50 µl of 2.0 µCi ^{3}H thymidine (25 Ci / mmol; Amersham) into each well and incubated with the cells for another 4–6 h. The cells were harvested in a Titertek Cell Harvester 530 with Printed Filtermat A (Pharmacia Fine Chemicals, Piscataway, NJ). The radioactivity in the harvest filters was counted in a 1205 Betaplate Scintillation counter (Wallac, Gaithersburg, MD).

In the cell counting assay, 1×10^4 cells were seeded into each well of the 24-well plates and cultured with standard medium (DMEM supplemented with 5% FBS) or medium containing different gene products. The cells were incubated at 37°C in a humidified incubator containing air with 5% CO_2. The cell numbers were determined at days 1, 2, and 3. Vector-transfected cell lines were used as a control and the experiments were done in quadruplicate.

RESULTS

Versican Stimulates Cell Growth Through Destabilizing Cell Adhesion

We have demonstrated previously that chicken versican (also called PG-M) stimulated cell proliferation by stably expressing the gene in NIH3T3 fibroblasts [Zhang et al., 1998]. Exogenous addition of gene product to the NIH3T3 fibroblast cell line or chicken fibroblasts (primary culture) also stimulated cell proliferation. It is known that versican binds to hyaluronan [LeBaron et al., 1992], a molecule that possesses the properties of stimulating cell proliferation by destabilizing cell adhesion. We wished to determine if versican has the same effects. We expressed stably a mini-versican construct in NIH3T3 fibroblasts. Integration of recombinant versican into the genome was demonstrated with PCR using genomic DNA as a template (data not shown). Four stably transfected cell lines were stored in liquid nitrogen. The cell lysate and growth medium were prepared for product analysis by Western blot. When the recombinant gene was expressed, the product in the growth medium showed a diffuse band, characteristic of proteoglycans. Figure 1B shows the typical results from a cell line transfected with the mini-versican and a cell line transfected with the control vector. In addition, some morphological changes were observed: transfected cells were not as flat as normal fibroblasts. Therefore, we performed a cell-substratum interaction assay. We found that all isolated cell lines exhibited decreased cell-substratum interaction compared to the cell lines that integrated the unmodified vector alone (Table I). A typical cell line that overexpressed versican had a decreased adherent ability as shown in Figure 1C. The versican-transfected cell line also showed increased proliferation (Fig. 1D), a result consistent with our previous observations [Zhang et al., 1998]. Versican-transfected cells also incorporated more ^{3}H thymidine (data not shown).

In order to determine whether the mini-versican product acts directly at the extracellular matrix to produce the observed effects, we tested whether exogenous addition of gene product to a cell line could destabilize cell-substratum adhesion and stimulate cell growth. Conditioned medium containing the mini-versican was collected and introduced into NIH3T3 fibroblast culture. Cell adhesion and proliferation were assayed as described above. This resulted in decreased cell adhesion (Fig. 2A) and enhanced cell proliferation (Fig. 2B).

TABLE I. Cell Adhesion of Different Cell Lines[a]

Vector		Versican	
Cell line	Adhesion	Cell line	Adhesion
1–3	+++	4–3	+
1–5	+++	4–12	+
1–7	+++	4–14	++
1–9	+++	4–10	+

[a]Cells were cultured in six-well tissue culture plates until 90% confluence. For cell adhesion assay, cells were incubated with 1× trypsin/EDTA and the other procedure was the same as described in Materials and Methods. + (very low adhesion), cells detached from plates within 1 min; ++ (low adhesion), cells detached from plates at about 2 min; +++ (high adhesion), cells detached from plates at about 4 min; ++++ (very high adhesion), cells detached at more than 6 min.

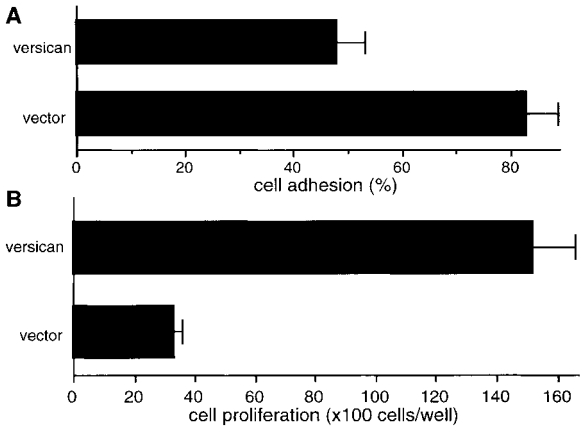

Fig. 2. Exogenous addition of growth medium containing the mini-versican gene product produced the same effects on cell adhesion and proliferation. **A**: To test the effect of gene products on cell-substratum interaction, NIH3T3 fibroblasts, transfected with pcDNA3 vector or untransfected, were seeded into 24-well plates. Growth media were collected from the cell lines transfected with versican mini-gene or vector alone and introduced into the cell cultures and incubated overnight in an incubator. Cell-substratum interaction was tested by introducing 0.10× concentration of trypsin/EDTA into the cultures and the assay was performed as described in Materials and Methods. The growth medium containing the mini-versican gene product inhibits cell attachment significantly ($n = 4$, $P < 0.001$). **B**: In cell proliferation assay, fibroblasts were seeded in 24-well tissue culture plates and incubated with growth medium containing the mini-versican gene product or growth medium from vector-transfected cell lines at 37°C for 3 days. Cell number was determined as described in the Methods. Growth medium containing versican stimulated cell proliferation significantly ($n = 4$, $P < 0.001$).

Deletion of the G1 Domain Reduced the Effect of Versican on Cell Adhesion and Cell Proliferation

We have demonstrated previously that aggrecan mediates cell-substratum interaction through the binding of its G1 domain to hyaluronan and link protein [Yang et al., 1998]. The versican G1 domain is structurally similar to the aggrecan G1 domain. To investigate the possibility that versican exerts its effects through the G1 domain, we deleted the G1 domain from the recombinant versican to derive the CSG3 construct as shown in Figure 1A. The signal peptide of link protein was linked to this construct to allow secretion of the gene product. The CSG3 construct, carried by pcDNA3, was transfected stably into NIH3T3 fibroblasts. Four cell lines, stably expressing the CSG3 construct (CSG3fa, CSG3fb, CSG3fc, and CSG3fd), were selected. To confirm secretion of CSG3, growth media from these cell lines were analyzed on Western blot using a monoclonal antibody (4B6) to an epitope in the leading peptide of the CSG3 construct. A smear of the bands (Mw. 150–200 kDa) on western blot indicated the attachment of GAG chains to the CSG3 core protein of the gene (Fig. 3A). We noted that the modification of GAG chains to the CSG3 core protein (Fig. 3A) is heavier than to the mini-versican core protein (Fig. 1B). These results suggest that the G1 domain is not required for GAG chain attachment, but the G3 domain may be important.

Cell-substratum interaction was analyzed in all cell lines expressing the CSG3 construct. The cell-substratum interaction was compared in cell lines expressing the mini-versican, the CSG3 construct, and vector alone (Fig. 3B). The reduction in cell-substratum interaction seen in the presence of the mini-versican was reversed when the G1 domain was deleted from the mini-versican. The reversal was not complete, however, and the cell-substratum interaction in cell lines expressing CSG3 was still lower than that in the vector-transfected cell lines. We have previously demonstrated that the G3 domain of aggrecan has no effect on cell-substratum interaction [Cao et al., 1998]. Thus, our results imply that the CS sequence has a negative effect on cell adhesion. This is supported by reports that the GAG chains of versican can play a negative role in cell adhesion [Yamagata et al., 1989].

Cell proliferation of cell lines expressing the CSG3 construct was analyzed. Increased cell proliferation was observed in CSG3-expressing cell lines, compared to a vector-transfected cell line. However, cell proliferation activity in the CSG3-transfected cell lines was lower than that in the mini-versican-transfected cell line (Fig. 4). We have demonstrated previously that the G3 domain of versican is an important element in stimulating cell proliferation [Zhang et al., 1998]. Thus, it seems that versican has at least two mechanisms to stimulate cell proliferation: one which acts through the G1 domain and one which acts via the G3 domain.

The Role of Versican G1 Domain on Cell Adhesion and Proliferation

To further study the role of the G1 domain in cell adhesion and proliferation, we cloned the versican G1 domain into a mammalian expression vector and transfected NIH3T3 fibroblasts with this construct. Cell lines stably expressing G1 were isolated. Four cell lines (G1fa, G1fb, G1fc, and G1fd) were tested for the stable ex-

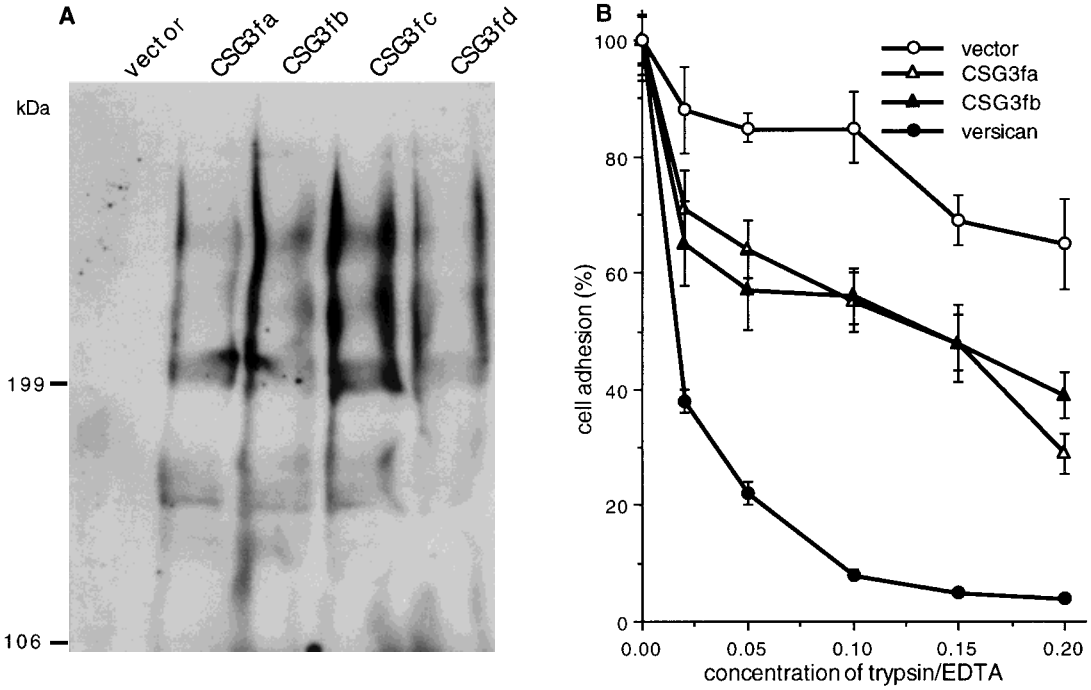

Fig. 3. Cell adhesion assay in cell lines expressing G1-deleted versican. **A**: The G1 domain was deleted from the mini-versican to generate a construct CSG3 as shown in Figure 1A. The gene products from four fibroblast cell lines expressing the CSG3 construct (CSG3fa, CSG3fb, CSG3fc, and CSG3fd) and a cell line transfected with vector alone were analyzed in western blot using the monoclonal antibody 4B6. All bands show a large smear, characteristic of proteoglycan. **B**: In cell adhesion assay, fibroblast cultures with 90% confluence were incubated with different concentrations of trypsin/EDTA. All cell lines demonstrated a decreased cell adhesion compared to the vector-transfected cell line. Shown is the cell attachment assay from two cell lines transfected with the CSG3 construct (CSG3fa and CSG3fb), one cell line transfected with vector and one cell line transfected with versican. Cell adhesion of CSG3-transfected cell lines was decreased compared to the vector-transfected cell line ($n = 4$, $P < 0.05$), but the versican-transfected cells showed the greatest decrease ($n = 4$, $P < 0.01$).

pression of the construct (Fig. 5A). Results from the cell-substratum interaction experiment indicated that all cell lines had decreased cell-substratum interaction compared to the vector-transfected cell line (Fig. 5B). However, the destabilizing effects of G1 alone were not as strong as the effects of the whole mini-versican (Fig. 3B compared to Fig. 1C). To ascertain that G1 could act extracellularly to decrease cell-substratum interaction, fibroblasts were incubated with growth medium containing different amounts of conditioned medium from G1-transfected cells prior to cell-substratum interaction assay. The results confirmed that exogenous addition of the G1 gene product decreased cell-substratum interaction in a dose-dependent manner (Fig. 5C). G1-transfected COS-7 cells were also incubated with standard medium (containing 5% FBS) and this medium had similar effect on reducing cell adhesion (data not shown). We also expressed the G1 domain in bacteria (pQE30-G1 construct) and purified G1 product using an affinity column. The effect of

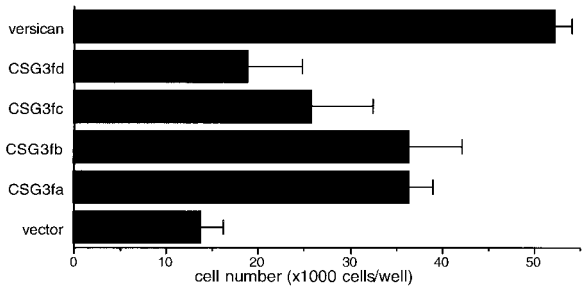

Fig. 4. Cell proliferation assay in cell lines expressing G1-deleted versican. Cell proliferation was assayed in four NIH3T3 fibroblast cell lines stably transfected with the CSG3 construct (the versican mini-gene lacking the G1 domain). A vector-transfected cell line and a versican-transfected cell line were used as controls. Cells were seeded in 24-well tissue culture plates at equal densities as described in Materials and Methods and allowed to grow for 3 days. Cell numbers were determined at day 3. The result demonstrated that all cell lines have decreased growth rate compared to the versican-transfected cell line ($n = 4$, $P < 0.01$). However, all CSG3-transfected lines showed increased growth rates compared to the vector-transfected cell line ($n = 4$, $P < 0.05$).

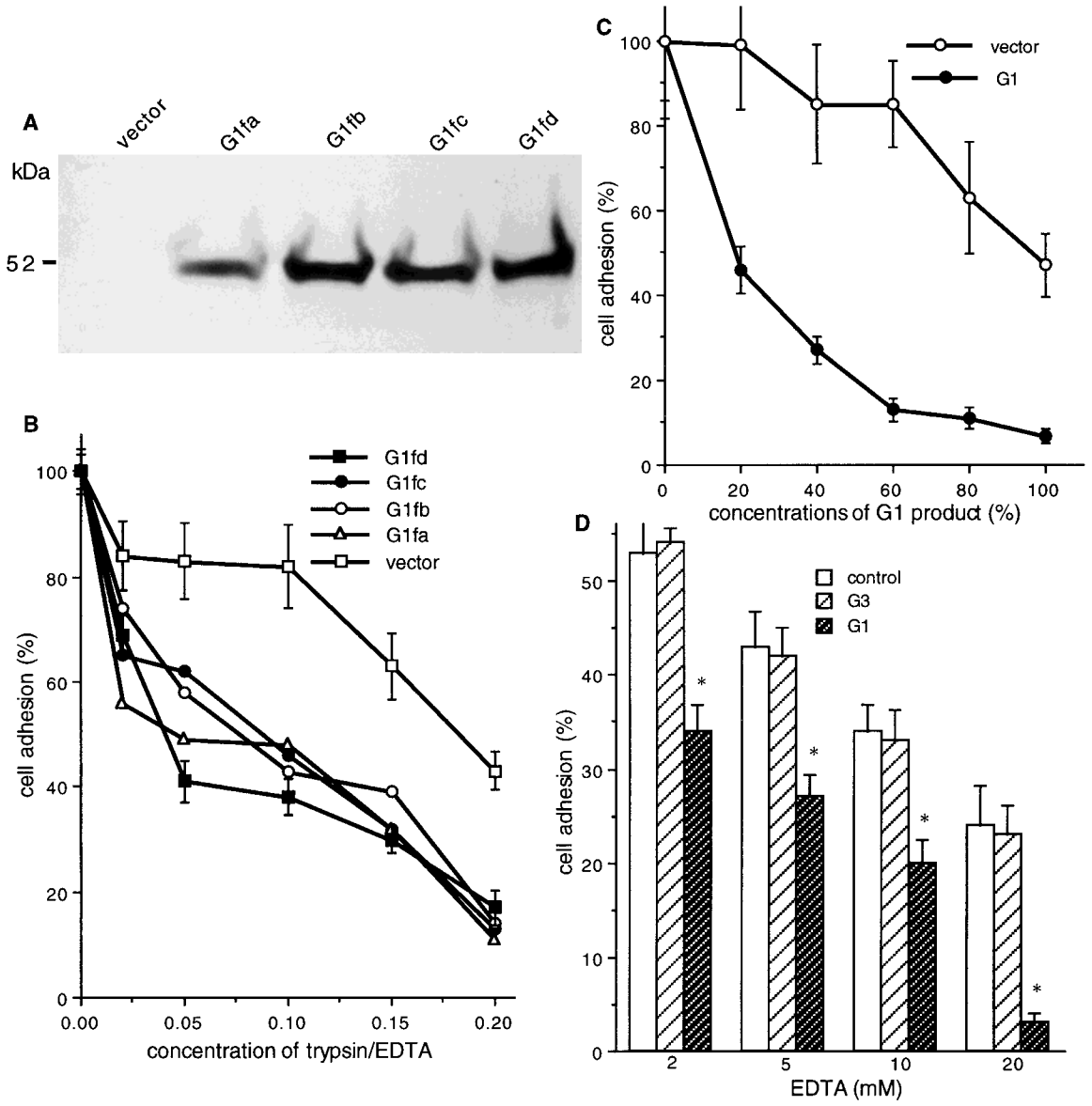

Fig. 5. Overexpression of G1 destabilizes cell adhesion. **A**: The G1 construct was generated as shown in Figure 1A. The construct was transfected stably into NIH3T3 fibroblasts as described earlier. The gene products from four cell lines expressing the G1 construct (G1fa, G1fb, G1fc, and G1fd) and a cell line transfected with vector alone were analyzed in western blot to ascertain the expression of G1 construct. **B**: In cell adhesion assay, cultures with 90% confluence were incubated with different concentrations of trypsin/EDTA. All cell lines transfected with the G1 construct had a decreased cell adhesion compared to the cell line transfected with vector. **C**: Conditioned medium from the G1-transfected cell line, or the vector-transfected cell line, was mixed with standard medium in different proportions as shown in the figure and introduced into NIH3T3 fibroblast cultures. The cultures were incubated overnight at 37°C. Cell adhesion assay was performed using trypsin/EDTA at 0.20× concentration. The gene product of the G1 destabilized cell attachment in a dose-dependent manner. **D**: The G1 and G3 gene products were purified from pQE30-G1- and pQE30-G3-transfected bacteria using Ni-NTA affinity columns (Qiagen) according to the manufacturer's instructions. Cells transfected with vector alone were used as a control. The effects of G1 and G3 gene products on cell adhesion was assayed by introducing different concentrations of EDTA as indicated in the figure. The purified G1 gene product reduced cell adhesion compared to G3 and control vector ($n = 4$, $P < 0.01$).

the purified G1 on cell adhesion was tested in NIH3T3 fibroblasts. We confirmed that the purified G1, but not G3 (from pQE30-G3 construct), reduced cell adhesion in a dose-dependent manner (Fig. 5D).

The effect of G1 on cell proliferation was assayed on all cell lines expressing the G1 construct (Fig. 6A). The G1-expressing cell lines had an increased cell growth rate compared to the vector-transfected cell line. However, the

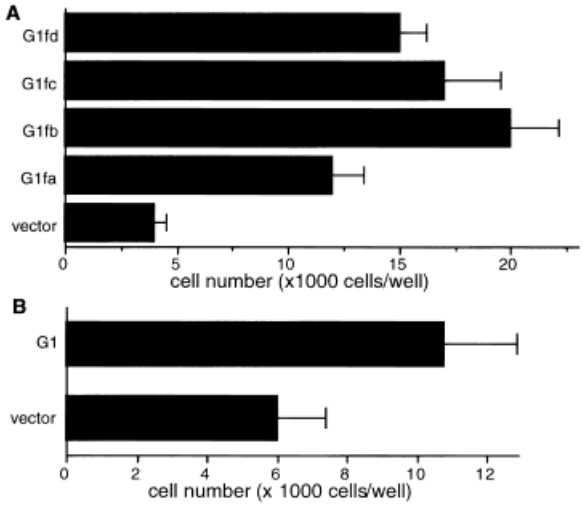

Fig. 6. The role of G1 domain on cell proliferation. **A**: Cell proliferation was assayed in four NIH3T3 fibroblast cell lines expressing the G1 construct (G1fa, G1fb, G1fc, and G1fd). A vector-transfected cell line was used as a control. The cells were seeded in 24-well tissue culture plates at equal concentrations. Each line was seeded in four wells. The cell number was determined at day 3. All the G1-transfected cell lines showed increased growth rate compared to the vector-transfected cell line ($n = 4$, $P < 0.001$). **B**: The G1 gene products were purified from G1-transfected bacteria using Ni-NTA affinity columns (Qiagen) according to the manufacturer's instructions. Cells transfected with vector alone were used as a control. The effect of the purified G1 gene products on fibroblast growth was tested in cultures seeded at 10^3 cells/well in 96-well plates. Each well contained 200 µl growth medium, to which 50 µl of purified gene products were added. Cell number was determined after 3 days. These experiments demonstrated that gene products enhanced NIH3T3 fibroblast growth significantly ($n = 4$, $P < 0.01$).

increase in growth rates differed among cell lines; this may be due to the presence of different copy numbers of transgene. We also purified G1 products from G1-transfected bacteria and tested the effect of the purified G1 on cell proliferation. We demonstrated that the purified G1 enhanced cell proliferation (Fig. 6B). Although the concentrations of the purified products added to cell cultures were at least 20-fold higher than that expressed by NIH3T3 fibroblast cell lines, the level of increase in proliferation enhanced by bacterial products was lower than that enhanced in the cell lines transfected with the G1 construct. This may suggest that most of the G1 products expressed by bacteria were not folded properly.

DISCUSSION

The large chondroitin sulfate proteoglycan versican is highly expressed in various tissues where the cells are actively metabolizing and proliferating, such as in the mesenchymal cells. In epidermis, versican is found only in the proliferating zone [Shinomura et al., 1990; Zimmermann et al., 1994]. In cultured cells, versican is expressed only when cells are actively proliferating; once cells reach confluence, versican is no longer expressed [Zimmermann et al., 1994]. Therefore, it has long been suspected that versican is associated with the process of cell proliferation.

In this study, we have presented evidence confirming that versican can promote cell proliferation, most probably by destabilizing cell-substratum adhesion, which seems necessary to permit proliferation. Cell adhesion is a very important phenomenon in normal tissue development and in pathogenic states, such as metastasis and wound healing, because it regulates cell attachment, cell proliferation and cell migration. Normally, cell-cell and cell-matrix interactions are required for cell proliferation. Cells need to be anchored to a substrate for proliferation and survival. If denied interaction, such cells will undergo mitotic arrest and apoptosis. However, although anchorage is necessary, activities inside and at the surface of the cells require that parts of the membrane be able to detach from the substrate to allow mitosis to proceed. Therefore, very strong cell-cell or cell-matrix adhesion will inhibit mitosis and prevent proliferation.

Using the techniques of molecular biology, we were able to determine that it is specifically the G1 domain of versican which is responsible for its destabilizing activity. A construct consisting of G1 alone was able to inhibit adhesion and increase proliferation. On the other hand, deletion of G1 from the mini-versican construct greatly reduced the previously observed effects of versican on adhesion and proliferation. Other domains of the versican may also play a role in reducing cell adhesion. It has been reported that the GAG chains of versican are involved in cell adhesion [Yamagata et al., 1989].

We have previously estimated that COS-7 cells transiently transfected with a G3 construct could express G3 product at a concentration of 1 ng per microliter [Zhang et al., 1998]. Using the same technique, we estimated that COS-7 cells transiently transfected the G1 construct could express G1 product at a concentration of 0.2 ng per microliter and NIH3T3 stably transfected with the G1 construct could

express G1 at a concentration of 0.02 ng per microliter. The G1 protein that was expressed by bacteria and added into the NIH3T3 fibroblast culture was at a final concentration of 1 ng per microliter, which is 50-fold of the G1 product expressed by NIH3T3 fibroblasts. However, the levels of reduced cell adhesion influenced by NIH3T3 cell lines expressing G1 is higher than bacteria-expressing G1. It could be explained that the bacterial expressed G1 products were not folded properly and that exogenous added G1 products did not interact with cell surface as sufficient as those expressed by the cells.

The molecular mechanism by which G1 exerts its effects is not yet known. However, it may involve hyaluronan. Hyaluronan is an important molecule found in the extracellular matrix. It has been shown to destabilize cell-cell and cell-matrix interactions, to enhance cell migration, and to stimulate cell proliferation. We have shown that G1 can bind to hyaluronan (data not shown). As well, tumors and tissues from organisms in early stages of development contain high levels of both versican and hyaluronan. A different mechanism by which G1 reduces cell adhesion could involve specific signaling pathway: the interaction of G1 with cell surface triggers a signal for reducing cell adhesion.

We propose the following scenario to explain the effects of versican and hyaluronan on cell proliferation: versican may bind to hyaluronan, resulting in a local increase in hyaluronan concentration, which will destabilize cell adhesion and increase cell proliferation. However, it is possible that versican destabilizes cell-cell and cell-matrix interactions by interacting with molecules other than hyaluronan. For example, the CRD (a lectin-like) domain of versican has been shown to bind tenascin [Aspberg et al., 1995] that was believed to be a modulator of cell growth [Eng et al., 1992]. Versican also interacts with type I collagen and fibronectin [Yamagata et al., 1986]. Thus, versican can compete for binding to matrix and disrupt or prevent binding of cell surface adhesion molecules.

It has been reported that chondroitin sulfate side chains play a critical role in inhibiting cell adhesion [Yamagata et al., 1989]. Neither the core protein nor the chains alone are sufficient; a native form, consisting of protein plus chains, is required for inhibition. We found that deleting G1 from versican, or transfecting G1 alone, reduced versican's effect on cell adhesion by half. This seems to indicate that versican-mediated destabilization is regulated by at least two independent mechanisms, one involving chondroitin sulfate chains and one involving the G1 domain.

Thus, the G1 domain of versican may have an important, but not the sole, destabilizing effect that allows proliferation to take place, but reducing cell adhesion may not be versican's only effect. Our cell adhesion assay was performed in the presence of EDTA, which removes Ca^{2+}, so the phenomenon which we report is Ca^{2+}-independent.

As our studies with the G1 deletion mutant of versican, the CSG3 construct, show, G1-mediated actions can only account for part of versican's enhancement of cell proliferation. Cells transfected with CSG3 still demonstrated increased proliferation, compared to control. We have previously demonstrated that versican can stimulate cell proliferation through its G3 domain, which contains two EGF-like motifs. Thus, the G3 domain can act on EGF receptors and enhance cell proliferation via the EGF-R-mediated pathway [Zhang et al., 1998]. Our study confirms that two distinct domains of versican can promote cell proliferation in two ways: G3, via the EGF-R pathway, and G1, via a reduction in cell adhesiveness.

Interestingly, we noted that the CSG3 construct produced approximately half of the cell proliferation seen when the whole versican gene was transfected. Since the G3 domain on its own produces the same effects as the G1 domain (unpublished data), this suggests that the CS sequence has a negative effect on cell proliferation. We have also observed that a G1-CS construct has very low activity on cell proliferation [Zhang et al., 1998], while the G1 domain alone has high activity to stimulate proliferation.

Versican is a large chondroitin sulfate proteoglycan with several distinct domains. Here we present a mini-versican expressed in a mammalian system, which allows us to observe new functions of versican that were not otherwise understood. However, to study such a large molecule, we also found it useful to express individual domains and to test their functions, separately and in combination. These investigations shed light on some aspects of the relationship between cell adhesion and all prolifera-

tion, and, we believe, may have implications for the study of cell differentiation (work currently in progress).

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