Identification of the Motif in Versican G3 Domain That Plays a Dominant-negative Effect on Astrocytoma Cell Proliferation through Inhibiting Versican Secretion and Binding*

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This study was designed to investigate the mechanisms by which mutant versican constructs play a dominant-negative effect on astrocytoma cell proliferation. Although a mini-versican or a versican G3 construct promoted growth of U87 astrocytoma cells, a mini-versican lacking epidermal growth factor (EGF) motifs (versicanΔEGF) and a G3 mutant (G3ΔEGF) exerted a dominant-negative effect on cell proliferation. G3ΔEGF-transfected cells formed smaller colonies, arrested cell cycle at G1 phase, inhibited expression of cell cycle proteins cdk4 and cyclin D1, and contained multiple nucleoli. In cell surface binding assays, G3 products expressed in COS-7 cells and bacteria bound to U87 cell surface. G3ΔEGF products exhibited decreased binding activity, but higher levels of G3ΔEGF products were able to inhibit the binding of G3 to the cell surface. G3ΔEGF expression inhibited secretion of endogenous versican in astrocytoma cells and also inhibited the secretion of mini-versican in COS-7 cells co-transfected with the mini-versican and G3ΔEGF constructs. The effect seems to depend on the expression efficiency of G3ΔEGF, and it occurred via the carbohydrate recognition domain.

Versican, a member of the large aggregating chondroitin sulfate proteoglycan family, was initially detected in the limb bud of chick embryo (1) and later cloned in human fibroblasts and chick embryo (2–5). It is also expressed in normal human central nervous system and brain tumors (6). RT-PCR reveals that transcripts of versican isoforms are present in astrocytomas, oligodendrogliomas, medulloblastomas, schwannomas, and meningiomas (6). Versican expression levels are low, however, because neuronal immunostaining for versican appears only pericellularly. Versican is highly expressed in the tissues flanking the regions where neural crest cells migrate in chick embryos, but it is absent from the actual migration pathways. Similar findings are noted for the outgrowing sensory and motor axons of chick embryos, because versican is notably absent in regions invaded by these axons (7). Versican is known to associate with a number of molecules in the extracellular matrix such as hyaluronan, tenasin, and fibronectin (8–10). In the central nervous system, versican has been observed to co-localize with tenasin and hyaluronan (9). Tenasin binds at the C-type lectin unit of versican (10, 11).

Structurally, versican is made up of an N-terminal G1 domain, a glycosaminoglycan attachment region, and a C terminus containing a selectin-like (or G3) domain. The latter contains two epidermal growth factor (EGF)-like repeats, a lectin-like motif (also known as carbohydrate recognition domain or CRD), and a complement binding protein (CBP)-like motif (3, 12, 13). Alternative splicing generates at least four versican isoforms (14–16), and some of these are highly expressed in brain tumors (6). The role of versican in brain tumor formation and progression is not clear.

We have previously demonstrated that a mini-versican construct promoted NIH 3T3 fibroblast proliferation through the G3 domain, and two EGF-like motifs in the G3 domain are involved in this effect (17). Deletion of the EGF-like motifs from the mini-versican construct significantly reduced the effect of the mini-versican on cell proliferation and differentiation (17, 18). Here we demonstrate that the mini-versican construct promotes astrocytoma cell proliferation through the G3 domain. To our surprise, deletion of these EGF-like motifs produced a dominant-negative effect on astrocytoma cell proliferation. We designed assays to uncover the mechanism associated with this dominant-negative effect. A G3 construct lacking the EGF-like motifs (G3ΔEGF) binds to the astrocytoma cell surface. This may have competed with endogenous versican for binding sites on the cell surface and blocked the function of versican in cell growth. Furthermore, we demonstrate that the mutant construct inhibited secretion of endogenous versican in glioma cells and the mini-versican in COS-7 cells. These two mechanisms may account for the dominant-negative effect of the mutant on cell growth. It appears that the effects of the G3ΔEGF on cell growth are concentration-dependent and occurred via the CRD motif.

EXPERIMENTAL PROCEDURES

Materials and Cell Cultures—Lipofectin, Geneticin (G418), Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Hank’s balanced salt solution, trypsin/EDTA were from Life Technolo-
digested CBP construct, in which the located at 3 purified. The purified products were inserted into the American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM supplemented with 10% (U87) or 5% (COS-7) FBS at 37°C in a humidified incubator containing 5% CO2.

**Construction of Recombinant Genes—** A mini-versican gene consisting of a complete G1 domain, a partial CS domain (15% in size of the entire sequence) and a complete G3 domain was introduced into glioma cells cultured in 96-well tissue culture plates at a density of 2 × 10^4 cells/well. The cultures were maintained in an incubator for 3 days, and cell number was counted using a cytometer. To test the effects of the purified products on cell proliferation, glioma cells were plated on 96-well dishes at a density of 2 × 10^5 cells/well, 200 μl/well. Purified products were added into each well (50 μl of column eluate/well). Lysate from vector-transfected bacteria was loaded onto Ni-NTA purification columns, and the eluate from these columns was used as a control. Cell proliferation was determined after 3 days of incubation. To test the effects of recombinant constructs on cell growth, glioma cells were transiently transfected with the constructs using the method described above. Four days after transfection, cells were counted. Cell lysate and culture medium were harvested for analysis of gene expression on Western blot. Cell proliferation assays and functional studies. Cell lines were monitored to ensure expression of the transgene for the duration of functional studies. Cell lines were maintained in an incubator at 37°C for 7 to 3 days, and cell number was counted as above.

**Cell Cycle Analysis—** U87 cells were plated on 6-well tissue culture plates at a density of 2 × 10^5 cells/well in DMEM containing 10% FBS at 37°C for 2 days. The cells were analyzed by flow cytometry. Briefly, the cells were collected and lysed in lysis buffer and resuspended in 1 ml of hypotonic propidium iodide solution (50 μg/ml) dissolved in 0.1% sodium citrate plus 0.1% Triton X-100. The cells were analyzed using a FACScan (Becton Dickinson).

** Colony Formation Assay—** Glioma cells were seeded to six-well plates at a density of 2 × 10^4 cells/well. The cells were allowed to attach and grow overnight in DMEM supplemented with 10% FBS to reach 70% confluence. Cultures in each well were transfected with 0.5–1 μg of G3ΔEGF plasmid or a control vector (pcDNA3) accompanied by 8 μl of Lipofectin as described above. Two days after transfection, a 0.5–1 μg of G3ΔEGF plasmid or a control vector (pcDNA3) was added to the media. The control plasmid was 5′-cagccctggagacagaggaagcc (producing a product of 297 base pairs). The primers for G3ΔEGF transgene were 5′-aattctagagcgccttgagtcctgccagct (complementary to nucleotides 10519–10531 of versican encoding the CBP motif) and the tail. The control primers were 5′-ccgcaagagagcatcgcgctg (complementary to β-actin nucleotides 247–683).

**Cell Surface Binding and Competition Assays—** Glioma cells transfected with G3 construct or the control vector (2.5 × 10^5 cells/well) were harvested, and total RNA were extracted with Qiagen RNeasy mini kit. RT-PCR assays were performed as previously described (18). Briefly, 2 μg of total RNA was used to synthesize cDNA, a portion of which (equal to 0.2 μg of RNA) was used as template. PCR with appropriate primers was performed. The products were analyzed in agarose gel electrophoresis and detected using ethidium bromide staining. The primers for endogenous versican were 5′-ccgctggagagagagacagaggaagcc (producing a product of 299 base pairs). The primers for G3ΔEGF transgene were 5′-aattctagagcgccttgagtcctgccagct (complementary to nucleotides 10519–10531 of versican encoding the CBP motif) and the tail.

**Analysis of Proteoglycans on Western Blot—** Cell lysate and growth medium that contained recombinant gene products or were subjected to SDS-PAGE electrophoresis and immunoblotting as described previously (17, 18). Primary antibodies were used at 1:1000 dilution, unless otherwise stated, and bound antibodies were visualized using an ECL kit according to the manufacturer's instructions. Because of its large size, electrophoresis of endogenous versican was performed in an agarose gel (agarose-Western blot assay). The agarose gel (4 cm height containing 1.5% agarose in a buffer containing 0.124 mM Tris-Cl, 27 mM barbituric acid, 1 mM EDTA, pH 8.7) was poured on top of a 1-cm conventional 10% polyacrylamide gel, which served to seal the bottom of the casting apparatus. This buffer was also used as a running buffer, and the electrophoresis was carried out at 40 V for 5 h at room temperature. Molecules (up to 2 million daltons in size) were able to enter the agarose gel, as shown by use of blue dextran 20000 as an internal control. Equal amounts of control and lysate (from U87 and COS-7) was adjusted to pH 7.4, and the cell lysates were prepared. The protein of recombinant mini-versican gene is 3.2 kilobases, which yields a core size, electrophoresis of endogenous versican was performed in an agarose gel. To allow transfer of such large molecules onto the nitrocellulose membrane, the blots took place in Tris-glycine buffer at 20 V overnight at 4°C. Western blotting was performed as above.

**Protein Purification—** G3 recombinant proteins containing a N-terminal His tag were purified using Ni-NTA affinity columns (17, 25). Briefly, the G3 domain was expressed in Escherichia coli strain M15 using the bacterial expression vector pQE30 (Qiagen Inc., Chatsworth, CA; catalog number 32149) as shown in Fig. 1A. The G3 domain was amplified in a PCR using two primers, 5′-aaatctagatgtccctttcttgcaggt (complementary to nucleotides 10519–10531 of versican encoding the CBP motif) and the tail. The control primers were 5′-ccgcaagagagcatcgcgctg (complementary to β-actin nucleotides 247–683).
cells expressing G3 (200 μl) were mixed with 0, 500, or 1500 μl of culture media from G3\(^{\text{DEGF}}\) transfected COS-7 cells, and the competition assay was performed as above.

**Immunostaining of Versican in Astrocytoma Cell Lines**—Astrocytoma cells U87 were cultured on glass slips to 80% confluence. The cells were fixed with 4% paraformaldehyde and stained with rabbit anti-versican polyclonal antibody, which we generated to recognize the CS sequence of the construct (17). The secondary antibody was goat anti-rabbit IgG antibody conjugated with horseradish peroxidase. 3'-Amino-9-ethylcarbazole (Sigma) was used for color development according to the manufacturer’s instructions. The stained cells were examined with a light microscope and photographed.

**RESULTS**

**Dominant-negative Effect on Cell Growth by Deletion of EGF-like Motifs**—To examine the role of versican in glioma cell growth, we generated a mini-versican gene and a number of mutants (Fig. 1A). We first confirmed the expression of versican in human glioma sample using Western blot. Our purified polyclonal antibody, originally raised against chicken versican, recognized a proteoglycan migrating as a large smear in agarose gel, characteristic of large aggregating chondroitin sulfate proteoglycans (Fig. 1B). Versican expression was also detected in the glioma cell line U87. The mini-versican gene was expressed in COS-7 cells, and its expression and secretion were confirmed by Western blot. Growth medium collected from COS-7 cells transiently transfected with versican\(^{\text{DEGF}}\), control vector, or the mini-versican construct was also analyzed on Western blot probed with 4B6. Deletion of the EGF-like motifs resulted in a smaller core protein, and this proteoglycan migrated slightly faster than the recombinant mini-versican. Growth medium collected from COS-7 cells transfected with versican\(^{\text{DEGF}}\) construct was mixed with DMEM containing 2.5% FBS, and the mixture was introduced into glioma cultures that had been seeded into 96-well tissue culture plates at a cell density of 2 \times 10^4 cells/well, 200 μl/well. Media collected from the vector-transfected cells were used as controls. After 3 days, cells were counted. Data represent the mean ± S.D. of four separate experiments (n = 4; *, p < 0.05). D, versican\(^{\text{DEGF}}\) was stably expressed in glioma cells. Three such cell lines and three cell lines stably transfected with a control vector were seeded in tissue culture plates for cell proliferation assay. Data represent the means ± S.D. of four separate experiments (n = 4; ***, p < 0.01). E, cells stably transfected with versican\(^{\text{DEGF}}\) or the control vector were seeded in 96-well tissue culture plates. Growth media from COS-7 cells transfected with the mini-versican construct or the control vector were introduced into the glioma cultures as indicated. The cultures were maintained in an incubator for 3 days, and cell number was determined. The dominant-negative effect of the versican\(^{\text{DEGF}}\) construct on cell proliferation was significantly reduced by addition of growth medium containing mini-versican products. Data represent the means ± S.D. of four separate experiments (n = 4; ***, p < 0.01).
growth medium from versican\(\Delta\)EGF-transfected cells exerted a dominant-negative effect, producing inhibition of glioma cell growth compared with the control (Fig. 1C). The dominant-negative effect of the mutant was further confirmed in cell lines stably transfected with versican\(\Delta\)EGF; three cell lines expressing the versican\(\Delta\)EGF construct exhibited a dominant-negative effect on cell growth compared with the control vector (Fig. 1D). One of the cell lines was cultured and incubated with growth medium from the mini-versican- and vector-transfected COS-7 cells. Addition of exogenous growth medium from the mini-versican-transfected cells reversed this effect somewhat but not completely (Fig. 1F).

Having determined that the effect of versican on glioma cell growth is mediated, at least in part, by its G3 domain, we sought to further characterize the molecular determinant(s) of the effect. Specifically, we tested whether the EGF-like motifs in G3 might play a role. We tested our hypothesis by using a G3 domain from which the EGF-like motifs had been removed (G3\(\Delta\)EGF). G3\(\Delta\)EGF construct was expressed in COS-7 cells (Fig. 2A), and culture medium containing G3\(\Delta\)EGF products was shown to have a weak inhibitory effect on cell growth (Fig. 2B), whereas purified G3\(\Delta\)EGF product produced a significant inhibitory effect on cell growth (Fig. 2C). The effect of G3\(\Delta\)EGF on cell proliferation was also obtained from colony formation assays. U87 cells transfected with G3\(\Delta\)EGF and a control vector were treated with Geneticin (1.5 mg/mL), and the cultures were maintained in this medium until individual colonies were formed. G3\(\Delta\)EGF transfection resulted in the formation of smaller colonies (Fig. 2D) than did transfection with control vector (Fig. 2D).

The results obtained from cell proliferation assays were confirmed by analyzing cell cycle progression. Overexpression of G3\(\Delta\)EGF caused arrest of a greater number of cells in G1 phase. A typical G3\(\Delta\)EGF-transfected cell line and a vector-transfected cell line are shown in Fig. 3A, in which 88% of G3\(\Delta\)EGF-transfected cells were arrested in G1 phase (7% in G2 phase and 4.7% in S phase). Only 65.5% of control vector-transfected cells were detected in G2 phase (24.8% in G1 phase and 9.7% in S phase). The effects of G3\(\Delta\)EGF expression on two cell cycle proteins are shown in Fig. 3B. Cell lysate harvested from
transfected cells was analyzed on Western blots probed with antibodies against cdk4 and cyclin D1 (Santa Cruz). Levels of cyclin D1 and cdk4 decreased dramatically in G3ΔEGF-transfected cell lines. The structure of nuclei was then examined, and it was observed that each nucleus of the vector-transfected cells contained one or two nucleoli (Fig. 3C), whereas each nucleus of the G3ΔEGF-transfected cells contained multiple nucleoli (Fig. 3D).

**Interaction of G3 Domain with Glioma Cell Surface**—There are a number of mechanisms by which the versicanΔEGF and G3ΔEGF constructs could exert a dominant-negative effect on cell growth. The simplest explanation is that these mutant gene products are able to bind to sites on the glioma cell surface and thus successfully block the proliferative effects of endogenous versican. Because the G3ΔEGF construct was alone sufficient to exert this effect, we used it in these studies to minimize complications arising from potential cell surface binding sites present in other versican domains (e.g. G1). We first demonstrated that the full-length G3 products were able to bind to the cell surface. Glioma cells were incubated in growth media from G3- and vector-transfected COS-7 cells. After extensive washing, cell lysate was harvested and analyzed on Western blot. G3 bound to the glioma cell surface, resulting in detection of a G3 band in the cell lysate (Fig. 4A). Using the same methods, we demonstrated that G3 produced by bacteria and added exogenously to U87 cells was also able to bind to the cell surface (Fig. 4A).

To test whether G3ΔEGF products were able to bind to glioma cells, U87 cells were incubated with growth media from COS-7 cells transfected with either G3ΔEGF or G3 construct, both of which were well expressed (Fig. 4B). The cells were washed extensively and lysed for Western blot analysis. The G3 signal was significantly more intense than that of G3ΔEGF, indicating that G3 had a higher affinity for the glioma cell surface (Fig. 4B). The media from G3- and G3ΔEGF-transfected cells were mixed with the cell lysate of bacteria expressing G3. The mixture was incubated with glioma cells, and the amount of His-tagged bacterial G3 product remaining on the cells was assessed. G3 and G3ΔEGF from COS-7 cells inhibited the binding of bacterial G3 product in a dose-dependent manner (Fig. 4C).

The finding that deletion of the EGF-like motifs from the mini-versican and the G3 construct has a dominant-negative effect on cell growth suggests that, at high concentrations, the products of the versicanΔEGF and G3ΔEGF constructs were able to compete with endogenous versican for binding sites on glioma cells, although their binding is apparently weaker. We tested whether G3ΔEGF could compete with G3 to bind to glioma cell surface. G3 was mixed, at a fixed concentration, with varying amounts of G3ΔEGF, and the mixtures were incubated with glioma cells. High concentration of G3ΔEGF inhibited G3 binding to glioma cell surface (Fig. 4D).

**Inhibition of Versican Secretion by the G3ΔEGF Construct**—To further characterize the mechanism of dominant-negative effect of G3ΔEGF on cell growth, we examined the expression of endogenous versican in cell lines stably transfected with the G3ΔEGF construct and the control vector. Interestingly, we observed that cells stably transfected with G3ΔEGF had a higher level of versican in their cytoplasm, as revealed by labeling with polyclonal anti-versican antibody (Fig. 5A) as compared with cells transfected with the control vector (Fig. 5B). This finding raised the possibility that the G3ΔEGF construct had no effect on the transcription and translation of endogenous versican, but in fact inhibited its post-translational processing. To test this, we analyzed culture media from cells stably transfected with G3ΔEGF or the vector on Western blot. Cells transfected with the vector did indeed secrete higher levels of versican into the growth medium as compared with the G3ΔEGF-transfected cells (Fig. 5C). It was then necessary to examine whether expression of G3ΔEGF had any effect on transcriptional regulation of the endogenous versican gene. RT-PCR was performed using RNA from astrocytoma cell lines transfected with G3ΔEGF or the vector. Levels of RT-PCR products were similar in both cell lines, implying...
that G3ΔEGF had no effect on versican transcription (Fig. 5D).

To further confirm the effect of G3ΔEGF on proteoglycan secretion, we co-transfected COS-7 cells with a mini-versican construct and one of the following three constructs: G3ΔEGF construct, CD44, or a control vector. Cell lysate and culture media were analyzed on Western blot probed with 4B6, which recognizes an epitope present in the G3ΔEGF and mini-versican. Cells co-transfected with the mini-versican/G3ΔEGF construct and one of the following constructs: G3ΔEGF, CD44, or a control vector. Cell lysate and culture media were analyzed on Western blot probed with 4B6 (Fig. 6A). Expression of CD44 was confirmed in cell lysate probed with a monoclonal antibody against CD44. The above G3ΔEGF-transfected cell lines were selected with Geneticin (1.5 mg/ml), and we only used those cell lines expressing high levels of G3ΔEGF. It is obvious that the effect of G3ΔEGF on cell proliferation depends on the levels of G3ΔEGF expression. To further confirm this, cell lines expressing low levels of G3ΔEGF were selected with low levels of Geneticin (0.5 mg/ml). Most of these cell lines expressed low levels of G3ΔEGF. Three cell lines expressing low levels of G3ΔEGF and three cell lines expressing high levels of G3ΔEGF (shown in Fig. 7A) were used for cell proliferation assay. Cell lines expressing high levels of G3ΔEGF had higher levels of inhibitory effect on cell proliferation as compared with the vector control, whereas cell lines expressing low level of G3ΔEGF had moderate inhibitory effect on proliferation (Fig. 7C). The former also had a significant inhibitory effect on cell elongation compared with the control, whereas the latter had a median effect (Fig. 7D).

The Effect of CRD and CBP Expression on Cell Proliferation—To dissect the motif in the G3ΔEGF construct that inhibited cell proliferation, constructs containing either CRD motif or CBP motif were produced as shown in Fig. 1A. Cell lines expressing CRD and CBP were selected, and their effects on cell proliferation were examined. Expression of CRD and CBP constructs were tested on Western blot probed with 4B6 (Fig. 8A). The cell lines expressing CRD had an inhibitory effect on cell proliferation as compared with the vector control, whereas expression of CBP had little effect on proliferation (Fig. 8C). Similarly, only the cell lines expressing CRD had a moderate effect on the alteration of cell morphology (Fig. 8D).

**DISCUSSION**

Versican is highly expressed in various tissues where the cells are metabolically active and proliferating, such as in the mesenchymal tissues. In epidermis, versican is found only in the proliferating zone (26, 27). In cultured cells, versican is expressed only when cells are actively proliferating; once cells reach confluence, versican expression decreases (27). Therefore, it has long been suspected that versican is associated with the process of cell proliferation. Immunohistochemical studies have revealed that versican is expressed in brain tumors. This
study was designed to investigate the role of versican in tumor cell growth.

In the central nervous system, chondroitin sulfate proteoglycans constitute the major proteoglycan component in the extracellular matrix. Versican is known to associate with a number of molecules in the extracellular matrix such as hyaluronan (8), tenascin (9–11), fibronectin (29), fibulin (30), and CD44 (31). Versican is excluded from focal contact and its distribution is similar to hyaluronan, CD44, and tenascin. Interestingly, this same study demonstrated that tracks left by migratory fibroblasts on culture plates exhibited versican immunoreactivity (32). Thus, versican may be involved in cell invasion. In astrocytomas, however, the expression of versican is not restricted to the invasive borders but is present in all grades of astrocytomas, indicating that all the tumors examined possess some invasive potential. Whereas low grade tumors contain scattered individual cells with versican expression, high grade tumors have large clusters of versican-immunoreactive cells, probably the result of clonal expansion of cells with invasive potential. Previous studies (33) of astrocy-

FIG. 7. The effect of G3ΔEGF levels on cell morphology and proliferation. Western blot analysis (A) and RT-PCR (B) of three cell lines expressing high levels of G3ΔEGF (E1, E2, and E3), three cell lines expressing low levels of G3ΔEGF (E4, E5, and E6), and three cell lines transfected with the control vector (V1, V2, and V3). The amounts of proteins loaded in E4, E5, and E6 were 3-fold that of E1, E2, and E3. Cell proliferation (C) and cell morphology (D) were assayed in these nine cell lines (n = 3; **, p < 0.01).

FIG. 8. The effect of CRD and CBP expression on cell proliferation. A, Western blot analysis of four cell lines expressing CRD, four cell lines expressing CBP, and one cell line transfected with the control vector (V). B, cell proliferation were assayed in these nine cell lines. C, cell morphology of cell lines transfected with CRD (D1, D2, D3, and D4) and CBP (P1, P2, P3, and P4) (n = 3; **, p < 0.01).
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Glioma cell lines have not been able to correlate the invasiveness of astrocytomas with their proliferative activity. But, clinically, grade III and IV astrocytomas with high proliferative activity are definitely highly invasive tumors that can infiltrate proximal tissue as well as the distant regions such as brain stem, the leptomeninges, and even the contralateral cerebral hemisphere through structures such as the corpus callosum. Our study suggests that there may be a correlation between astrocytoma proliferative activity and density of versican expression within these tumors, because we showed that versican can stimulate glioma cell growth. High histological grades or MIB-1 labeling index would be associated with a greater propensity to invade.

One other finding is the expression of versican in areas surrounding tumor necrosis. This suggests that the up-regulation of versican expression could be either associated with factors produced by necrotic tumor tissue such as cytokines (34) or linked to tumor necrosis.

Our studies made use of an in vitro system by using glioma cells transfected with a mini-versican construct. We demonstrated that the mini-versican construct promoted glioma cell proliferation and that this occurred through the G3 domain in the mini-versican gene. We have previously demonstrated that two EGF-like motifs in the mini-versican construct were involved in enhancing NIH 3T3 fibroblast proliferation. In this report, we found that deletion of the EGF-like motifs not only abolished the effect of mini-versican on cell growth, but the resultant mutant also exerted a dominant effect on glioma cell proliferation. The G3ΔEGF construct had the same effect. This indicated that motifs in G3 other than the EGF-like motifs were important in producing this effect. This result allowed us to use mutant G3 construct (G3ΔEGF) to investigate the mechanisms by which deletion of the EGF-like motifs from the mini-versican generated a dominant-negative effect on glioma cell proliferation.

The products of the G3ΔEGF construct was small enough to analyze even trace amount of G3ΔEGF product in Western blot assay. This has made the cell surface binding assay possible because the levels of protein bound to the cell surface was very low in some cases. Low levels of mini-versican, which migrated as a smear in the gel of SDS-PAGE, were impossible to be detected on Western blot. As well, in the assay that the G3ΔEGF construct inhibited endogenous versican secretion, the small G3ΔEGF products were easily separated from the endogenous versican. Otherwise, we would see the versicanΔEGF products overlapped by the endogenous versican. In the COS-7 cell transfection assays, we also benefited from the fact that the small mutant construct G3ΔEGF was able to inhibit the secretion of the mini-versican products.

We have previously demonstrated that a mini-versican construct also enhanced the growth of NIH 3T3 cells (17) and chicken chondrocytes (35). Deletion of two EGF-like motifs from the mini-versican significantly reduced the effect of the mini-versican on cell proliferation but did not completely abolish this effect. A dominant-negative effect on NIH 3T3 cell proliferation was not seen. Perhaps in NIH 3T3 cells, the endogenous versican has only a minimal effect on cell growth. Consequently, expression of exogenous mini-versican enhanced cell proliferation, but deletion of the EGF-like motifs did not result in a dominant-negative effect. In the studies reported here, we demonstrated that deletion of the EGF-like motifs from the mini-versican or the G3 construct produced a dominant-negative effect on glioma cell proliferation. Thus, this effect is apparent specific to the glioma U87 cell line. In glioma cells, endogenous versican probably plays an important functional role in enhancing cell proliferation, and the mutant constructs likely interfere with a process that is crucial for cell proliferation. In other cell types this process may be less crucial or nonexistent, and so the effect of the mutant versican is less profound.

In cell surface binding assays, we demonstrated that the products of mutant G3 construct (G3ΔEGF) were bound to the glioma cell surface. High levels of G3ΔEGF were able to inhibit the interactions of native G3 products with the cell. Thus, the G3ΔEGF mutant may suppress the role of endogenous versican in enhancing cell proliferation by hindering its interaction with the cell surface. Other G3 motifs such as CRD and/or CBP may bind to the cell surface, and this binding may promote interaction of the EGF-like motifs with molecules on the cell surface such as signal transduction molecules (e.g. the EGF receptor EGFR). The mutant G3ΔEGF, which still contains CRD and CBP regions, would retain a binding ability but be inactive, because it lacks EGF-like motifs. G3ΔEGF would thus compete with endogenous versican for binding. This represents a potential molecular mechanism to account for the dominant-negative effect of G3ΔEGF.

Another possible explanation for the dominant-negative effect is that the G3ΔEGF products might inhibit the production of endogenous versican. To test this, we analyzed the secretion of endogenous versican and observed that less versican was secreted from cells transfected with G3ΔEGF, compared with control. Immunostaining revealed that the endogenous versican was synthesized at similar levels in both types of cells. Thus, it appears that G3ΔEGF expression does not inhibit the synthesis of endogenous versican but does suppress its secretion. This was further confirmed in co-transfection studies. In COS-7 cells co-transfected with G3ΔEGF and mini-versican, secretion of the mini-versican was inhibited, but synthesis was not affected. These results strongly suggested that the mutant G3ΔEGF construct plays a dominant-negative effect on cell proliferation through suppressing the secretion of endogenous versican, and this represents a second mechanism for dominant-negative effect of the mutant G3ΔEGF. This was further confirmed in our study that only those cell lines expressing high levels of G3ΔEGF had a lower rate of proliferation and shortened cell morphology. On the other hand, the cell lines expressing low levels of G3ΔEGF had little effect on cell proliferation and morphology. Because the major motifs in the G3ΔEGF construct are CRD and CBP, their effect on cell proliferation was investigated, and our studies suggested that the inhibitory effects of G3ΔEGF on cell proliferation and morphology occurred via the CRD motif. The effect of CBP motif on cell activity was not clear. Our previous study indicated that CBP plays a role in glycosaminoglycan chain attachment and proteoglycan secretion (28, 36).

Our studies have demonstrated two possible mechanisms that may underlie the dominant-negative effect of G3ΔEGF on glioma cell proliferation: competition from cell surface binding sites and suppression of secretion of endogenous versican. We cannot exclude a third possibility: that G3ΔEGF binds to tenascin-C, a molecule that is believed to play a role in cell proliferation. It has been shown that the CRD motif can bind to tenascin-C (10, 11). The effect of this binding on cell proliferation awaits further investigation.

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