β₁-Integrin-mediated Glioma Cell Adhesion and Free Radical-induced Apoptosis Are Regulated by Binding to a C-terminal Domain of PG-M/Versican

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Integrins are cell-surface glycoproteins that mediate cell activities, including tissue morphogenesis, development, immune response, and cancer, through interaction with extracellular proteins. Here we report a novel means by which integrin signaling and functions are regulated. In pull-down assays and immunoprecipitation, β₁-integrin bound to the C-terminal domain of PG-M/versican, an extracellular chondroitin sulfate proteoglycan. This was confirmed by cell-surface binding assays. Binding was calcium- and manganese-dependent. Upon native gel electrophoresis, β₁-integrin cosedimented with the C-terminal domain of PG-M/versican. The interaction of β₁-integrin with the C-terminal domain of PG-M/versican activated focal adhesion kinase, enhanced integrin expression, and promoted cell adhesion. As a result, cells expressing the C-terminal domain of PG-M/versican were resistant to free radical-induced apoptosis. As the PG-M/versican peptide used in this study does not contain the RGD consensus-binding motif for integrins, the mechanism of the observed binding represents an entirely new function.

Brain tumors (15–17). This proteoglycan is made up of a hyaluronan-binding N terminus (G1 domain) and a selectin-like C terminus (G3 domain) with a central sequence for glycosaminoglycan chain modification (14, 18, 19). The G1 domain is composed of an immunoglobulin-like motif and two proteoglycan tandem repeats (11, 14). PG-M/versican is known to associate with a number of molecules in the ECM and on the cell surface, including hyaluronan (20), tenasin (24, 25), fibulin-1 and fibulin-2 (26, 27), fibronectin (28), and CD44 and L-selectin (29). Binding of the G1 domain to hyaluronan affects cell adhesion (20, 30). Our previous studies also demonstrated that PG-M/versican stimulates cell proliferation and migration and inhibits cell adhesion and differentiation (30–35), but the signaling pathway for this role is not known. Recently, we observed that a PG-M/versican mutant exerts a dominant-negative effect on cell proliferation by inhibiting secretion of endogenous PG-M/versican and can interact with intact astrocytoma cells (33). Our studies suggest that PG-M/versican functions through binding to cell-surface receptors. This study was designed to investigate whether PG-M/versican can act via an integrin-mediated pathway. We show here that PG-M/versican interacts with β₁-integrin. This interaction activates focal adhesion kinase (FAK) activity, mediates cell adhesion, and protects the cells from free radical-induced cell apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Lipofectin, Geneticin (G418), Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Hanks’ balanced salt solution, and trypsin/EDTA were from Invitrogen (Burlington, Ontario, Canada). The ECL Western blot detection kit was from Amersham Biosciences. Horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit secondary antibodies, and all chemicals were from Sigma. Tissue culture plates were from Nunc Inc. Oligonucleotides were synthesized by BioBasic Inc. (Scarborough, Ontario). Astrocytoma U87 and COS-7 cells were obtained from 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.

Protein-Protein Interaction—The cell lysate was prepared from the astrocytoma cell line U87 stably transfected with the mini-versican construct or the G3ΔEGF construct (the versican G3 domain lacking two EGF-like motifs). Both constructs contain a His tag that binds to Ni²⁺-NTA resin. The lysis buffer contained 20 mM Tris-Cl (pH 8.0), 300 mM NaCl, 30 mM imidazole, 1 mM MnCl₂, 1 mM CaCl₂, and 1.5% Triton X-100. After lysis, the cell extract was subjected to centrifugation at 10,000 × g for 30 min in a cold room. The supernatant was incubated overnight with Ni²⁺-NTA beads at 4 °C under native conditions (QIA-GEN purification system). The beads were washed with washing buffer containing 45 mM imidazole and eluted with elution buffer. The eluate was precipitated with trichloroacetic acid, boiled in 1× protein loading...
dye for 5 min, subjected to Western blot analysis, and probed with anti-β3-integrin antibody JB1a (Chemicon International, Inc.).

**Immunoprecipitations**—The cell lysate was prepared in lysis buffer for protein interaction (20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml phenylmethylsulfonyl fluoride), followed by incubation with protein G that had been presaturated overnight with anti-FAK monoclonal antibody (Transduction Laboratories) at 4 °C. After washing, the beads were boiled in 1× protein loading dye for 5 min, followed by Western blot analysis and probing with anti-FAK or anti-phosphotyrosine (clone PY-20, Transduction Laboratories) at 37 °C and 1–1.5 h as described in the figure legends. Unattached cells were removed by washing, and the remaining cells were photographed. Ni-NTA-purified His6-G3 was also used to coat six-well plates. The coated wells were washed with DMEM, followed by incubation with vector-transfected cells (2 × 10⁵ cells) in DMEM at 37 °C for 7 h. The cultures were photographed, and attached cells were counted.

To test whether cell adhesion is proportional to a decreasing concentration of G3ΔEGF product, the culture media collected from vector- and G3ΔEGF-transfected U87 cells were diluted with phosphate-buffered saline to 100, 50, 25, 12.5, and 6.25% and used to coat six-well tissue culture dishes at 4 °C overnight. U87 cells (2 × 10⁵ cells/well) were seeded on the wells in DMEM containing 1% FBS and incubated at 37 °C for 1 h. The medium was removed, and the cells were washed with phosphate-buffered saline. The cells remaining on the plate were counted.

**Blocking Assay**—Vector-transfected cells (1.5 × 10⁵ cells) were incubated in 100 μl of DMEM in the presence or absence of antibody JB1a at a concentration of 30 μg/ml at room temperature for 30 min. The cells were then seeded on 96-well culture plates precoated with purified His6-G3 and maintained at 37 °C for 7 h. Unattached cells were removed by washing. The remaining cells were photographed, and cell attachment was determined by cell counting.

**Competition Assay**—Nylon membranes were incubated with the U87 cell lysate (membrane preparation) or heat-denatured bovine serum at room temperature for 1 h, followed by blocking with heat-denatured bovine serum at room temperature for 1 h. After washing, the nylon membranes were incubated at room temperature with the culture medium from G3ΔEGF-transfected U87 cells for 1 h to absorb G3ΔEGF. The treated medium, untreated G3ΔEGF-containing medium, or medium containing BSA was incubated with vector-transfected U87 cells for attachment assays as described above.

U87 cells (5 × 10⁵ cells) were also incubated with the culture medium containing purified His6-G3 or eluate from the vector control in the presence of 2 μg/ml each CaCl₂ and MnCl₂ at room temperature for 30 min. The cells were then inoculated on 12-well tissue culture dishes precoated with purified His6-G3 and maintained at 37 °C for 7 h. Unattached cells were removed, and attached cells were photographed and counted.

**Apoptosis Assay**—U87 cells (2 × 10⁵ cells/well) in monolayer culture on six-well plates or in suspension culture were treated with H₂O₂ (at 1.5 mM for 6 h or at 0.1 mM for 48 h, respectively). Trypan blue staining was used to determine viability. Cells were also subjected to annexin V-FITC staining (15 min in the dark) using an annexin V apoptosis detection kit (Santa Cruz Biotechnology, Inc.) to monitor apoptosis as described (37, 38).

**RESULTS**

**β3-Integrin Binds to the C-Terminal Domain of PG-M/Versican**—A mini-versican (Fig. 1A) containing an engineered His₃ tag was stably expressed in human astrocytoma cell line U87. The His₃ tag was purified on Ni-NTA affinity columns and dialyzed overnight against TBS containing CaCl₂ at room temperature. Equal numbers of cells were incubated with 0.1% BSA in Hanks’ balanced salt solution as a negative control. The cells were washed three times with cold TBS containing CaCl₂ and MnCl₂ (2 mM each), followed by antibody 46B6 staining as described above.

**Action of PAK**—Cell culture plates were precoted with the culture medium from vector- or G3ΔEGF-transfected cells or with purified His₃-G3. Plates were then seeded with cells transfected with G3ΔEGF or the vector control and incubated at 37 °C for 1 h. The cell lysate was prepared in lysis buffer for phosphorylation assay (50 mM HEPES (pH 7.5), 0.5% Triton X-100, 100 mM NaF, 10 mM sodium phosphate, 4 mM EDTA, 2 mM sodium vanadate, 2 mM molybdate, 2 mM ATP, 2 μM aprotinin, 2 μM leupeptin, and 2 μM phenylmethylsulfonyl fluoride), followed by incubation with protein G that had been presaturated overnight with anti-FAK monoclonal antibody (Transduction Laboratories) at 4 °C. After washing, the beads were boiled in 1× protein loading dye for 5 min, followed by Western blot analysis and probing with anti-FAK or anti-phosphotyrosine (clone PY-20, Transduction Laboratories) at 37 °C and 1–1.5 h as described in the figure legends. Unattached cells were removed by washing, and the remaining cells were photographed. Ni-NTA-purified His6-G3 was also used to coat six-well plates. The coated wells were washed with DMEM, followed by incubation with vector-transfected cells (2 × 10⁵ cells) in DMEM at 37 °C for 7 h. The cultures were photographed, and attached cells were counted.

**Adhesion Assay**—Vector-transfected cells (2 × 10⁵ cells) were seeded on six-well plates precoated with the culture medium from vector- or G3ΔEGF-transfected cells and incubated on the plates at 37 °C for 1–1.5 h as described in the figure legends. Unattached cells were removed by washing, and the remaining cells were photographed. Ni-NTA-purified His6-G3 was also used to coat six-well plates. The coated wells were washed with DMEM, followed by incubation with vector-transfected cells (2 × 10⁵ cells) in DMEM at 37 °C for 7 h. The cultures were photographed, and attached cells were counted.

**Western Blotting**—Protein samples were subjected to SDS-PAGE on separating gel containing 10–12% acrylamide. The buffer system was 1X Tris/glycine buffer (Amresco) containing 1% SDS. Separated proteins were transblotted onto a nitrocellulose membrane in 1X Tris/glycine containing 20% methanol and room. The membrane was blocked in TBS (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) containing 10% nonfat dry milk powder (TBSTM) for 30 min at room temperature to saturate nonspecific binding sites on the membrane. The membrane was then incubated overnight at 4 °C with monoclonal antibody 46B6 (which recognizes an epitope at the leading peptide of the link protein, which was engineered in all recombinant constructs) or with the anti-His tag monoclonal antibody prepared in TBSTM. The membranes were washed with TBS (3 × 30-min washes) and then incubated for 2 h under horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:50,000 dilution) in TBSTM. After washing as described above, the bound antibodies were visualized by chemiluminescence (ECL kit).

**Protein Purification**—G3 recombinant proteins containing an N-terminal His tag were purified on Ni-NTA affinity columns (catalog no. 30230, Qiagen Inc., Chatsworth, CA) as previously described (31). Briefly, the G3 domain was amplified by PCR using two primers, 5′-aaagatcggacaggatccatgcaaa and 5′-aaagcatgcgcgccttgagtcctgc-3′, and the resulting construct contained a N-terminal NcoI His tag. The G3 construct encoded G3ΔEGF in Escherichia coli strain M15 using pQE30.

**Cell-surface Binding Assays**—G3ΔEGF- and vector-transfected cells harvested by trypsinization were washed three times with TBS (20 mM Tris-HCl and 150 mM NaCl (pH 7.4)) containing 2 mM CaCl₂ and probed with antibody 46B6 for 1 h on ice in the presence of 2 mM CaCl₂, followed by staining with FITC-conjugated secondary antibody for 1 h on ice in the dark. Cells were washed after each treatment. Binding of G3ΔEGF to the cell surface was analyzed by flow cytometry. Vector-transfected U87 cells were also incubated with the culture medium from G3ΔEGF- or vector-transfected cells at 37 °C for 1 h or with purified His₃-G3 (expressed by bacteria) at room temperature for 1 h to assess binding of G3ΔEGF or His₃-G3 products to cells. CaCl₂ and MnCl₂ were included in the system at a concentration of 2 mM each. Bacterially expressed His₃-G3 was purified on Ni-NTA affinity columns and dialyzed overnight against TBS containing CaCl₂ at room temperature. Equal numbers of cells were incubated with 0.1% BSA in Hanks’ balanced salt solution as a negative control. The cells were washed three times with cold TBS containing CaCl₂ and MnCl₂ (2 mM each), followed by antibody 46B6 staining as described above.
sion of the mini-versican was confirmed by Western blotting (Fig. 1C). Our previous studies suggested that U87 cells interact with a versican mutant construct containing only the CRD and CBP motifs (G3ΔEGF) expressed by COS-7 cells and with the versican G3 domain expressed by bacteria (HisG3) (33, 34). To examine the molecular sites of interaction at the subdomain level, the cell lysate prepared from G3ΔEGF-transfected cells was subjected to the pull-down assay using Ni-NTA resin as described above. Probing with antibody JB1a indicated that β1-integrin coprecipitated with G3ΔEGF (Fig. 1, B and C). HisG3 expressed in bacteria was purified and incubated with intact U87 cells for 2 h, followed by pull-down assay. The results indicated that HisG3 bound to β1-integrin (Fig. 1D). To confirm this binding, co-immunoprecipitation was performed on the lysate from G3ΔEGF-transfected cells using monoclonal antibodies BJ1a and 4B6 (which recognizes an epitope at the leading peptide of G3ΔEGF). Consistent with the above results, β1-integrin was coprecipitated with G3ΔEGF (Fig. 1E), and G3ΔEGF was co-immunoprecipitated with antibody BJ1a (Fig. 1F). As a final experiment, the lysate prepared from G3ΔEGF- or vector-transfected cells was subjected to native gel electrophoresis and Western blotting and then probed with antibody JB1a or 4B6. Both antibodies detected bands with similar patterns, migrating at sizes ranging from 250 to 500 kDa (Fig. 1G). This indicated that the majority of the cell-associated G3ΔEGF interacts with β1-integrin under native conditions and suggested that versican is an important partner in regulating the integrin signaling pathway, although G3ΔEGF does not contain the RGD consensus-binding sequence for integrins (14).

To examine binding of G3ΔEGF to the cell surface, G3ΔEGF- and vector-transfected cells were analyzed by flow cytometry. Labeling with antibody 4B6 demonstrated that cells transfected with G3ΔEGF expressed and interacted with this protein strongly (77% binding) (Fig. 2A). When untransfected U87 cells were incubated with the culture medium from G3ΔEGF-or vector-transfected COS-7 cells, large amounts of bound G3ΔEGF were detected (51% binding) (Fig. 2B). Incubation with HisG3 purified from a bacterial expression system produced similar results (Fig. 2C). In the presence of either calcium (Fig. 2D) or manganese (Fig. 2E), binding of HisG3 to the cell surface was concentration-dependent. However, manganese had the greater effect on binding, and 5 mM manganese had the same effect on the binding activity as a combination of calcium and manganese (2 mM each) (data not shown). The binding activity disappeared when EDTA was added (Fig. 2F), confirming the requirement for calcium or manganese. Binding of G3ΔEGF to the cell surface was also confirmed by confocal microscopy. Labeling with antibody 4B6 showed that G3ΔEGF bound to the surface of G3ΔEGF-transfected U87 cells (Fig. 2G), but no staining was detected in vector-transfected cells (Fig. 2H).

The G3 Domain of PG-M/Versican Activates FAK and Enhances Cell Adhesion—To investigate the biological effect of versican-β1-integrin interaction, we examined its effect on FAK activity, the immediate intracellular step in the integrin-medi-
FIG. 2. Interactions of G3ΔEGF with the cell surface. Flow cytometry analysis indicated that the transfected cells expressed high levels of G3ΔEGF (red; 77% staining) compared with untransfected control cells (black; no staining) and vector-transfected cells (green; probed with secondary antibody alone) (A). The culture medium from G3ΔEGF-transfected COS-7 cells also interacted with the cell surface as indicated by the antibody 4B6 labeling (red; 51%) (B). The G3 domain expressed by bacteria (His₆-G3) also interacted with the U87 cell surface (red; 33% staining; green; background staining) (C). This binding was calcium-dependent (D) and manganese-dependent (E), and the interaction was abolished when EDTA (5 mM) was added (F). G3ΔEGF-transfected (G) and vector-transfected (H) U87 cells were immunostained with antibody 4B6 and examined with a confocal microscope. G3ΔEGF labeled the cell surface.

FIG. 3. G3ΔEGF induces FAK activity. The cell lysate prepared from U87 cells incubated with the culture medium from G3ΔEGF- or vector-transfected U87 cells was incubated with anti-FAK antibody-saturated protein G beads. The precipitates were analyzed by Western blotting and probed with PY-20, an anti-phosphotyrosine antibody. G3ΔEGF transfection enhanced FAK phosphorylation. The cell lysate prepared from U87 cells treated with or without His₆-G3 was also subjected to anti-FAK immunoprecipitation (IP). Treatment with His₆-G3 enhanced FAK phosphorylation, but FAK expression remained similar in treated and untreated cells.

FIG. 4. G3ΔEGF enhances cell adhesion. In cell attachment assays, G3ΔEGF- and vector-transfected cells were cultured in DMEM containing 10% FBS on tissue culture plates for 1.5 h. G3ΔEGF-transfected cells attached and spread on the plates (A), but vector-transfected cells did not (B). G3ΔEGF- and vector-transfected cells were cultured in DMEM containing 10% FBS on bacterial Petri dishes for 4 days. G3ΔEGF-transfected cells attached to the dishes and grew as monolayer cultures (C), whereas vector-transfected cells aggregated and remained in suspension (D). Tissue culture plates coated with the G3ΔEGF-containing medium (E) or purified His₆-G3 (F) enhanced cell attachment compared with plates coated with the medium from vector-transfected cells (G) or BSA alone (H).

FIG. 5. PG-M/Versican Binds to Integrin 12297
Dilution of the G3ΔEGF product reduced its ability to enhance cell attachment (Fig. 5E).

To test the function of mini-versican and G3ΔEGF in the attachment of different cell types, we compared their effects on the attachment of U87 cells, NIH3T3 fibroblasts, the MDA-MB-231 breast cancer cell line, and the Jurkat tumor cell line. The products of mini-versican and G3ΔEGF enhanced the attachment of all types of cells (Fig. 6A). Both mini-versican and G3ΔEGF had the highest activity to enhance the attachment of U87 cells, but the lowest activity to enhance the attachment of Jurkat cells. The enhancement by these products of the attachment of NIH3T3 fibroblasts is shown in Fig. 6 (B–D).

In blocking assays, U87 cells pretreated with or without anti-integrin antibody were seeded on plates precoated with purified His$_6$-G3. Antibody treatment reduced cell attachment (Fig. 7A). In competition assays, U87 cells were also incubated with the G3ΔEGF-containing medium or the G3ΔEGF-containing medium pre-absorbed by integrin. Integrin treatment partially abolished the enhanced adhesion effects of the G3ΔEGF-containing medium (Fig. 7B). Purified His$_6$-G3 or eluate from the vector control was incubated with U87 cells before they were seeded on His$_6$-G3-coated plates (24), and the incubation of free His$_6$-G3 inhibited cell attachment (Fig. 7C). U87 cells were also incubated with the G3ΔEGF-containing medium in the presence (Fig. 7D) or absence (Fig. 7E) of free His$_6$-G3, followed by immunostaining with antibody 4B6. Treatment with free His$_6$-G3 reduced G3ΔEGF binding to the cells. Taken together, our results provide strong evidence that G3ΔEGF or purified His$_6$-G3 plays an important role in enhanced cell adhesion through binding to integrin and activation of FAK.

**The G3 Domain of PG-M/Versican Enhances β$_1$-Integrin Expression and Protects Cells from Free Radical-induced Apoptosis**—As integrin expression is always associated with cell adhesion, we examined whether G3ΔEGF-induced cell adhesion affects integrin expression. G3ΔEGF- and vector-transfected U87 cells were seeded on tissue culture plates for 1, 2, 3, and 24 h. Analysis of integrin expression by Western blotting indicated that G3ΔEGF-transfected cells expressed higher levels of integrin during cellular attachment compared with vector-transfected cells (Fig. 8). However, no difference could be detected after 24 h of cell inoculation, when both types of cells were well spread on the plates.

As integrin-mediated cell adhesion is essential for cell survival (1–7), we investigated whether G3ΔEGF-induced cell adhesion and integrin expression can promote cell viability. We used the free radical H$_2$O$_2$ to induce cell death and examined the effects of G3ΔEGF. H$_2$O$_2$ was added to monolayer and suspension cultures of G3ΔEGF- and vector-transfected cells maintained in DMEM containing 10% FBS. H$_2$O$_2$-induced apoptosis was reduced in G3ΔEGF-transfected cells as measured by cell survival (Fig. 9, A and B) and by labeling with annexin
In a blocking assay, treatment with antibody JB1a reduced cell attachment to His6-G3-coated plates (Fig. 7A). In competition assays, U87 cells were incubated with one of the following: G3C/V (Fig. 9, panel A, and Fig. 12B, D), or medium containing BSA. Treatment with free His6-G3 reduced cell attachment (Fig. 7B), or medium containing BSA. Treatment with integrin antibody against an epitope located at the leading peptide of G3ΔEGF was able to coprecipitate G3ΔEGF and β1-integrin. Similarly, antibody against β1-integrin was able to coprecipitate the G3ΔEGF product. Integrins have been well known to interact with ECM molecules such as fibronectin, collagen, and vitronectin and with soluble ligands such as fibrinogen (39). Integrins also interact with other cell-surface receptors such as ICAM (intercellular adhesion molecule) and tetraspan (40, 41). The chondroitin sulfate proteoglycan PG-M/versican has been shown to interact with a number of ECM and cell-surface glycoproteins, including hyaluronan, fibronectin, tenascin, fibulin-1, fibulin-2, CD44, and L-selectin (20, 24–28). However, the binding activity reported here represents the first observation of interaction between PG-M/versican and integrin. Furthermore, a well known consensus-binding sequence for integrins is the RGD peptide (8, 9). The PG-M/versican G3 domain does not contain an RGD peptide; thus, the binding observed in this study may represent a novel mechanism of integrin binding.

The diverse binding activities of PG-M/versican are achieved via its multiple heterogeneous domains: hyaluronan binds to the N-terminal fragment of PG-M/versican; the CD44, L-selectin, and P-selectin interactions involve the glycosaminoglycan chains of PG-M/versican; and tenascin and fibulin-1 bind to the CRD motif of PG-M/versican. The minimal sequence present in all products studied here is G3ΔEGF, and our aim was to dissect the motif in G3ΔEGF (e.g., CRD or CBP) that interacts with β1-integrin. However, we did not obtain any binding activity with the CRD or CBP construct. Only in the presence of both CRD and CBP (the G3ΔEGF construct) was the interaction with β1-integrin observed. It seems that the binding requires the cooperation of CRD and CBP to generate the optimal conformation for binding to β1-integrin.

To test whether G3ΔEGF can directly interact with β1-integrin, we used yeast two-hybrid assays. We generated constructs containing different fragments of β1-integrin for the assays, but no positive result was obtained. It could be that the binding requires a certain conformational structure and/or glycosylation modification of β1-integrin that can be obtained only in mammalian cells. The binding might also require the presence of the partner of β1-integrin, the α chain. We have tried (α2 and α5) to identify the α chain involved without success. From the binding results, we could not exclude the possibility that the interaction of PG-M/versican with integrin requires other components in forming complexes for their binding. Nevertheless, our competition results that anti-β1-integrin antibody blocked cell attachment to G3 domain-coated plates suggest a direct interaction between β1-integrin and G3.

When the cell lysate prepared from cells stably transfected with the G3ΔEGF construct was subjected to native gel elec-
The stable U87 cell line transfected with G3 cells interacted with U87 cells, the binding affinities varied. Highest affinity for G3 suspension (His6-G3) in tissue culture plates precoated with purified transfected cells. U87 cells seeded on tissue-transfected cells, but not in G3 cells in DMEM containing 10% FBS and 0.1 mM H2O2 were seeded on tissue culture plates and incubated for 0 or 10 min or 4 h (E). Probing with antibody JB1a showed that integrin expression increased in G3EGF-transfected cells, but decreased in vector-transfected cells. G3EGF- and vector-transfected U87 cells were cultured on tissue culture plates in DMEM containing 10% FBS (F). After 24 h, H2O2 (0.1 mM) was added, and the treatment lasted for 30 min or 1 or 4 h. Integrin expression decreased in vector-transfected cells, but not in G3EGF-transfected cells. U87 cells seeded on tissue culture plates precoated with purified His-G3 (blue) or BSA (red) were treated with 1.5 mM H2O2 at 37°C for 4 h, followed by labeling with annexin V-FITC (G). The presence of His-G3 protected the cells from H2O2-induced apoptosis.

...and apoptosis assays. Plates coated with the E. coli-expressed G3 domain showed reduced cell attachment compared with those coated with the COS-7 cell-expressed product (Fig. 4), and the E. coli-expressed G3 product only partly protected U87 cells from free radical-induced cell apoptosis (Fig. 9). Nevertheless, these results indicated that the purified G3 product interacted with U87 cells, enhanced cell attachment, and reduced apoptosis of cells exposed to free radicals.

Although the products of mini-versican and G3EGF enhanced the attachment of different cell types, including U87 cells, NIH3T3 fibroblasts, MDA-MB-231 breast cancer cells, and Jurkat tumor cells, the degree of enhancement varied. This might be due to the difference in the expression levels of β1-integrin, which interacts with the G3 domain of versican. At the moment, it is not known which α chain of the integrins is required to combine with the β1 chain and binds versican. It is possible that only one α chain is involved in this binding. It is also not clear if other surface proteins of U87 cells play roles in binding to the versican G3 domain. Although CD44 has been reported to bind versican (29), it does not bind to the G3 domain.

It was interesting to observe that the expression of β1-integrin decreased greatly in vector-transfected U87 cells after free radical treatment. These data support the hypothesis that β1-integrin plays an important role in protecting the cells from free radical-induced apoptosis. However, the results shown did not exclude the possibility that the decreased detection levels upon Western blotting were due to an increase in protein degradation rather than a decrease in protein synthesis. To examine this, we harvested proteins in the culture medium by trichloroacetic acid precipitation and analyzed them by Western blotting. We did not detect a difference in the degraded fragment of β1-integrin (data not shown). It seems that in the presence of free radical, integrin expression was reduced, although degradation of the protein was not excluded. In all of these cases, the presence of G3EGF elevated the levels of cell-associated β1-integrin in addition to increasing signaling, and this protected the cells from free radical-induced apoptosis. This was further demonstrated by coating the culture plate with the purified G3 domain, which decreased cell apoptosis in response to free radical treatment.
PG-M/Versican Binds to Integrin

In summary, our study has demonstrated that G3ΔEGF interacts with β1-integrin and binds to the cell surface. These interactions are correlated with FAK activation, enhanced cell adhesion, and protection of cells from free radical-induced apoptosis. The exact molecular mechanism of this binding and its physiological significance are prospects for future study. It has been established that PG-M/versican also binds to hyaluronan in vivo: the PG-M/versican-hyaluronan interaction (via the G1 domain) may counteract the effects of the G3 domain-integrin interaction. When the G1 domain is not present, the G3-integrin interaction increases cell adhesion and therefore promotes cell survival. These counteractive effects of the G1 and G3 domains have also been extensively studied in the product processing of versican and aggrecan (43–49). It would be interesting to understand how the G1 and G3 domains modulate the functions of these chondroitin sulfate proteoglycans. Also of great importance is the fact that the G3 ΔEGF-integrin binding reported here represents an entirely new mechanism for integrin binding because G3ΔEGF does not contain an RGD sequence, the consensus-binding sequence for integrins. Determination of the sequence involved in the reported interaction will require further studies.

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