Glypican and Biglycan in the Nuclei of Neurons and Glioma Cells: Presence of Functional Nuclear Localization Signals and Dynamic Changes in Glypican During the Cell Cycle

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Abstract. We have investigated the expression patterns and subcellular localization in nervous tissue of glypican, a major glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan that is predominantly synthesized by neurons, and of biglycan, a small, leucine-rich chondroitin sulfate proteoglycan. By laser scanning confocal microscopy of rat central nervous tissue and C6 glioma cells, we found that a significant portion of the glypican and biglycan immunoreactivity colocalized with nuclear staining by propidium iodide and was also seen in isolated nuclei. In certain regions, staining was selective, insofar as glypican and biglycan immunoreactivity in the nucleus was seen predominantly in a subpopulation of large spinal cord neurons. The amino acid sequences of both proteoglycans contain potential nuclear localization signals, and these were demonstrated to be functional based on their ability to target β-galactosidase fusion proteins to the nuclei of transfected 293 cells. Nuclear localization of glypican β-galactosidase or Fc fusion proteins in transfected 293 cells and C6 glioma cells was greatly reduced or abolished after mutation of the basic amino acids or deletion of the sequence containing the nuclear localization signal, and no nuclear staining was seen in the case of heparan sulfate and chondroitin sulfate proteoglycans that do not possess a nuclear localization signal, such as syndecan-3 or decorin (which is closely related in structure to biglycan). Transfection of COS-1 cells with an epitope-tagged glypican cDNA demonstrated transport of the full-length proteoglycan to the nucleus, and there are also dynamic changes in the pattern of glypican immunoreactivity in the nucleus of C6 cells both during cell division and correlated with different phases of the cell cycle. Our data therefore suggest that in certain cells and central nervous system regions, glypican and biglycan may be involved in the regulation of cell division and survival by directly participating in nuclear processes.

GLYPICAN, whose primary structure was first reported based on its cloning from human lung fibroblasts (David et al., 1990), was the initial member of a rapidly expanding family of glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans that currently includes four other vertebrate proteins (cerebroglycan, Stipp et al., 1994; OCI-5, Filmus et al., 1995; K-glypican, Watanabe et al., 1995; and glypican-5, Veugelers et al., 1997) as well as the *dally* gene product of *Drosophila* (Nakato et al., 1995). In earlier biochemical studies of the brain (Klinger et al., 1985) and PC12 pheochromocytoma cells (Gowda et al., 1989), we described a major heparan sulfate proteoglycan that we later cloned and identified as the rat homologue of glypican (Karthikeyan et al., 1992). Immunocytochemical studies (Karthikeyan et al., 1994) demonstrated that glypican is present in the marginal layer (prospective white matter) and in the dorsal root entry zone of E13-16 spinal cord, as well as in the optic nerve and retina at this stage, but does not appear in significant levels in the brain until approximately E19. The proteoglycan shows a wide distribution in the gray matter and axonal projections of postnatal brain, including the hippocampal formation, the parallel fibers of cerebellar granular cells, and in the medulla and brainstem. Northern analysis demonstrated high levels of glypican mRNA in the brain, skeletal muscle, and in rat PC12 pheochromocytoma cells, and in situ hybridization histochemistry showed that glypican mRNA was especially prominent in cerebellar granule cells, large motor neurons in the brainstem, and CA3 pyramidal cells of the hippocampus (Karthikeyan et al., 1994). Based on these findings, we concluded that glypican is predominantly a neuronal membrane proteoglycan in the late embryonic

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and postnatal rat central nervous system, and in situ hybridization studies of glypican expression in adult rat nervous tissue led to similar conclusions (Litwack et al., 1994). Although glia do not appear to be a major source of glypican in the central nervous system, its mRNA has been detected in cultured oligodendroglia (Bansal et al., 1996), and glypican is present on peripheral nerve Schwann cells (Carey et al., 1993).

We have now extended our studies of glypican in nervous tissue to an analysis of its subcellular localization, and in view of evidence for a nuclear pool, we carried out parallel studies of biglycan, a small, leucine-rich chondroitin sulfate proteoglycan for which we have also found nuclear immunoreactivity. Biglycan and the closely related proteoglycan decorin (for reviews see Kresse et al., 1993; Iozzo and Murdoch, 1996) are most prominently associated with connective tissue and related elements such as endothelial cells, fibroblasts, and blood vessels, but they have also been detected in the central and peripheral nervous tissue. Immunocytochemical studies have shown that both decorin and biglycan occur in adult rat brain parenchyma and that their expression levels are increased after injury, although these changes in expression occur in somewhat different locations and follow different time courses (Stichel et al., 1995). Biglycan is synthesized by astrocytes (Koops et al., 1996), and has also been identified as the chondroitin sulfate proteoglycan secreted by meningeal cells that is capable, at nanomolar concentrations, of sustaining the survival of cultured rat neocortical neurons (Junghans et al., 1995). The potent neurotrophic activity of biglycan, even after its isolation and purification in the presence of denaturing agents such as 8 M urea and 4 M guanidine HCl, together with the nuclear localization demonstrated in the studies described here, suggest that in certain cells and central nervous system regions, both biglycan and glypican may be involved in the regulation of cell division and survival.

Materials and Methods

Antibodies

Antibodies were raised to amino acids 1–423 of glypican, expressed in pGEX2T as a glutathione *S*-transferase fusion protein from a Kspl-Kpnl restriction fragment of rat glypican cDNA (Karthikeyan et al., 1992), and then isolated by SDS-PAGE after thrombin cleavage of the fusion protein. For the initial immunization, rabbits were injected with crushed gel slices containing the recombinant glypican band, as well as with glypican transferred to a nitrocellulose membrane after electrophoresis and untransferred protein electroeluted from gel slices and emulsified in Freund's complete adjuvant (~100 μg total protein/rabbit). The first and second boosts (at 2-wk intervals, 40–50 μg protein/rabbit) used gel slices and nitrocellulose membrane, and only gel slices (~25 μg protein/rabbit) were used for subsequent boosts at 1-mo intervals. Antibody titers were followed by a dot-binding assay (Rauch et al., 1991) and showed serum dilution endpoints in the 1:1,000,000 range.

Antipeptide antibodies were raised to a COOH-terminal sequence of human and bovine biglycan (Roughley et al., 1993) in which all of the 13 biglycan residues are also identical to the corresponding rat sequence (Dreher et al., 1990). These antibodies do not cross-react with decorin (Roughley et al., 1993) and were used as affinity-purified IgG. A rabbit antiserum to decorin from cultured human skin fibroblasts (Glössl et al., 1984) was provided by Dr. Hans Kresse (University of Münster) and has been shown to also recognize rat decorin (Stichel et al., 1995). Affinity-purified rabbit antibodies to a 16-kD recombinant fragment of rat syndecan-3 (Carey et al., 1992) were provided by Dr. David Carey (Geisinger Clinic, Danville, PA). Monoclonal and rabbit polyclonal antibodies to *Escherichia coli* β-galactosidase were obtained from Promega (Madison, WI) and 5 Prime—3 Prime, Inc. (Boulder, CO), respectively, and were used for immunocytochemistry at a dilution of 1:500. mAbs to the HSV and human Fc tags were obtained from Novagen (Madison, WI) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively.

Electrophoresis and Western Blotting

Proteins were electrophoresed on 10% SDS-PAGE minigels and transferred to nitrocellulose membranes. After blocking in 5% BSA for 2 h at room temperature, membranes were incubated with primary antibodies at dilutions of 1:1,000 (glypican) or 1:500 (biglycan) for 1–1.5 h at room temperature or at 4°C overnight. Bound antibody was then detected using peroxidase-conjugated second antibody (1 hr at room temperature) followed by enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL).

Cell lysates or tissue homogenates were treated with heparitinase (E.C. 4.2.2.8; Seikagaku America, Rockville, MD) in 0.1 M Tris-HCl buffer (pH 7.2) containing protease inhibitors (Kato et al., 1985) for 4–6 h at 37°C, and treatment with protease-free chondrointinase ABC (Seikagaku America) was performed for 1 h at 37°C in 0.1 M Tris-HCl buffer (pH 8.0) in the presence of protease inhibitors.

Isolation of Nuclei

Cells were lysed in a hypotonic 10 mM Tris-HCl buffer (pH 7.2) containing protease inhibitors (1 μg/ml pepstatin A, 10 mM *N*-ethylmaleimide, 1 mM PMSF) by two freeze–thaw cycles at −70°C, and the pellet was washed once with the same buffer, followed by centrifugation for 5 min at 4°C. For isolation of nuclei (Mazzoni et al., 1992), the cells were allowed to stand for 2 min at room temperature in hypotonic buffer containing 2 mM $MgCl_2$, and then for 5 min at 4°C. NP-40 was added to a concentration of 0.5%, and the solution was passed twice through a 22-gauge needle. The $MgCl_2$ concentration was then adjusted to 5 mM, and the crude nuclei were pelleted by centrifugation (3 min, 700 *g*) followed by two washes with Tris buffer containing 5 mM $MgCl_2$. Possible contamination of the isolated nuclei with plasma membranes was minimized by mild trypsin treatment before cell lysis and by detergent treatment of the isolated nuclei. C6 cells were treated with trypsin (10 μg/ml) in serum-free F10 medium for 15 min at 37°C. Cell layers were then washed twice with cold PBS containing 50 μg/ml each of egg white trypsin inhibitor and tosyllysine chloromethyl ketone before isolation of nuclei, which were treated with 1% followed by 2% Triton X-100 before being plated on slides and fixed with methanol for immunocytochemistry.

Transfections

293 cells and COS-1 cells were plated overnight in 35-mm dishes and transfected with 0.5 μg DNA using Lipofectamine (GIBCO BRL, Gaithersburg, MD). After 6 h, the transfection medium was replaced with fresh complete medium for 6 h, and cells were moved overnight to 26-well slides (Cel-Line Associates, Newfield, NJ) before using for immunocytochemistry. Transfection of C6 cells followed the same procedure, with the exception that the recovery time on slides was decreased to 5–6 h.

Cell Synchronization and Flow Cytometry

For serum starvation, C6 cells were plated overnight in 60-mm dishes at 80–90% of confluency, and were then grown for 3 d in F10 medium containing 0.4% serum. For some experiments, basic fibroblast growth factor (bFGF)[1] (10 ng/ml) or cAMP (1 mM) was added for 1 d in the low serum medium. Serum-starved cells were released for different periods by replacing the low serum medium with complete medium. To obtain cells arrested at the G_1/S boundary, serum release was in the presence of 1 mM hydroxyurea for 24 h.

To determine the relative proportions of C6 cells in different phases of the cell cycle by flow cytometry, cells synchronized at different phases were collected and washed once with PBS. Thy were then resuspended in 200 μl of PBS, overlayed with 1.8 ml cold methanol followed by gentle mixing, and allowed to stand for 15 min at 4°C. The cells were then washed once with cold PBS, incubated for 0.5–1 h in the dark at 37°C in PBS containing 100 μg/ml RNase B and 20 μg/ml propidium iodide, and stored at 4°C.

1. *Abbreviation used in this paper*: bFGF, basic fibroblast growth factor.

Construction of Plasmids

To generate β-galactosidase fusion proteins, cDNAs encoding amino acids 356–423 of glypican and amino acids 70–147 of biglycan were amplified by PCR. A partial rat biglycan DNA (Dreher et al., 1990) was kindly provided by Dr. Kevin Dreher (U.S.-E.P.A., Research Triangle Park, NC). The sense and antisense primers used were 5′-TCTGAATTCGAGGAGAAGCGT-3′ and 5′-AACCTCGCCGGCTTACCGGCCCTT-3′ (for glypican) and 5′-CCCTGAATTCCGCAAGGAT-3′ and 5′-AATGCAGCCGGCTTACCGGAGCCC-3′ (for biglycan). Both antisense primers contained a stop codon. The PCR products were purified by Qiagen gel (QIAGEN Inc., Chatsworth, CA) and ligated into the COOH-terminal EcoRI site of the *LacZ* gene. PCR amplifications were performed for 30 cycles with denaturation for 30 s at 94°C, primer annealing for 1.5 min at 57°C, and extension for 1 min at 72°C. All constructs were in the pcDNA3 vector (Invitrogen, La Jolla, CA) under the control of a CMV promoter.

For mutation of the glypican basic cluster, the primers 5′-CCTGAATTCAACAGTAGCAGTGCCAAACTG-3′ (sense) and 5′AGTCCACATCATTTCCACC-3′ (antisense) were used (annealing, 56°C, 1.5 min; extension, 72°C, 1.5 min; 40 cycles). A deletion mutant was also constructed by PstI digestion to remove the sequence after the basic cluster that was mutated in the construct described above (see Fig. 11).

To construct a full-length glypican–Fc fusion protein including the signal peptide (designed pcGLYPFc), the cDNA encoding amino acids 424–499 of glypican was amplified by PCR (annealing, 55°C, 1.5 min; extension, 72°C, 1 min; 30 cycles) using as sense and antisense primers 5′-CCGCTGCTGGAATGGG-3′ and 5′-ACGGATCCACTTACCTGTACAGGCGTCATCT-3′, respectively. The PCR product and the BamHI-NotI fragment from the PIG-1 vector (kindly provided by Dr. James Salzer, New York University Medical Center) containing the human IgG1 Fc region were ligated into the KpnI site of the glypican cDNA.

The 68–amino acid glypican sequence used to produce the β-galactosidase fusion protein described above was deleted from the glypican–Fc fusion protein (construct designated pcGDFc) by a PCR reaction (annealing, 62°C, 1.5 min; extension, 72°C, 1.5 min; 40 cycles) using 5′-GGGGATGCCCCTCGAGAACTG-3′ and 5′-ACGCTGGTACCCAGGCCCAGAGCCATG-3′ as sense and antisense primers, respectively.

To produce an HSV epitope–tagged glypican, the NH_2-terminal signal sequence of glypican and the HSV-tag (Novagen) were amplified by PCR (annealing, 70°C, 1 min; extension, 72°C, 1 min; 30 cycles), and the product was ligated into the KspI site of glypican. The sense and antisense primers used were 5′-CGAATTCGCCATGGCCATCCGGGCCCGAG-3′ and 5′-CGAATCCCGCGGTCTTCCGGATCCTCTGGAGCGAGTTC-3′.

Immunocytochemistry

C6 cells were plated for 3–5 h on 26-well Cel-Line slides coated with poly-D-lysine (10 μg/ml, Sigma, mol wt >300,000; Sigma Immunochemicals, St. Louis, MO). After fixation for 10 min in 4% formalin or paraformaldehyde, cells were permeabilized with methanol for 2 min at room temperature, or in some cases, were treated directly with methanol without previous fixation. After fixation/permeabilization, the slides were rinsed with PBS and blocked with 1% BSA/PBS for 1 h at room temperature or overnight at 4°C, and were incubated with primary antibody for 1.5–2 h at room temperature (1:400 dilution for antiglypican and 1:200 for antibiglycan). The cells were washed again with PBS and incubated for 1 h at room temperature with FITC- or rhodamine-conjugated second antibody (1:100 dilution) before coverslipping under Vectashield (Vector Laboratories, Burlingame, CA).

For propidium iodide staining of nuclei, cells or tissue sections were blocked with BSA and treated for 20–30 min at 37°C with RNase B (1 mg/ml in PBS; Sigma). After incubation with the primary antibody and blocking, propidium iodide was mixed with the second antibody (at a concentration of 1 μg/ml for tissue sections and 20 μg/ml for cells) followed by staining for 1 h at room temperature.

Fresh frozen sections of adult spinal cord were mounted on slides and processed for immunocytochemistry as described by Meyer-Puttlitz et al. (1996). Confocal optical sections of cultured cells and spinal cord were recorded using a laser scanning microscope (Molecular Dynamics, Inc., Sunnyvale, CA). The 488-nm laser line was used for simultaneous propidium iodide and fluorescein staining, and in other cases, double immunofluorescence microscopy was performed using rhodamine and fluorescein. Vibratome sections were prepared from perfusion-fixed brain and stained with peroxidase-diaminobenzidine, as described previously (Rauch et al., 1991).

Results

Characterization of Antibodies to Glypican and Biglycan

The specificity of antibodies to glypican and biglycan was demonstrated by several methods. In rat brain homogenates and rat C6 glioma cells, antibodies to recombinant glypican recognized a major 64-kD glypican core protein

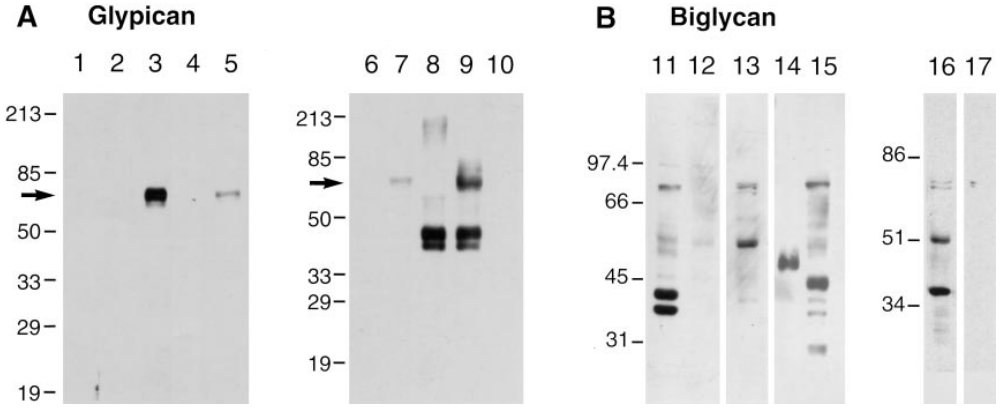


Figure 1. Reactivity of antisera to glypican and biglycan. (*A*) Glypican immunoreactivity in a C6 glioma cell lysate (lanes *1–3*), in isolated C6 cell nuclei (lanes *4* and *5*), in a homogenate of 1-mo postnatal rat brain (lanes *6* and *7*), and in a partially purified preparation of rat brain heparan sulfate proteoglycans (lanes *8–10*), which contains glypican and two proteolytic degradation products of its core protein (Karthikeyan et al., 1994). Samples in lanes *1, 3, 5, 7, 9,* and *10* have been treated with heparitinase, whereas those in lanes *2, 4, 6,* and *8* were incubated for the same period in buffer alone. Detection was by enhanced chemiluminescence after transfer to nitrocellulose, and antibody that had been preadsorbed with recombinant rat glypican gave no signal (lanes *1* and *10*). (*B*) Biglycan immunoreactivity in a chondroitinase-treated postnuclear supernatant fraction of C6 glioma cells in the absence (lane *11*) and presence (lane *12*) of the biglycan peptide used for immunization (100 μg/ml). Lane *13* shows biglycan immunoreactivity in purified C6 cell nuclei without chondroitinase treatment (additional bands at 38 and 42 kD were seen in postnuclear fractions of C6 cells), and lanes *14* and *15* show purified biglycan before and after incubation with a C6 cell lysate. Lanes *16* and *17* show the reactivity of proteins in a nuclear fraction of C6 cells with IgG purified on a peptide affinity matrix after subsequent adsorption to and elution (by 100 mM glycine, pH 2.5) from nitrocellulose-immobilized cartilage biglycan (lane *16*), compared with that of the unbound antibodies (lane *17*).

that was generated by heparitinase treatment, and this immunoreactivity could be abolished by adsorbing the antibodies with the glypican fusion protein (Fig. 1 *A*). Immunocytochemical staining was not seen using adsorbed serum (Figs. 2 and 3) or preimmune serum (data not shown).

Affinity-purified IgG raised to a synthetic peptide sequence from the COOH terminus of biglycan predominantly stained several bands on immunoblots of C6 cells (Fig. 1 *B*) and brain (data not shown) that apparently represent partial proteolysis products of the proteoglycan, insofar as they were smaller than the size of the biglycan core protein (Fig. 1, lane *11*), and bands of less than the expected size were also obtained without chondroitinase treatment (Fig. 1, lane *13*). The high susceptibility of biglycan to proteolytic degradation by C6 cell extracts was further demonstrated by incubating bovine cartilage biglycan (kindly provided by Dr. Lawrence Rosenberg, Montefiore Hospital and Medical Center, New York) with a C6 cell lysate in the presence of protease inhibitors under the conditions used for chondroitinase treatment (Fig. 1, lanes *14* and *15*).

In all cases, staining of immunoblots, cultured cells, and tissue sections was prevented by preincubation of the antibody with the biglycan peptide (Figs. 1, 3, and 4). The biglycan antibodies also recognized a doublet that migrated at ~75 kD, which is above the 51-kD biglycan core protein (Fig. 1 *B*). Although these appear to be proteolytic products of biglycan dimers (see below), it was possible that they were not derived from biglycan but instead represented other proteins that contained an epitope present in a neoprotein (i.e., the synthetic peptide immunogen) with an atypical linkage. We therefore demonstrated that adsorption of the antibodies with an equivalent molar concentration of authentic cartilage biglycan also abolished staining (Fig. 4 *J*).

In another experiment, IgG that had been purified on a peptide affinity column was further purified by binding to cartilage biglycan on a nitrocellulose membrane followed by elution with 100 mM glycine (pH 2.5). The eluted antibodies (after neutralization with 1 M Tris) still showed reactivity with the ~75-kD proteins in a nuclear fraction of C6 cells, whereas the unbound fraction produced no significant staining of immunoblots (Fig. 1, lanes *16* and *17*). In immunocytochemical studies, the bound and eluted antibodies retained the ability to strongly stain C6 cell nuclei, whereas the unbound fraction produced little or no nuclear staining (Fig. 5). These results indicate that all of the immunoreactive bands, including those larger than the biglycan core protein, are derived from biglycan. The biglycan core protein is known to self associate to dimers and higher oligomers under physiological conditions, and although based on Coomassie blue staining it would appear that aggregates of purified cartilage biglycan are dissociated on SDS-PAGE (Liu et al., 1994), our results suggest that (at least in tissue extracts) a small proportion of proteolytic products derived from these dimers, and that are resistant to dissociation by SDS, can be revealed by more sensitive immunochemical detection techniques.

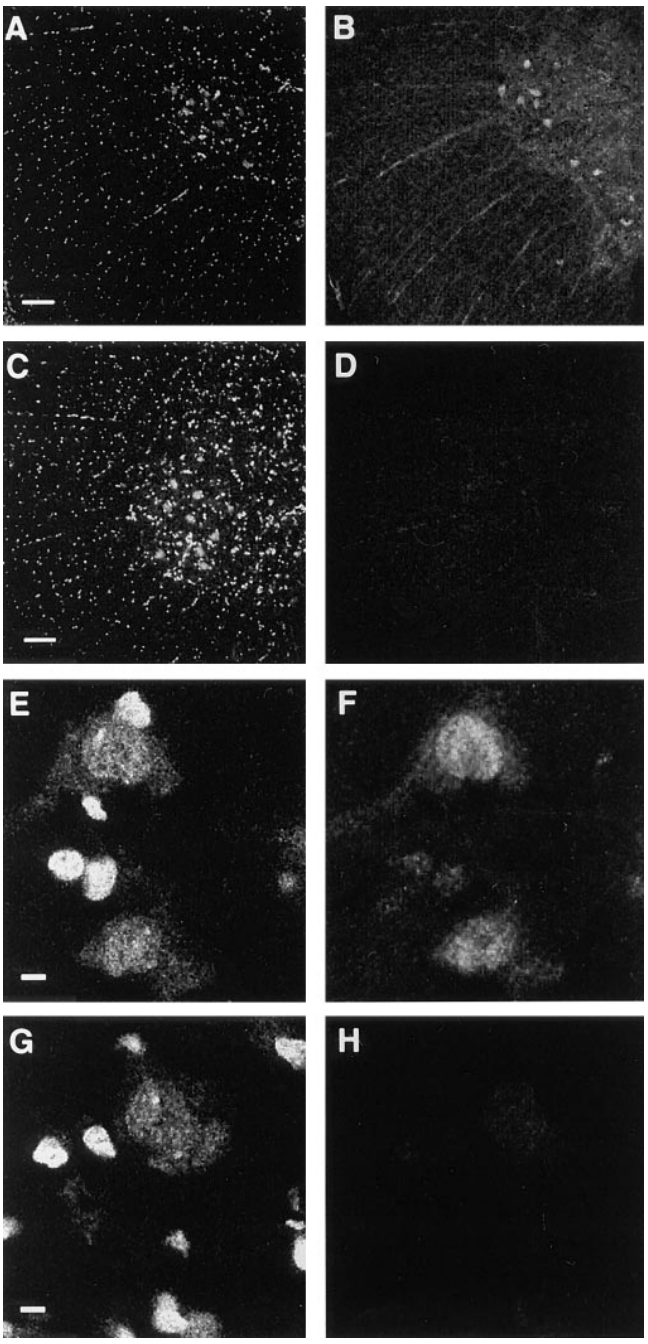


Figure 2. Confocal laser scanning photomicrographs of coronal sections of adult rat spinal cord stained with propidium iodide (*A, C, E,* and *G*) and antiglypican antibodies (*B, D, F,* and *H*). Only large neurons representing a small portion of the cells that can be identified by propidium iodide staining of their nuclei (seen as bright dots at lower magnification) also show nuclear glypican immunoreactivity. Very faint staining is seen using adsorbed antibody (*D* and *H*), and the nuclei of neurons showing glypican immunoreactivity are usually larger than average and stain weakly with propidium iodide. Bars, *A* and *C*, 100 μm; *E* and *G*, 5 μm.

Presence of Glypican and Biglycan in the Nucleus

Immunocytochemical studies of spinal cord, brain, and cultured cells showed unexpectedly that both glypican and biglycan immunoreactivity was present in the nucleus. We have previously demonstrated that in nervous tissue, glypican is a component of the neuronal plasma membrane (Karthikeyan et al., 1994), and it is known that the small,

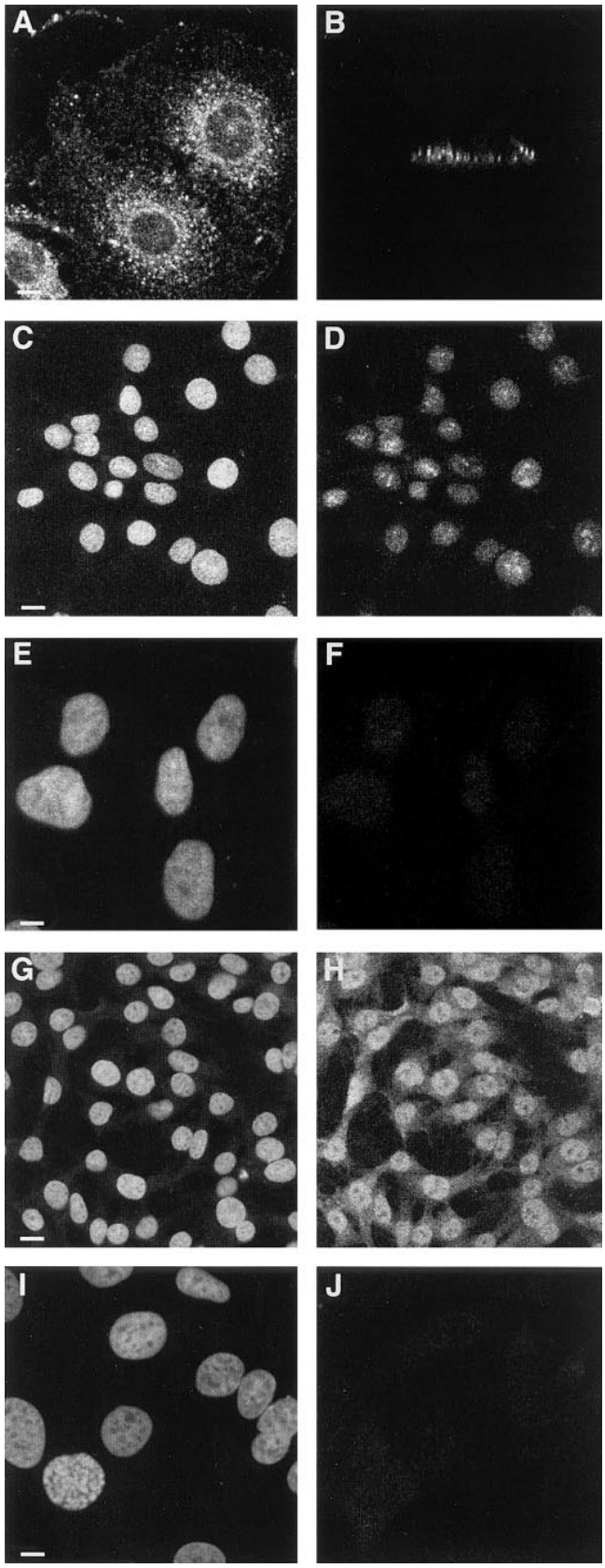


Figure 3. Rat C6 glioma cells stained with antibodies to either glypican (*A, B, D,* and *F*) or biglycan (*H* and *J*). Nuclei of the antibody-stained cells shown in *D, F, H*, and *J* were also stained with propidium iodide (*C, E, G,* and *I*). Glypican immunoreactivity in the plasma membrane is seen in horizontal and vertical sections of nonpermeabilized cells (*A* and *B*, respectively), whereas all other cells were permeabilized with methanol. In *F*, the antibodies were adsorbed with recombinant glypican before use, and in *J*, the antibiglycan antibodies were used in the presence of 25 μg/ml biglycan peptide. Bars, *A, E,* and *I*, 5 μm; *C* and *G*, 10 μm.

leucine-rich proteoglycan biglycan is secreted into the extracellular matrix or culture medium by fibroblasts and other cell types (Kresse et al., 1993). However, examination of coronal sections of adult rat spinal cord demonstrated that glypican and biglycan immunoreactivity was also present in a relatively small proportion of cell bodies and their nuclei, being found most frequently in large cells of the ventral gray matter that are probably motor neurons (Figs. 2 and 4). These cells have correspondingly large nuclei that were less intensely and uniformly stained by nuclear stains such as propidium iodide (Figs. 2 and 4) or DAPI in comparison with the nuclei of surrounding cells. In other central nervous system areas (e.g., the Purkinje cells and deep nuclei of the cerebellum, the brainstem, and the cerebral cortex, hippocampus, and thalamus), most cells showed nuclear staining of glypican or biglycan (Fig. 6).

Horizontal sections obtained by confocal laser scanning microscopy after combined immunostaining and propidium iodide staining of nuclei demonstrated that the nuclear staining was distinct from the cytoplasm and intracellular membranes (Figs. 2, 3, and 4). This conclusion was further supported both by examination of vertical sections, which showed that the glypican and biglycan immunoreactivity was present primarily in the nuclear matrix rather than in the surrounding membrane (data not shown), and by staining of isolated nuclei prepared by various types of detergent treatment (Fig. 7). However, the matrix usually showed a heterogeneous rather than uniform staining pattern, frequently with one or more intensely stained spots, some of which are probably nucleoli. No nuclear staining was observed with antibodies to *N*-syndecan/syndecan-3, another heparan sulfate proteoglycan of nervous tissue (Fig. 8), or to decorin, a small, leucine-rich chondroitin/dermatan sulfate proteoglycan with ~55% sequence identity to biglycan (data not shown).

Dynamics of Glypican Immunoreactivity in C6 Cell Nuclei

Although glypican immunoreactivity in interphase C6 cell nuclei was seen throughout the nuclear matrix, when the chromosomes begin to condense the focal staining disappears and glypican immunoreactivity, which increases in intensity, is excluded from the prophase chromosomes (Fig. 9 *B*). This perichromosomal pattern continues through metaphase (Fig. 9 *D*) and anaphase (Fig. 9 *F*) until the chromosomes are decondensed after separation into two daughter cells.

Because there was considerable variability in the pattern of glypican nuclear immunoreactivity in asynchronous cultures of C6 cells, we examined possible correlations between the nuclear staining pattern and the cell cycle. After serum starvation, the pattern of focal staining seen in cells from a regular culture almost disappeared, whereas nearly

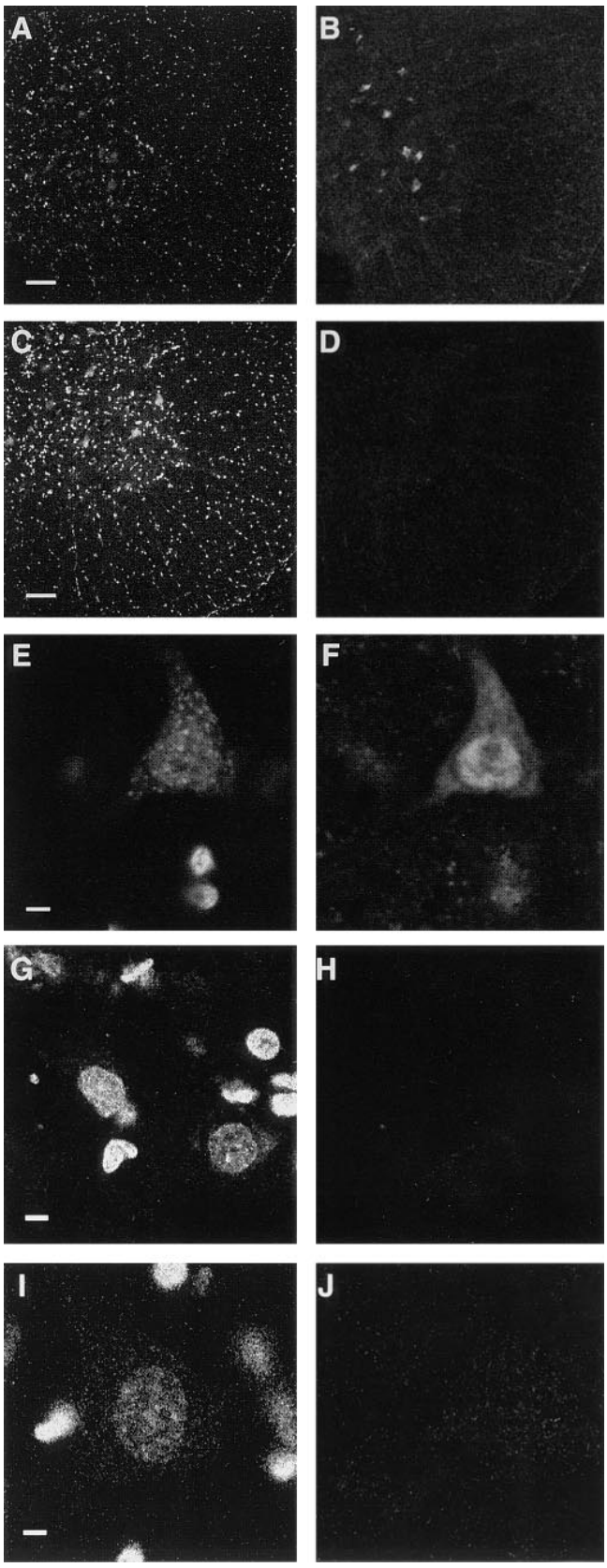


Figure 4. Nuclear staining by propidium iodide (*A, C, E,* and *G*) and antibodies to biglycan (*B, D, F,* and *H*) in adult rat spinal cord. Biglycan staining is completely inhibited by preincubation of the antibodies with 12 μM peptide (*D* and *H*) and 13 μM cartilage biglycan (*J*). The biglycan concentration is calculated on the basis of an average molecular mass of 93,000 (Liu et al., 1994). The cytoplasmic staining seen in *E* represents autofluorescent granules that are present in spinal cord neurons (see Fig. 8 *C*). Bars, *A* and *C*, 100 μm; *E, G,* and *I*, 5 μm.

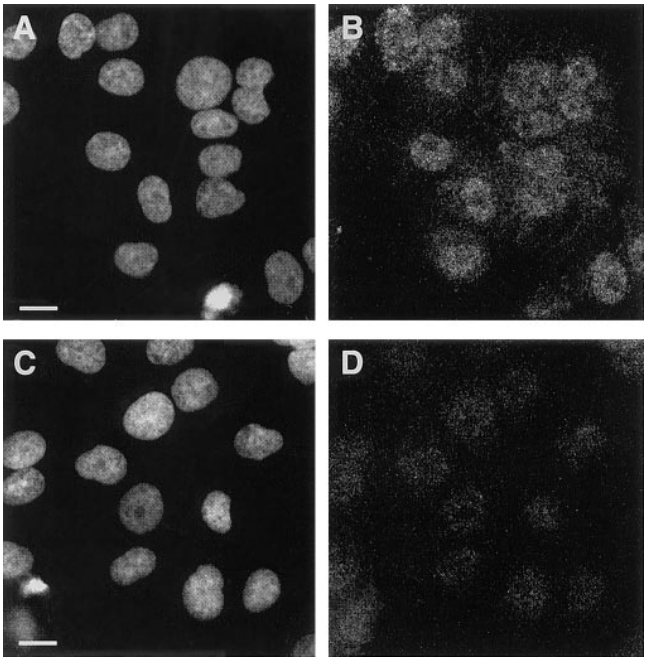
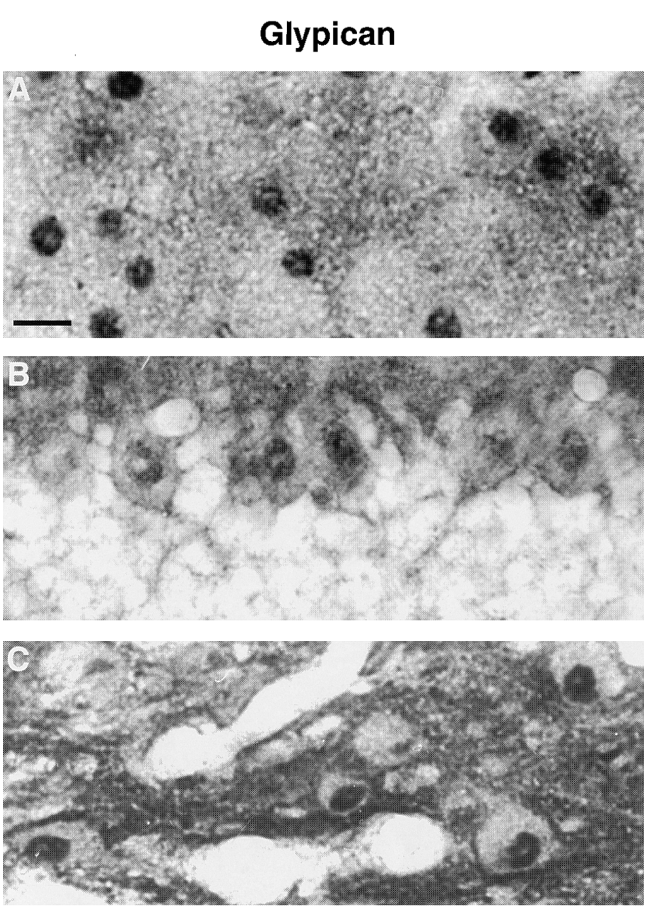


Figure 5. Staining of C6 cells with propidium iodide (*A* and *C*) and antibodies to biglycan (*C* and *D*). (*B*) Strong nuclear staining by peptide affinity-purified IgG that was further adsorbed to and eluted from nitrocellulose-bound cartilage biglycan (Fig. 1, lane *16*) compared to the much weaker nuclear staining (*D*) produced by the antibodies that did not bind to biglycan (Fig. 1, lane *17*). Bars, 10 μm.

all cells showed this pattern after arrest at the G_1/S boundary by treatment for 24 h with 1 mM hydroxyurea. This focal staining pattern is also similar to that observed in cells after serum release for 16 h (data not shown) or 24 h, and in serum-starved cells after treatment with 10 ng/ml bFGF (Fig. 10), which has a proliferative effect on C6 cells. In contrast, cAMP treatment did not change the flow cytometry profile of serum-starved C6 cells and had no effect on glypican nuclear staining (data not shown). It is known that exogenous bFGF can stimulate C6 cell proliferation in serum-free medium (Okumura et al., 1989), and we demonstrated by flow cytometry that after serum starvation, more C6 cells were at the S and G_2/M phases after bFGF treatment (Fig. 10). In view of the finding that C6 cells arrested at the G_1/S boundary or released from serum starvation showed the same staining pattern, it would appear that glypican begins to concentrate at some "hot spots" in the nucleus during G_1 progression.

Translocation of Glypican and Biglycan to the Nucleus of Transfected Cells

Inspection of glypican and biglycan amino acid sequences revealed that both contain potential nuclear localization signals (Silver, 1991; Dingwall and Laskey, 1991). Glypi-



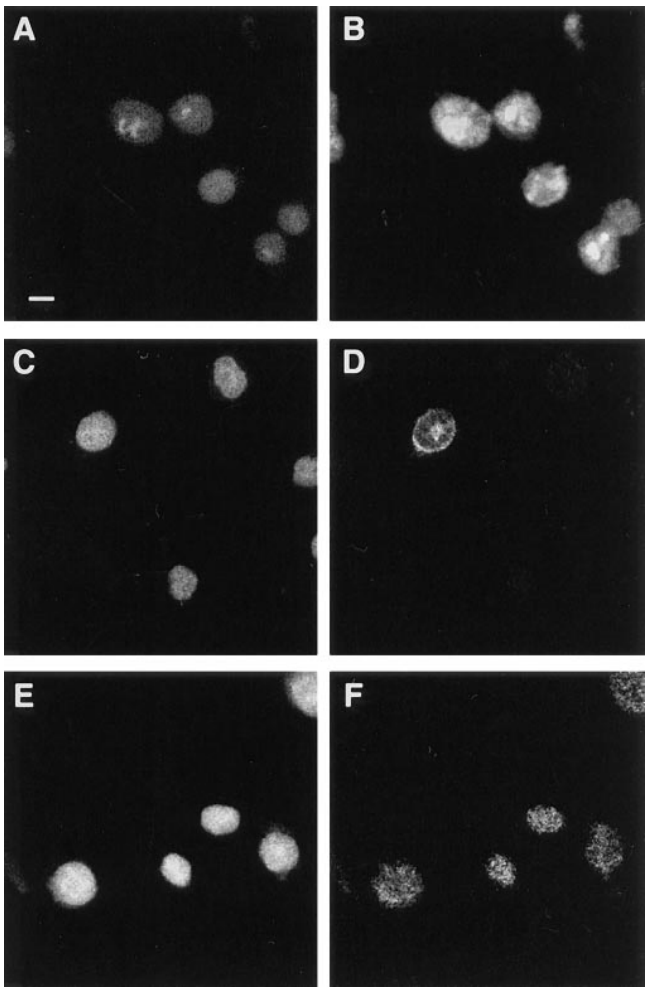


Figure 7. Staining of isolated C6 cell nuclei with propidium iodide (*A, C,* and *E*) and antibodies to glypican (*B*) or biglycan (*F*). For the experiment shown in *A* and *B*, C6 cells were trypsinized before isolation of nuclei, which were then treated successively with 1% and 2% Triton X-100 to further reduce contamination by intracellular and plasma membranes. (*C* and *D*) Nuclei isolated from nontrypsinized C6 cells that were first incubated with antibodies to glypican (1 h at room temperature), after which the isolated nuclei were stained with second antibody alone. Bar, 5 μm.

can contains the sequence KRRRAK, which is in excellent agreement with the proposed hexapeptide signal consisting of four K or R residues that are not interrupted by acidic (D or E) or bulky (W, F, or Y) amino acids (Boulikas, 1994), whereas rat, mouse, and human biglycan all contain two basic amino acids (RK, residues 102/103 in mature rat biglycan) followed by a 30–amino acid spacer and a cluster of seven amino acids consisting of four basic residues (RIRKVPK). This corresponds to the "bipartite motif" for nuclear localization signals proposed by Dingwall

Figure 6. Peroxidase-diaminobenzidine staining of glypican (*A–C*) and biglycan (*D–F*) in Vibratome sections of 9-d rat cerebrum and cerebellum: *A*, cerebral cortex; *B*, Purkinje cell layer of cerebellum; *C*, deep cerebellar nuclei; *D*, thalamus; *E*, hippocampus (hilus); *F*, cingulum. Bar, 20 μm.

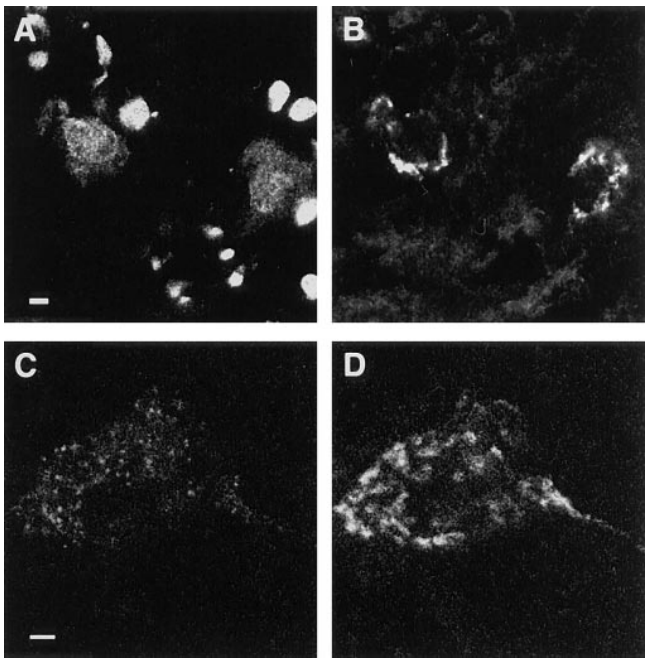


Figure 8. Syndecan-3 immunoreactivity is not present in the nuclei of rat spinal cord neurons. Confocal laser scanning photomicrographs of adult rat spinal cord stained with propidium iodide (*A*) and antibodies to syndecan-3 (*B* and *D*) show that immunoreactivity is restricted to the plasma membrane. The section shown in *C* and *D* was not stained with propidium iodide, and the signal seen in the propidium iodide channel (*C*) represents cytoplasmic autofluorescent granules. Bars, 5 μm.

and Laskey (1991), in which the length of the "spacer" region can be variable (Robbins et al., 1991).

In view of these features (summarized in Fig. 11), we transfected 293 cells with constructs in which β-galactosidase was fused to 68–78 amino acid sequences of glypican and biglycan containing their potential nuclear localization signals. Immunocytochemistry using both monoclonal and polyclonal antibodies to β-galactosidase demonstrated that these putative nuclear localization signals are in fact capable of targeting the fusion proteins to 293 cell nuclei, whereas untransfected cells and control cells transfected with only *E. coli* β-galactosidase showed very little or no nuclear staining (Fig. 12). It is perhaps significant in this regard that decorin, which is structurally very similar to biglycan and is also present in nervous tissue, does not contain a potential nuclear localization signal and was not detected in nuclei (see above).

When the basic KRRRAK sequence of the glypican–β-galactosidase fusion protein was mutated by PCR to nonbasic amino acids and the cDNA transfected into 293 cells, the nuclear localization of the resulting fusion protein was greatly decreased although not entirely eliminated (Fig. 12 *H*). The presence of some residual nuclear immunoreactivity probably does not result from effects of the remainder of the glypican sequence but rather to the nature of the β-galactosidase fusion protein itself, insofar as a second mutant in which most of the remaining glypican sequence was deleted from the nonbasic mutant fusion protein (Fig. 11 *B*) also showed the same small degree of nuclear immunoreactivity (Fig. 12 *J*). Although full-length glypican was

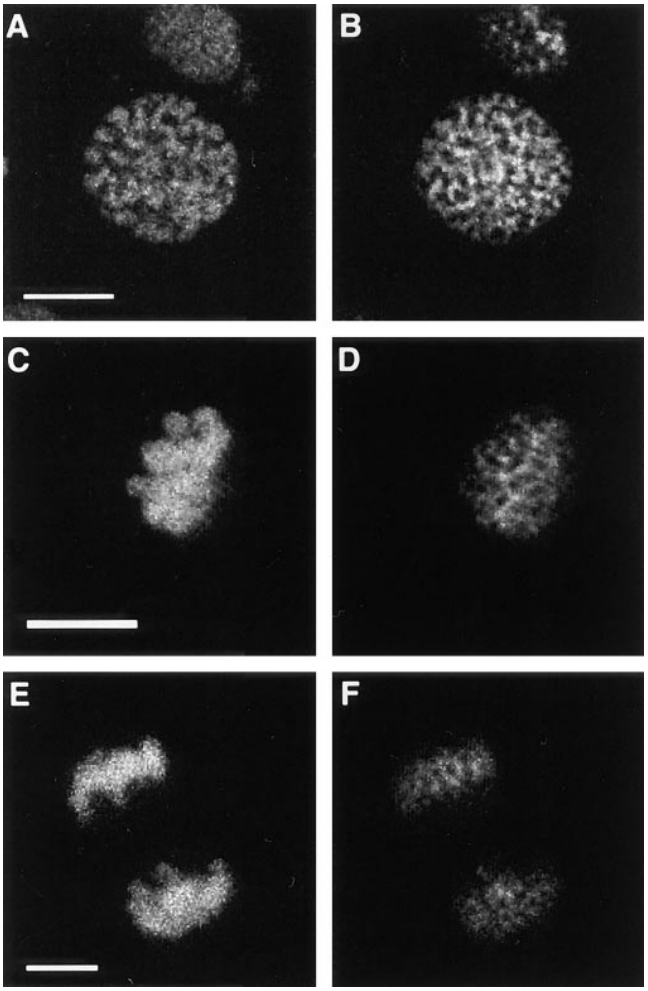


Figure 9. Confocal laser scanning photomicrographs showing C6 cells at different stages of cell division stained with propidium iodide (*A, C,* and *E*) and an antiserum to recombinant glypican (*B, D,* and *F*). Bars, *A* and *C*, 10 μm; *E*, 5 μm.

not transported to the nucleus of 293 cells (data not shown), we did find nuclear expression of glypican in COS-1 cells transfected with an HSV epitope-tagged glypican cDNA (Fig. 13).

The localization of glypican overexpressed in C6 cells transfected with either a full-length HSV-tagged glypican cDNA (including the signal peptide) or the corresponding glypican-Fc fusion gene was also examined using mAbs against the HSV-tag or human IgG Fc (Fig. 14). Using both cDNA constructs, almost half of the transfected cells showed distinct foci of nuclear immunoreactivity similar to that of endogenous glypican in C6 cell nuclei. When the basic 68–amino acid glypican sequence used for the β-galactosidase fusion protein was deleted from the glypican-Fc fusion protein and this deletion mutant was transfected into C6 cells, only a small proportion of cells showed the characteristic focal pattern of nuclear immunoreactivity seen in nontransfected cells or cells transfected with the full-length fusion protein (Fig. 14). Therefore, since both mutation and deletion of the potential nuclear localization signal of glypican in two different types of constructs (i.e., β-galactosidase and Fc fusion proteins) produced highly significant

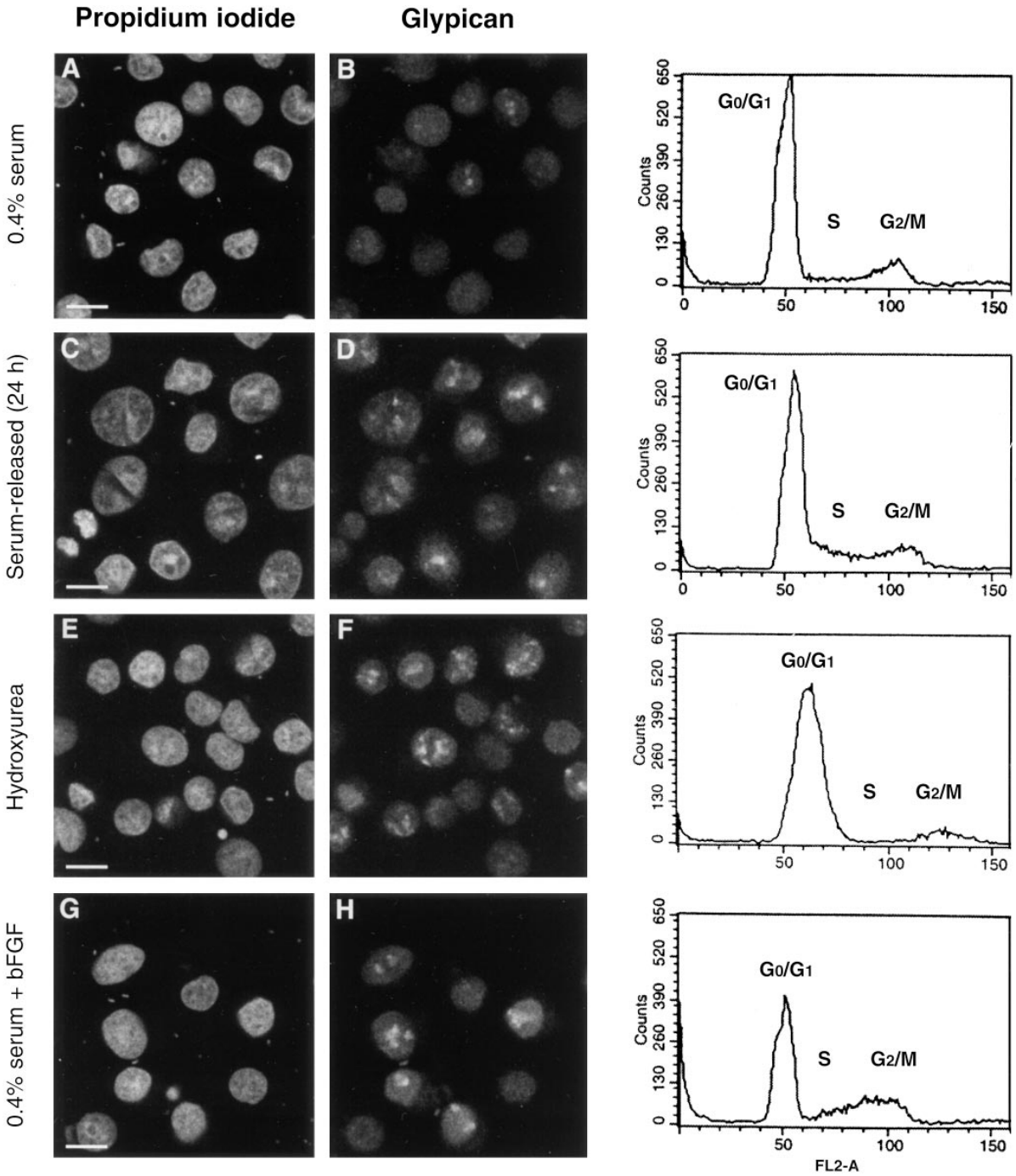


Figure 10. C6 cells were serum-starved for 2 d and transferred for 24 h to either new medium also containing 0.4% serum (*A* and *B*), complete medium (*C* and *D*), complete medium with 1 mM hydroxyurea (*E* and *F*), or medium containing 0.4% serum and 10 ng/ml bFGF (*G* and *H*). Cell aliquots were then used either for double staining with propidium iodide (*A, C, E,* and *G*) and antiglypican antibodies (*B, D, F,* and *H*) or for flow cytometry. The right-hand portion of the figure shows the corresponding flow cytometry profiles, presented as cell number on the ordinate and propidium iodide fluorescence intensity (in arbitrary units) on the abscissa. Each graph represents the analysis of 10,000 cells. Bars, 10 μm.

decreases in their nuclear localization, it is likely that this basic sequence is a functional nuclear localization signal.

Discussion

By now, there is extensive literature concerning glycosaminoglycans in the nucleus (Bhavanandan and Davidson, 1975; Stein et al., 1975; Margolis et al., 1976; Fromme et al., 1976; Furukawa and Terayama, 1977; Fedarko and Conrad, 1986; Ishihara et al., 1986; Ripellino et al., 1988; Busch et al., 1992). Most of these reports have described biochemical studies in which labeled glycosaminoglycans were detected in purified nuclei. Although it has recently been demonstrated that a large portion of the labeled glycos-

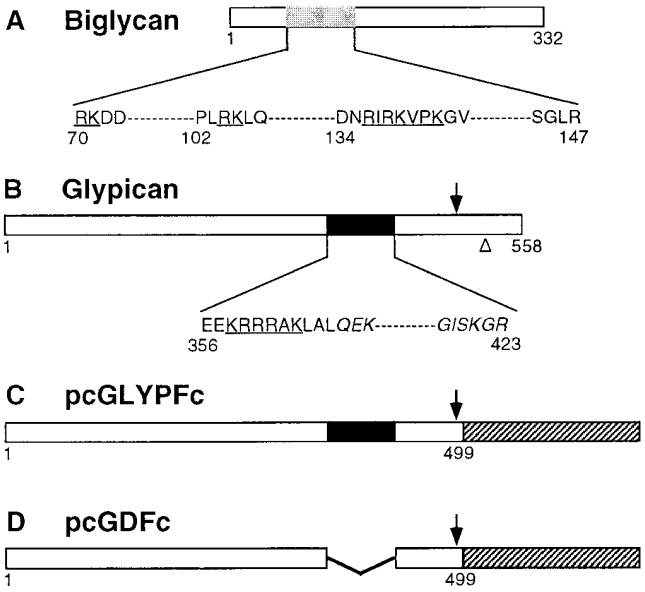


Figure 11. The location of potential nuclear localization signals in biglycan (*A*) and glypican (*B*) is indicated by the shaded regions, that were used for the construction of β-galactosidase fusion proteins. The clusters of basic amino acids are underlined. In one glypican mutant, the basic cluster was mutated to NSSSAN, and the sequence indicated by italics was deleted in a second mutant. The attachment sites for the phosphatidylinositol anchor and the COOH-terminal heparan sulfate chain of glypican are indicated by an open triangle and an arrow, respectively. In two fusion proteins, amino acids 499–558 of glypican were replaced by the human IgG Fc sequence (shown as hatched regions in *C* and *D*), and in a deletion mutant, the potential nuclear localization signal used for the β-galactosidase fusion protein was removed from the glypican–Fc fusion protein (*D*).

aminoglycans found in nuclei isolated from primary cultures of rat ovarian granulosa cells may represent material derived from the plasma membrane (Hiscock et al., 1994), our studies of glypican and biglycan demonstrate considerable cellular specificity with regard to their nuclear localization. Even in ovarian granulosa cells, not all of the glycosaminoglycans associated with the nuclei could be ascribed to possible contamination from other subcellular fractions, and earlier mass analytical studies on the concentrations of glycoconjugates in rat brain nuclei demonstrated negligible contamination of an unlabeled crude nuclear fraction after mixing with [^{35}S]sulfate-labeled proteoglycans present in an 850-*g* postnuclear supernatant fraction before purification of the nuclei (Margolis et al., 1976). Moreover, auto-

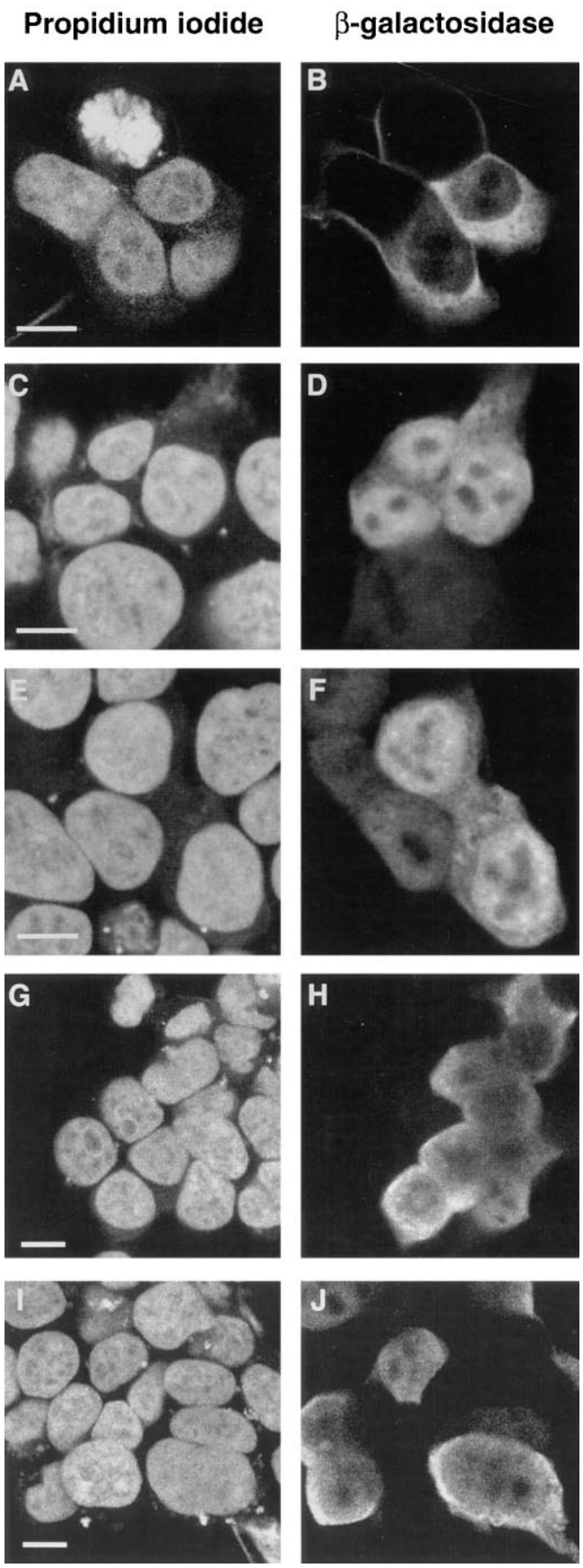


Figure 12. Localization of β-galactosidase immunoreactivity in transfected 293 cells. 293 cells were transfected with β-galactosidase alone (*A* and *B*) or β-galactosidase fusion proteins containing either the putative nuclear localization signals of glypican (*C* and *D*) or biglycan (*E* and *F*). In a third fusion protein, the basic cluster of the glypican fragment was mutated to nonbasic amino acids (*G* and *H*), and in a fourth fusion protein (*I* and *J*), there was a further deletion of the amino acids shown in italics in Fig. 11 *B*. *A, C, E, G,* and *I* show propidium iodide staining; *B, D, F, H,* and *J* show β-galactosidase immunoreactivity. Bars, 10 μm.

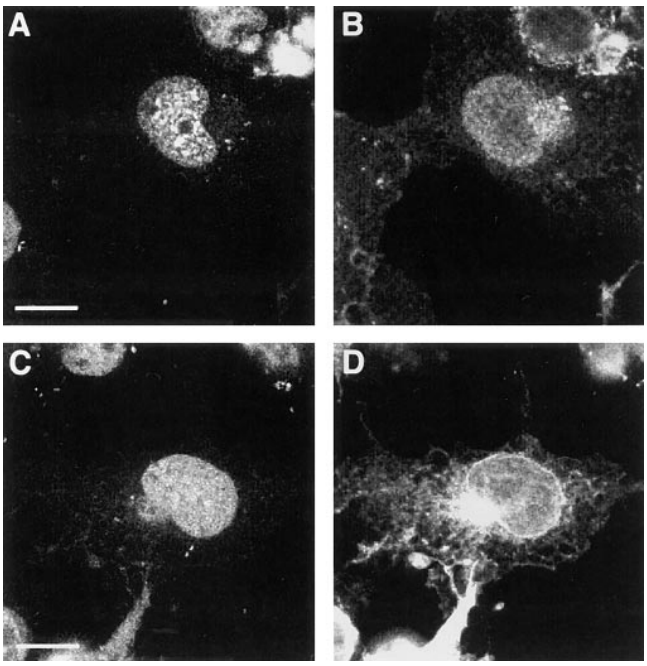
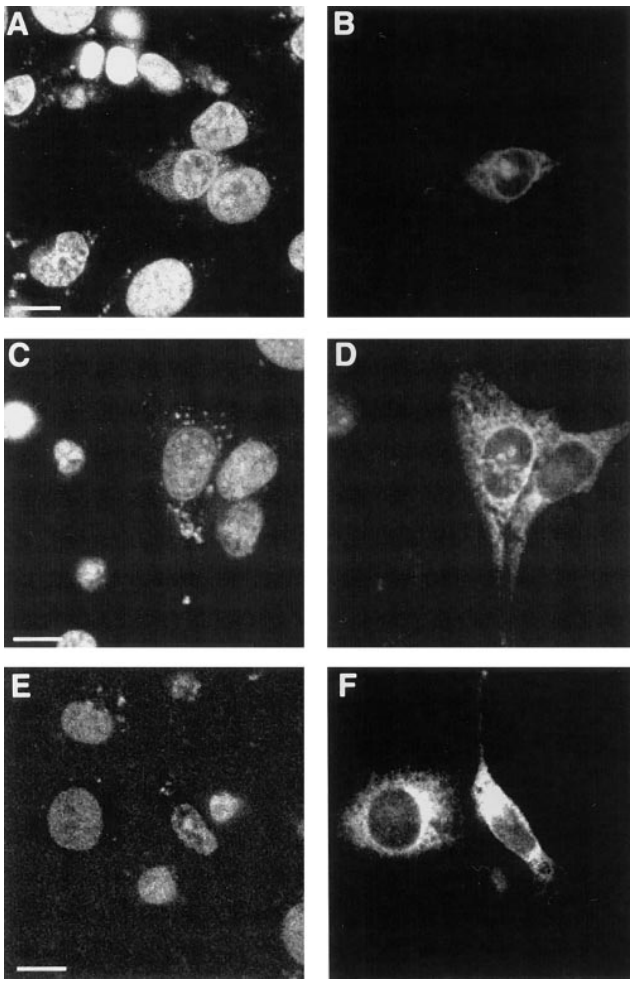


Figure 13. Transport of HSV-tagged glypican to the nuclei of transfected COS-1 cells. Cells were transfected with full-length glypican cDNA containing an HSV epitope tag and stained with propidium iodide (*A* and *C*) or an mAb to the HSV tag (*B* and *D*). Staining is seen in the nuclei and in the surrounding ER and Golgi membranes. Bars, 50 μm.

radiographic (Fromme et al., 1976), immunocytochemical (Aquino et al., 1984; Ripellino et al., 1989), and histochemical studies using a specific biotinylated probe for hyaluronan (Ripellino et al., 1988) have also demonstrated the presence of sulfated glycosaminoglycans, chondroitin sulfate proteoglycans, and hyaluronan in the nucleus.

Glypican was detected both on Western blots and immunocytochemically in nuclei isolated from C6 cells treated with trypsin to reduce possible contamination by cell-surface proteoglycans, and in nuclei prepared from trypsinized cells, there was no reduction in glypican immunoreactivity after their treatment with 25 mM potassium iodide to depolymerize actin and thereby reduce cytoskeletal–membrane interactions (Hiscock et al., 1994), or by incu-



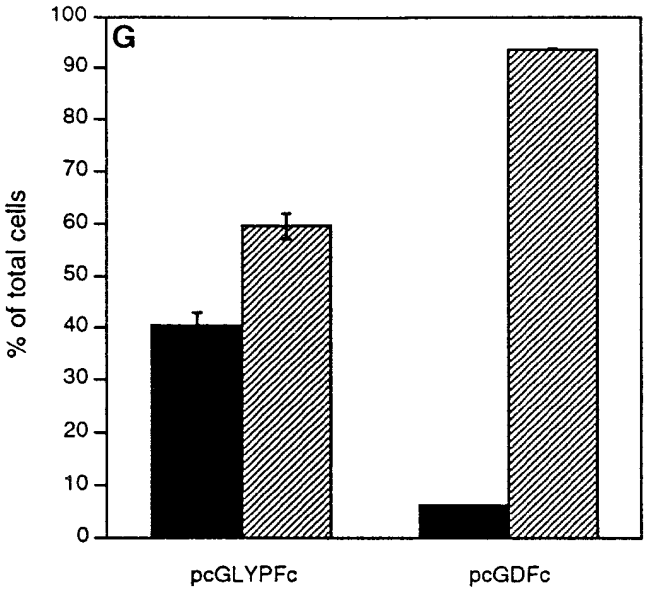


Figure 14. Localization of glypican fusion proteins in C6 glioma cells. Cells were transfected with an HSV fusion protein (*A* and *B*) or Fc fusion proteins containing either the complete glypican cDNA (pcGLYPFc, *C* and *D*), or glypican cDNA from which the nuclear localization signal had been deleted (pcGDFc, *E* and *F*). The cells were then stained with both propidium iodide (*A, C,* and *E*) and mAbs to either the HSV tag (*B*) or to the human Fc region (*D* and *F*). For the Fc fusion protein studies, two independent transfections were performed, and more than 200 cells were scored in each experiment. The transfected cells were divided into two groups; one in which the nuclei contained discrete foci of glypican immunoreactivity (e.g., *D*, in which only the cell on the left side shows nuclear staining), and a second group with either no nuclear staining or a very weak immunoreactivity (*F*). A quantitation of these results is shown in the bar graph (*G*), where it can be seen that whereas ~40% of the cells transfected with the full-length cDNA (*pcGLYPFc*) show the characteristic pattern of discrete nuclear immunoreactivity (*solid bars*), this pattern was seen in only 5% of cells transfected with DNA from which the glypican nuclear localization signal had been deleted (*pcGDFc*). The hatched bars represent the proportion of cells that do not show foci of nuclear staining. Cells with abnormal morphology or condensed nuclei were not scored. Bars, 10 μm.

bation of the isolated nuclei with phosphatidylinositol-specific phospholipase C, 0.5 M NaCl, heparin (10 mg/ml), or heparitinase (data not shown). Because nuclei isolated even from nontrypsinized cells pretreated with antibodies to glypican showed no significant plasma membrane contamination after staining with secondary antibody (Fig. 7 *D*), an adventitious association of cell-surface glypican with isolated nuclei appears very unlikely, and the conclusions from these studies are in excellent agreement with our confocal immunocytochemical data demonstrating glypican immunoreactivity in the nuclear matrix in situ.

The recent cloning of two hyaluronan-binding proteins has identified one of these (which also binds chondroitin sulfate and heparin in vitro) as the vertebrate homologue of the essential cell cycle control protein Cdc37 (Grammatikakis et al., 1995), while a 68-kD hyaluronan-binding protein from human fibroblasts was shown to be identical to P-32 (Deb and Datta, 1996), a protein that copurifies with the human pre-mRNA splicing factor SF2. Both of these proteins contain glycosaminoglycan-binding motifs previously described in several hyaluronan-binding proteins, and they provide further support for a role for glycosaminoglycans and proteoglycans in nuclear processes such as the control of cell division. Our finding that glypican and biglycan are components of some nuclei raises the obvious possibility that they might modulate the functions of other nuclear proteins with which they may interact, such as transcription factors or histones.

The nuclear localization of chondroitin sulfate and heparan sulfate proteoglycans extends the rapidly growing number of proteins that have been found at unexpected sites. Examples include the cell surface and/or nuclear localization of cytoskeletal proteins such as actin, tubulin, tau, and glycosyltransferases and kinases (reviewed by Smalheiser, 1996), and the nuclear translocation or localization of growth factors and their receptors (Maher et al., 1996), structural protein 4.1 (Krauss et al., 1997), myelin basic protein (Pedraza et al., 1997), myosin I (Nowak et al., 1997), and ferritin (Cai et al., 1997), to mention only a few of the many proteins that fall into this category. It has also recently become apparent that a number of generally and genuinely nuclear proteins can be recruited to contribute to the assembly of different types of junctional plaques. These include the desmosomal plaque proteins plakophilin 1 and plakophilin 2 (Mertens et al., 1996), the adherens junction plaque protein β-catenin (Funayama et al., 1994; Karnovsky and Klymkowsky, 1995), and symplekin, a novel type of tight junction plaque protein (Keon et al., 1996).

It is not clear how glypican and biglycan are transported across membranes insofar as they both contain NH_2-terminal signal sequences and cotranslationally traverse the lumen of the ER. However, the HSV-1 structural protein VP22, which (like a small group of unusual proteins such as IL-1β, the HIV-1 Tat protein, and basic fibroblast growth factor) contains no signal peptide, was recently found to be transported intercellularly to neighboring cells after endogenous synthesis (Elliott and O'Hare, 1997). Although glypican and biglycan both contain signal sequences, they may share certain other features with these proteins that permit their translocation across the plasma membrane. Numerous other cell membrane and secreted proteins also contain nuclear localization signals (Boulikas, 1994), but whether any of these are actually transported to the nucleus remains to be examined.

Because the HSV tag in glycosylphosphatidylinositol-anchored glypican (which is continually shed into the culture medium) and the Fc tag in the secreted form both showed a similar pattern of nuclear immunoreactivity, it is likely that soluble rather than membrane-anchored glypican is the species that is transported into the nucleus. However, because cells surrounding transfected C6 cells expressing the glypican Fc fusion protein did not show any Fc immunoreactivity, it would appear that glypican released from the cell surface uses an autocrine pathway for entry into the nucleus.

C6 cells expressing the glypican deletion mutant showed a significant reduction in the number of cells with foci of nuclear immunoreactivity, indicating the importance of the basic cluster in its nuclear targeting. However, the cytoplasmic form of glypican lacking the signal peptide showed a rather uniform distribution throughout the nucleus (data not shown), suggesting that in addition to the nuclear localization signal, certain posttranslational modifications may be necessary for its targeting to nuclear subdomains. Although glypican immunoreactivity was not seen in the nuclei of 293 cells transfected with either tagged full-length or cytoplasmic glypican, tagged full-length glypican was transported to the nuclei of COS-1 cells (Fig. 13), indicating a cell type specificity for this process. The absence of any detectable expression of glypican in 293 cells suggests that its nuclear targeting may require an interaction with one or more other proteins expressed only by certain cell types.

The glypican and biglycan immunoreactivity seen in the ventral horn of the spinal cord is most prominent in the nuclei of large cell bodies with a morphology typical of motor neurons, and their large nuclei are also distinguished by a relatively weaker staining with both DAPI (data not shown) and propidium iodide (Figs. 2 and 4). Although we are not aware of any previous reports calling attention to different patterns of nuclear staining in spinal cord neurons, differences in propidium iodide staining have been described in relation to the organization of nuclear DNA and as a function of different physiological conditions of the cells. For example, propidium iodide staining is increased after 0.7 M NaCl or low pH extraction of histone H1 (Giangarè et al., 1989) or mild nuclease digestion to relax DNA supercoils (Prosperi et al., 1991), and both DAPI and propidium iodide staining of nuclei decreases after erythroid differentiation of Friend leukemia cells (Darzynkiewicz et al., 1984). It is therefore possible that the pattern of nuclear glypican and biglycan immunoreactivity that we observed in the spinal cord is associated with a subpopulation of more highly differentiated neurons having a more compact chromatin structure.

C6 cells entering the G_1 phase acquire prominent foci of nuclear glypican immunoreactivity, suggesting that this nuclear localization may be related to cell proliferation. The dynamic changes in the glypican staining pattern resemble those of certain conventional nuclear components, as well as that of some unexpected proteins, such as protein 4.1, which was first identified as a crucial protein in the mature red cell membrane skeleton but redistributes to several structural zones during the cell cycle (Krauss et al., 1997). Glypican may also serve as a structural compo-

nent of the nucleus, although the specific compartment(s) corresponding to the foci of glypican immunmoreactivity seen in C6 cell nuclei have not yet been identified. The detailed pathways leading to the nuclear localization of glypican and biglycan and their physiological role in the nucleus therefore remain to be elucidated, as does the basis for the cellular specificity that we have observed, which may be regulated either by cell type–specific mechanisms for entry of the proteoglycans into the cytoplasm or at the nuclear level. However, the similar nuclear localization of glypican and biglycan, but not of related proteoglycans such as decorin and syndecan-3, emphasizes the potential importance of both chondroitin sulfate and heparan sulfate proteoglycans in nuclear processes.

We thank Drs. David Carey, Hans Kresse, Kevin Dreher, James Salzer, and Lawrence Rosenberg for generously providing antibodies, DNAs, and proteins, and Markus Mevissen and Susanna Popp for skillful technical assistance.

This work was supported by research grants NS-09348, NS-13876, and MH-00129 from the National Institutes of Health.

Received for publication 3 June 1997 and in revised form 2 September 1997.

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