Journal of Cellular Biochemistry 98:115–127 (2006)

Molecular Basis of Interaction Between NG2 Proteoglycan and Galectin-3

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Abstract Previous work has demonstrated the ability of the NG2 proteoglycan, a component of microvascular pericytes, to stimulate endothelial cell motility and morphogenesis. This function of NG2 depends on formation of a complex with galectin-3 and $\alpha 3\beta 1$ integrin to stimulate integrin-mediated transmembrane signaling. In addition, the co-expression of galectin-3 and NG2 in A375 melanoma cells suggests that the malignant properties of these cells may be affected by interaction between the two molecules. Here, we extend the theme of co-expression and interaction of NG2 and galectin-3 to human glioma cells. We also establish a molecular basis for the NG2/galectin-3 interaction. The C-terminal carbohydrate recognition domain of galectin-3 is responsible for binding to the NG2 core protein. Within the NG2 extracellular domain, the membrane-proximal D3 segment of the proteoglycan contains the primary binding site for interaction with galectin-3. The interaction between galectin-3 and NG2 is a carbohydrate-dependent one mediated by N-linked rather than O-linked oligosaccharides within the D3 domain of the NG2 core protein. These studies establish a foundation for attempts to reduce the aggressive properties of tumor cells by disrupting the NG2/galectin-3 interaction. J. Cell. Biochem. 98: 115–127, 2006.

Key words: NG2; galectin-3; glycosylation; carbohydrate-dependent protein–protein interaction; glioma

The NG2 chondroitin sulfate proteoglycan is a membrane-spanning protein expressed by immature progenitor cells in several types of developing tissues [Levine and Nishiyama, 1996; Stallcup, 2002]. NG2 expression is typically maximal during the period when progenitor cells are mitotic and motile, and then is downregulated when progenitors undergo terminal differentiation. In addition to serving as a marker for various types of progenitor cells [Nishiyama et al., 1996a; Ozerdem et al., 2001; Fukushi et al., 2003; Legg et al., 2003], the NG2 proteoglycan and its homologs have been implicated as functional contributors to both cell proliferation and migration [Harper and Reisfeld, 1983; Burg et al., 1997; Burg et al., 1998; Grako et al., 1999; Stallcup and Dahlin-Huppe, 2001; Ozerdem and Stallcup, 2004]. Interestingly, NG2 is also expressed by several types of malignant tumor cells, including melanomas, glioblastomas, chondrosarcomas, and lymphomas [Schrappe et al., 1991; Leger et al., 1994; Smith et al., 1996; Campoli et al., 2004]. The ability of the proteoglycan to potentiate cell proliferation and motility is believed to contribute to tumor growth and metastasis.

While evidence exists for the ability of NG2 to function directly as a mediator of transmembrane signaling [Fang et al., 1999; Tillet et al., 2002; Majumdar et al., 2003], the proteoglycan also serves important roles as a co-receptor or

Abbreviations used: CRD, carbohydrate recognition domain; SAD, self-association domain; EC, extracellular; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; BAG, benzyl 2-acetamido-deoxy-α-D-galactopyranoside; CNBr, cyanogen bromide.

Grant sponsor: National Institutes of Health (to W.B.S.); Grant numbers: RO1 CA95287, PO1 HD25938.

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Received 19 October 2005; Accepted 16 November 2005
DOI 10.1002/jcb.20768

facilitator of signaling via other well-known transmembrane receptors such as receptor tyrosine kinases [Grako and Stallcup, 1995; Nishiyama et al., 1996b; Grako et al., 1999] and integrins [Iida et al., 1995; Eisenmann et al., 1999; Yang et al., 2004]. In this regard, we have recently reported that NG2 promotes vascular endothelial cell motility and morphogenesis via an integrin-dependent mechanism [Fukushi et al., 2004]. This is significant due to the intimate apposition of endothelial cells to NG2-positive perivascular cells [Ozerdem et al., 2001, 2002; Ozerdem and Stallcup, 2003]. The ability of NG2 to activate endothelial cells depends on its formation of a tri-molecular complex on the endothelial cell surface with α3β1 integrin and galectin-3 [Fukushi et al., 2004]. We have proposed that the interaction between galectin-3 and NG2 serves as a mechanism for multimerization of cell surface complexes as a means of amplifying or focusing transmembrane signaling via the integrin.

Whereas vascular endothelial cells do not themselves express NG2 and require stimulation by exogenous proteoglycan from another source such as pericytes, we noted that A375 human melanoma cells endogenously express all three components of the signaling complex, namely α3βl integrin, galectin-3, and NG2 [Fukushi et al., 2004]. We speculated that tumor cells might have co-opted this signaling mechanism as a means of promoting their malignant behavior. In this respect, it is noteworthy that galectin-3 is well studied for its ability to influence tumor progression via effects on cell transformation, proliferation, adhesion, motility, and apoptosis [Hughes, 2001; Liu and Rabinovich, 2005]. The galectins are a family of mammalian lectins that contain conserved carbohydrate recognition domains (CRDs) responsible for oligosaccharide-dependent ligand binding. In addition to a single β-galactoside-binding CRD module in its C-terminus, galectin-3 (MAC-2) is unique in that it contains an N-terminal self-association domain (SAD) with multiple PGAYPG repeats. This domain mediates the multimerization of galectin-3 and its ligands [Liu and Rabinovich, 2005].

The apparent contributions of both NG2 and galectin-3 to the malignant properties of tumor cells, along with the co-expression of both molecules on melanoma cells, suggest that the interaction between the two molecules merits further investigation. In this report, we extend the theme of NG2/galectin-3 co-expression and physical interaction to an additional malignant tumor cell type, human glioma. Furthermore, we use discrete recombinant protein domains to establish the molecular basis for interaction between the two molecules. We show that the galectin-3 CRD mediates carbohydrate-dependent interaction with NG2. In turn, the membrane-proximal D3 segment of the NG2 ectodomain is primarily responsible for binding to galectin-3. N-linked, rather than O-linked, oligosaccharides within the D3 domain are required for recognition of NG2 by galectin-3. These studies set the stage for future attempts to reduce the malignant properties of tumor cells by disrupting the NG2/galectin-3 interaction.

MATERIALS AND METHODS

Cell Lines

Cell lines used in this study include the U251 human astrocytoma [Ponten and Westermark, 1978] and the U87 human glioblastoma [Schrappe et al., 1991]. NG2-transfected U251 cells (U251/NG2) have been described previously [Makagiansar et al., 2004]. All cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Tissue Culture Biologicals, Tulare, CA), 2 mM glutamine, 100 IU/ml penicillin, and 100 ug/ml streptomycin sulfate (CalBiochem, La Jolla, CA).

Reagents and Antibodies

Polyclonal rabbit antibodies against rat NG2 [Ozerdem et al., 2001; Makagiansar et al., 2004], and a cocktail of mouse monoclonal anti-NG2 antibodies [Nishiyama et al., 1996a] have been described previously. The mouse monoclonal anti-human NG2 antibody 9.2.27 was obtained from Dr. Ralph Reisfeld (The Scripps Research Institute, La Jolla, CA), while the B5 hybridoma was purchased from the American Type Culture Collection. Rat anti-mouse galectin-3 monoclonal antibody M3/38 was purified from hybridoma TIB-166 (ATCC). Purified polyclonal rabbit anti-human galectin-3 antibody has been described previously [Liu et al., 1995]. The *O*-glycosylation inhibitor benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BAG), the *N*-glycosylation inhibitor tunicamycin, and CNBr-activated Sepharose beads were purchased from Sigma (St. Louis,

MO). *N*-glycanase was obtained from Genzyme (Cambridge, MA). Lys-type plasminogen was purchased from Calbiochem (San Diego, CA).

Construction of Recombinant NG2 and Galectin-3 Species

Recombinant forms of the NG2 ectodomain (see Fig. 3B) were obtained from transfected 293 cells as previously described [Tillet et al., 1997]. In some cases, 293 cells producing the NG2/D3 species were treated with 2 mM BAG to inhibit O-linked glycosylation or with 5 μg/ml tunicamycin to inhibit N-linked glycosylation.

Full-length cDNA coding for human galectin-3 was synthesized by polymerase chain reaction [Fukushi et al., 2004]. Additional polymerase chain reactions were conducted to produce cDNAs for the N-terminal (amino acids 1–122) and C-terminal (amino acids 114–250) halves of human galectin-3 (Fig. 3A). In both cases, the sense primers contained a 5′ BamH1 site, while the anti-sense primers contained an *Eco R1* site. This facilitated cloning of all three cDNAs into the pGEX-2T vector (Amersham Biosciences, Piscataway, NJ) for the purpose of producing GST fusion proteins. Full length galectin-3 cDNA was also ligated into the pcDNA 3.1-hygromycin (–) vector (Invitrogen, San Diego, CA) for transfection of mammalian cells.

Stable Transfection of Galectin-3 and NG2 in U251 Cells

Stable expression of galectin-3 and NG2 in U251 cells was accomplished by sequential lipofectamine (Invitrogen) transfections. U251 cells were first transfected with the pcDNA3.1 hygromycin/galectin-3 vector followed by selection with 400 μg/ml hygromycin. Hygromycin-resistant colonies were screened by immunoflorescence for galectin-3 expression. Positive colonies were then processed by limiting dilution in 96-well plates to obtain galectin-3-positive clones. These cells were then co-transfected with the pcDNAampI/NG2 and pSV2neo plasmids and selected in the presence of 400 mg/ml G418 (Invitrogen). Positive colonies were enriched for NG2-positive cells by immunopanning [Stallcup and Dahlin-Huppe, 2001]. Co-expression of NG2 and galectin-3 was confirmed by immunoflorescence double staining and Western blotting with anti-NG2 and anti-galectin-3 antibodies.

Binding of Galectin-3-GST Fusion Proteins to Recombinant NG2 Species

Gal-3F-GST, Gal-3C-GST, Gal-3N-GST, and GST alone were purified from bacterial extracts using glutathione-agarose beads as previously described [Ausubel et al., 1997]. The various GST beads were used as matrices for testing binding to specific fragments of the NG2 ectodomain. Incubations of recombinant NG2 species with galectin-carrying beads were carried out for 2 h at 4°C in PBS containing 0.1% NP-40 and 0.05% SDS. In the carbohydrate–ligand competition experiments, lactose and cellobiose (Sigma), ranging from 0 to 30 mM, were included during the incubation period. For some experiments, CNBr-activated Sepharose beads derivatized with lys-type plasminogen according to the manufacturer's instructions were used as a pull-down matrix. In all cases, beads were washed five times with the 0.1% NP-40/0.05% SDS solution, followed by boiling in 2× SDS–PAGE loading buffer (Invitrogen). Samples were subjected to SDS–PAGE analysis on 4–20% gradient gels, followed by transfer to Immobilon-PVDF membranes (Millipore) and immunoblotting with anti-NG2 or anti-galectin-3 antibodies.

Immunoprecipitation and Immunoblotting

Transfected U251/Gal-3 cells, U251/NG2/Gal-3 cells, and U87 cells were extracted for 1 h in ice-cold lysis buffer (pH 7.4) containing 1% NP-40, 0.05 mM EDTA, 50 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail. For direct immunoblotting with anti-NG2 antibody, the lysate was treated with chondroitinase ABC (Seikagaku) (0.01 U/μg of protein) for 1 h at room temperature. For immunoprecipitation, cell lysates were incubated with continuous mixing for 1 h at 4°C with rabbit anti-NG2, monoclonal anti-NG2 9.2.27, or control IgG, followed by an additional 1 hr incubation with protein A- or Protein G-Sepharose (Sigma). Immunoprecipitates were washed five times with PBS containing 0.1% NP-40 and 0.05% SDS, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail. For immunoblotting, samples were separated by SDS-PAGE on 4–20% gradient gels, transferred to PVDF membranes, and blocked with 5% BSA in Tris-buffered saline. Membranes were probed with primary antibodies, followed by incubation with peroxidase-labeled second

antibodies. Detection of reactive bands was accomplished using an ECL kit (Amersham Pharmacia, Buckinghamshire, United Kingdom).

Immunoflorescence Microscopy

For analysis of NG2 and galectin-3 expression in U87 cells and transfected U251 cells (Fig. 1A), cells were fixed for 10 min in 4% paraformaldehyde followed by blocking for 30 min in DMEM containing 2% FCS. Immunoflorescence labeling was performed as described previously [Stallcup and Dahlin-Huppe, 2001]. Briefly, cells were incubated with primary antibodies for 1 h at room temperature in DMEM/2% FCS containing 0.1% Triton-X 100. After three washes, incubation with florescein- or rhodamine-coupled second antibodies (Biosource International, Camarillo, CA) was carried out for an additional hour. Following final washing and fixation with 95% ethanol, specimens were air dried and cover-slipped in Immumount (Shandon, Pittsburg, PA).

For the detection of cell surface expression of NG2 and galectin-3 (Fig. 2A), unfixed, living cells were immunostained at room temperature, using 30 min incubations for both primary and secondary antibodies. In another group of cells, the labeling of living cells was preceded by a 3-h incubation at 37°C with rabbit anti-galectin-3 antibody to promote cell surface clustering. A Nikon Optiphot epiflorescence microscope (Garden City, NY) equipped with a Nikon 40× PlanApo objective (oil immersion, 1.0 numerical aperture) was used for all imaging. Data acquisition of each image was performed with Image-Pro Digital 3.0 (Nikon).

Prediction of N- and O-Linked Glycosylation Sites

Prediction of sites for O-linked and N-linked glycosylation in the NG2 core protein was performed using the programs NetOGlyc 3.1 and NetNGlyc 1.0 from the Center for Biological Sequence Analysis at the Technical University of Denmark.

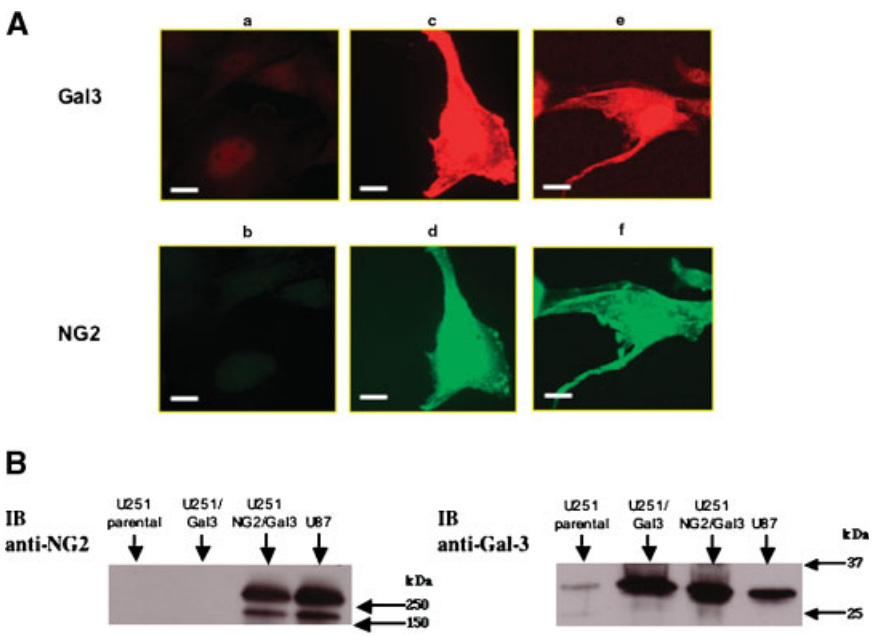

Fig. 1. Expression of NG2 and galectin-3 in human glioma cells. **A**: Immunoflorescence double staining was performed to detect the expression of galectin-3 (**a**, **c**, **e**), and NG2 (**b**, **d**, **f**) in parental U251 cells (a, b), U251 cells doubly transfected with galectin-3 and NG2 (c, d), and U87 cells (e, f). Cells were fixed with 4% paraformaldhyde and permeabilized with 0.1% Triton-X 100 prior to immunostaining. A rabbit anti-NG2 antibody was used to label the proteoglycan, followed by a goat-anti-rabbit IgG conjugated with florescein. A rat anti-galectin-3 antibody was used for labeling galectin-3, followed by a goat-anti-rat IgG conjugated with rhodamine. Scale bars = 10 μm. **B**: Immunoblotting for NG2 and galectin-3 was performed on detergent extracts of U251 parental cells, U251/Gal-3 cells, U251/NG2/Gal-3 cells, and U87 cells. To enable quantitative comparison of expression levels between cells, 50 μg of total cellular protein was loaded in each lane. NG2 immunoblots (IB) were performed with the B5 mAb, which recognizes both rat and human NG2. Galectin-3 immunoblots were performed with rabbit anti-galectin-3. U87 cells endogenously express both NG2 and galectin-3, while U251 cells express large quantities of these proteins only after transfection.

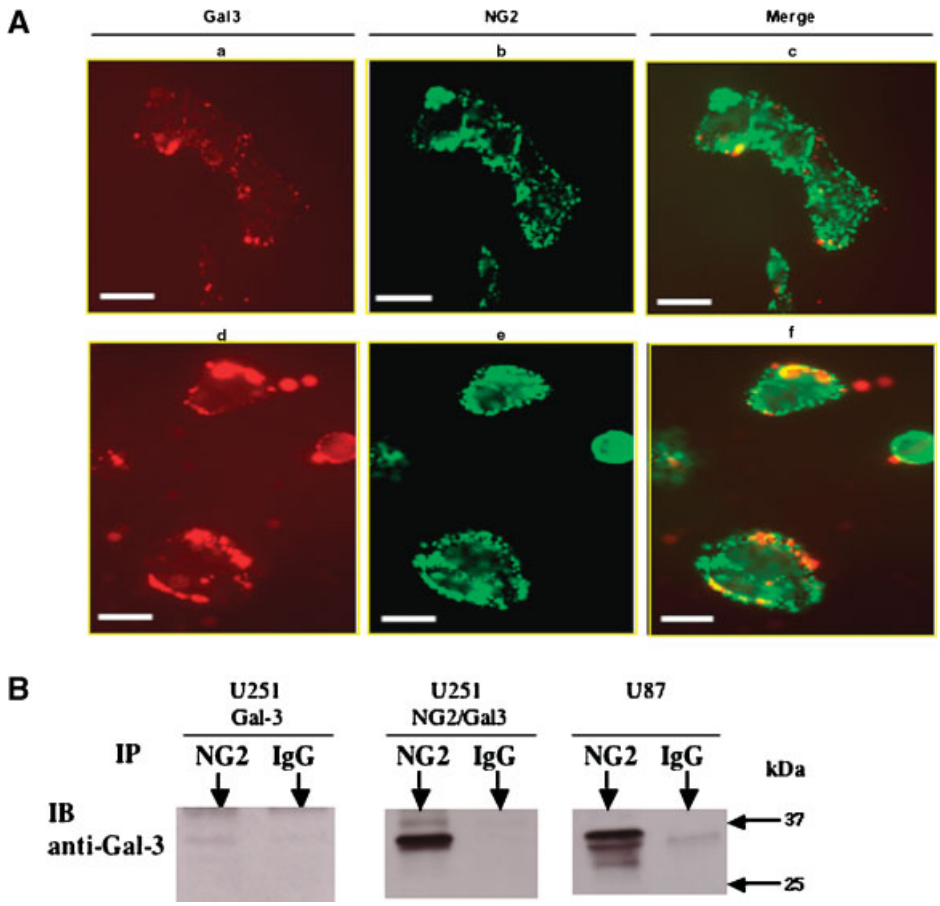

Fig. 2. Physical association between NG2 and galectin-3 on the cell surface. **A**: To demonstrate cell surface expression of galectin-3 and NG2, living U251/NG2/Gal-3 cells were immunoflorescently double-labeled at room temperature with rabbit antibody against galectin-3 (**a**) and a mouse monoclonal antibody cocktail against NG2 (**b**). The merged images are shown in (**c**). In a separate immunopatching experiment, living U251/NG2/Gal-3 cells were incubated for 3 h at 37°C with the rabbit anti-galectin-3 antibody, followed by double immunostaining for galectin-3 (**d**) and NG2 (**e**). The merged image (**f**) demonstrates a significant degree of antibody-mediated co-clustering of NG2 and galectin-3 on the cell surface. Scale bars = 10 μm. **B**: Detergent extracts of U251/Gal-3 cells, U251/NG2/Gal-3 cells, and U87 cells were used for immunoprecipitation (IP) with a rabbit antibody against rat NG2 (for U251 cells), or mouse monoclonal 9.2.27 anti-human NG2 antibody (for U87 cells). Non-immune rabbit IgG (for U251 transfected cells) and mouse IgG (for U87 cells) are applied as isotype controls. Anti-NG2 immunoprecipitates were immunoblotted (IB) with an antibody against galectin-3. The 33 kDa galectin-3 band is present in anti-NG2 immunoprecipitates from U87 cells and doubly transfected U251 cells, but not from NG2-negative U251/Gal-3 cells.

RESULTS

Association Between NG2 Proteoglycan and Galectin-3 in Human Glioma Cells

We previously showed that NG2 and galectin-3 are endogenously expressed and physically associated with each other in A375 melanoma cells [Fukushi et al., 2004]. In order to extend these observations to another malignant cell type, we investigated the NG2/galectin-3 interaction in U87 human glioblastoma cells, which also endogenously express both molecules. In addition, we tested U251 human astrocytoma cells, which normally express extremely low levels of both molecules, but which were transfected to express both rat NG2 and human galectin-3 (U251/NG2/Gal-3). High levels of galectin-3 and NG2 expression in both U87 and U251/NG2/Gal-3 cells are demonstrated in Figure 1A by immunoflorescence labeling with anti-NG2 and anti-galectin-3 antibodies. Immunoblotting was used to assess expression levels of these two proteins more quantitatively (Fig. 1B). From this analysis, U87 cells appear to have a somewhat higher ratio of NG2 to galectin-3 expression than U251/NG2/Gal-3 cells.

The high levels of cytoplasmic and nuclear galectin-3 expression revealed by immunostaining of fixed, permeabilized U87 and U251/NG2/

Gal-3 cells (Fig. 1A) are consistent with expression patterns for this protein that have been described previously [Hughes, 2001; Liu and Rabinovich, 2005]. In addition to these intracellular pools of galectin-3, we also detected lesser amounts of the protein co-localized with NG2 on the surfaces of live, unpermeabilized cells. Weak cell surface labeling of galectin-3, along with much higher levels of NG2, can be detected by room temperature double immunoflorescence staining of living U251/NG2/Gal-3 cells (Fig. 2A, a–c). Significantly, incubation of these cells at 37°C for 3 h with rabbit antibody to galectin-3, followed by NG2/galectin-3 double immunoflorescence labeling, reveals co-clustering of galectin-3 and NG2 on the U251/NG2/Gal-3 cell surface (Fig. 2A, d–f). The fact that these complexes are localized on the cell surface, rather than internally, is assured by the use of living, unpermeabilized cells for the entire labeling protocol. The incomplete overlap of NG2 and galectin-3 labeling in these antibody-induced clusters may reflect the existence of subpopulations of the two molecules that do not interact on the cell surface. Similar patterns of antibody-induced co-clustering of NG2 and galectin-3 are observed with U87 cells (data not shown). This type of antibody-mediated co-patching is highly indicative of a physical association between the two proteins on the cell surface. In support of this possibility, co-clustering of the two molecules is also induced by pre-incubation of the cells with mouse antibody against NG2 (data not shown).

In order to further demonstrate the association between NG2 and galectin-3, we used anti-NG2 antibody to co-immunoprecipitate galectin-3 from detergent extracts of the two glioma cell lines. Figure 2B shows that galectin-3 is co-immunoprecipitated from extracts of U87 cells and U251/NG2/Gal-3 cells by anti-NG2 antibody, but not by control immunoglobulin. As an additional control, the NG2 antibody does not immunoprecipitate galectin-3 from NG2-negative U251/Gal-3-transfected cells.

The Carbohydrate Recognition Domain of Galectin-3 Binds to NG2

To understand the mechanism of association between NG2 and galectin-3, we identified the functional domains of the two molecules that mediate their interaction. For this purpose, we utilized three different galectin-3 constructs prepared as GST fusion proteins (Fig. 3A). The full-length galectin-3 species (Gal-3F) contains both the N-terminal self-association domain (SAD) and the C-terminal carbohydrate recognition domain (CRD). Gal-3C contains only the

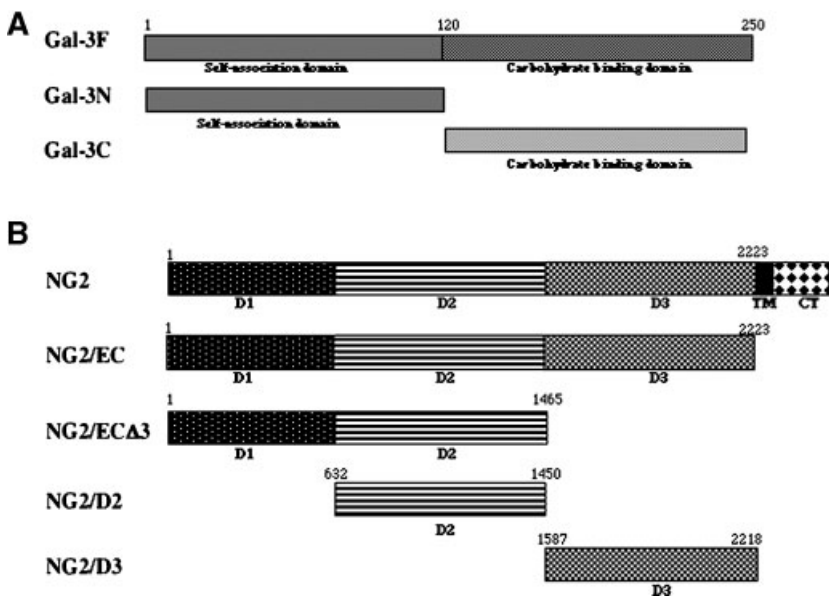

Fig. 3. Recombinant Galectin-3 and NG2 proteins. **A**: Three recombinant galectin-3 species were prepared as GST fusion proteins: full-length galectin-3 (Gal-3F), the N-terminal self-association domain (Gal-3N), and the C-terminal carbohydrate recognition domain (Gal-3C). **B**: Four recombinant NG2 species were prepared as purified proteins. The domain structure of full-length NG2 is shown, along with those of the recombinant NG2 species NG2/EC, NG2/ECΔ3, NG2/D2, and NG2/D3. TM, transmembrane domain; CT, cytoplasmic domain. Numbers above the constructs denote amino acid residues.

C-terminal CRD domain, while Gal-3N contains only the N-terminal SAD domain. In turn, the four recombinant NG2 species (Fig. 3B) were prepared from transfected 293 cells, as previously described [Tillet et al., 1997]. NG2/EC represents the entire NG2 ectodomain containing the D1, D2, and D3 segments. NG2/D2 and NG2/D3 represent the respective isolated D2 or D3 segments, while NG2/ECΔ3 represents the ectodomain lacking D3. The NG2/EC and NG2/ECΔ3 species were purified by DEAE chromatography as core glycoproteins lacking the chondroitin sulfate chain [Tillet et al., 1997].

The NG2-binding capability of the galectin-3 species was tested via pull-down assays utilizing GST fusion proteins attached to glutathione-agarose beads. The four purified galectin-3-GST fusion species are shown in Figure 4A. When these four species were compared for their ability to pull down the 260 kDa recombinant NG2/EC protein, we found that neither Gal-3N-GST nor GST alone were effective in binding to NG2/EC (Fig. 4B). In contrast, Gal-3F-GST and Gal-3C-GST had roughly equivalent NG2-binding capabilities. We, therefore, conclude that the galectin-3 CRD is the functional domain with regard to interaction with NG2.

The D3 Domain of NG2 Is Primarily Responsible for Galectin-3 Binding

To determine which portion of the NG2 ectodomain is responsible for interaction with galectin-3, we next performed pull-down assays in which Gal-3F-GST beads were tested for their ability to pull down the four different recombinant NG2 species NG2/EC, NG2/D2, NG2/D3, and NG2/ECΔD3. The respective electrophoretic mobilities of these species are illustrated in the loading control shown in Figure 5A. The pull-down results show that while none of the four NG2 species are recognized by control GST beads

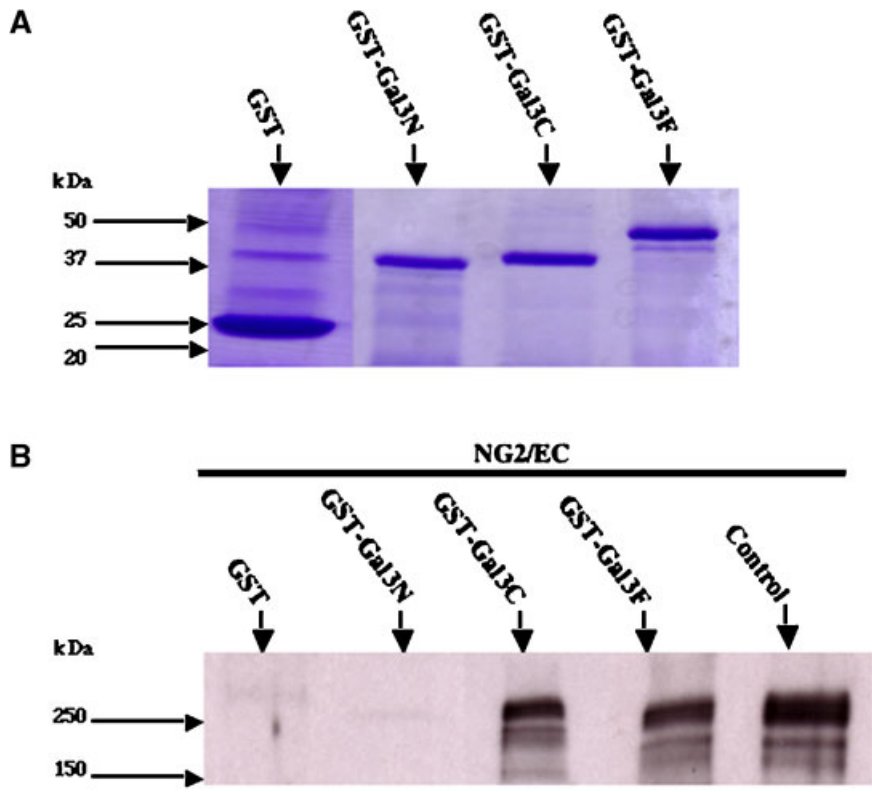

Fig. 4. The galectin-3 CRD mediates binding to NG2. **A**: The purity and electrophoretic mobility of GST alone and the galectin-3-GST fusion proteins (GST-Gal3N, GST-Gal-3C, and GST-Gal-3F) are illustrated by Coomassie Blue staining. **B**: Glutathione agarose beads coated with GST protein alone and with the three GST-galectin-3 fusion proteins were compared for their ability to pull down recombinant NG2/EC. Following SDS-PAGE, bound NG2/EC was detected by immunoblotting with rabbit antibody against NG2. A sample of NG2/EC was included as a positive control in the far right-hand lane. Both full-length galectin-3 (GST-Gal3F) and the CRD (GST-Gal3C) are effective in binding NG2, whereas neither the galectin-3 SAD (GST-Gal3N) nor GST alone interact with NG2/EC. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

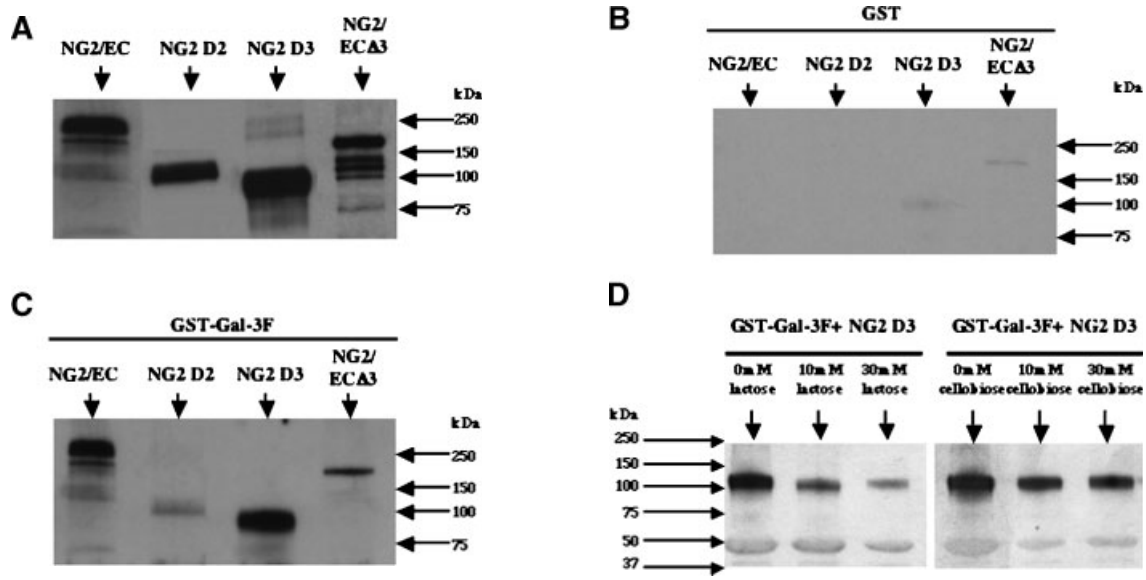

Fig. 5. The D3 segment of NG2 is primarily responsible for binding to galectin-3. Recombinant NG2 species were used to identify the functional domain of NG2 responsible for binding to galectin-3. Electrophoretic mobilities of the NG2 species, detected by immunoblotting, are shown in (**A**). Pull-down assays with each recombinant NG2 species were conducted using glutathione agarose beads carrying full length galectin-3 (**B**) or GST alone (**C**). Bound NG2 species were detected by immunoblotting with rabbit antibody against NG2. The membrane-proximal D3 domain is much more effective than the central D2 domain in binding to galectin-3. NG2/ECΔ3 retains a small degree of binding activity, possibly due to recognition of galectin-3 by the amino terminal D1 domain. **D**: The carbohydrate dependence of the NG2/galectin-3 interaction is demonstrated by inhibition via lactose competition. Binding of NG2/D3 to Gal3F/GST beads is inhibited in a dose-dependent fashion by the presence of lactose at concentrations over the range from 0 to 30 mM. In contrast, cellobiose has little effect on D3 binding to Galectin-3.

(Fig. 5C), both NG2/EC and NG2/D3 exhibit strong binding to Gal-3F-GST beads (Fig. 5B). By comparison, NG2/D2 is very poorly reactive, while NG2/ECΔ3 exhibits a low level of binding. As expected from the data shown in Figure 4B, this same pattern of strong binding to NG2/D3 was reproduced when Gal-3C-GST beads were used for the pull-down assay, while Gal-3N-GST beads were ineffective in recognizing any of the NG2 species (data not shown). These results indicate that the galectin-3-binding capacity of NG2 resides primarily in the membrane-proximal D3 domain. The low level of binding by NG2/ECΔ3 may indicate that domain D1 also makes a minor contribution to galectin-3 recognition.

To further establish the carbohydrate dependence of the interaction between galectin-3 and the D3 domain of NG2, we used the pull-down assay to conduct ligand competition experiments with lactose (*O*-β-D-galactopyranosyl-(1,4)-β-D-glucopyranose) and cellobiose (*O*-β-D-glucopyranosyl-1,4)-β-D-glucopyranose) (Fig. 5D). Whereas a significant reduction of D3/galectin-3 interaction was observed in the presence of 30 mM lactose, inclusion of cellobiose had little effect on binding. In addition to confirming the carbohydrate dependence of D3/galectin-3 binding, these tests also establish the sugar specificity of the interaction. The galactoside dependence of the interaction is apparent from the comparison of the two disaccharides.

N-linked Glycosylation in the D3 Domain Is Required for NG2/Galectin-3 Interaction

As an initial step towards determining whether galectin-3 binding to NG2 is dependent on N- or O-linked glycosylation within the D3 domain of the NG2 core protein, we evaluated the presence of N- and O-linked glycans within this segment of NG2. Analysis with the glycosylation prediction programs NetOGlyc 3.1 and NetNGlyc 1.0 suggests that NG2/D3 might be especially rich in glycosylation sites. Asparagine residues at positions 1650, 1914, 2021, 2039, 2045, and 2080 are predicted to be potential sites of N-linked glycosylation. Threonine residues at positions 2154, 2191, 2194, 2198, 2200, and 2213, along with serine residues at positions 2197, 2209, and 2217, are predicted as possible sites of O-linked glycosylation.

In order to test these predictions, we used oligosaccharide removal strategies to evaluate the actual presence of N- and O-linked glycans within the NG2 core protein as a whole and within the D3 segment in particular. Treatment of NG2-expressing U251 cells with the *O*-glycosylation inhibitor BAG resulted in production of an NG2 core protein with increased electrophoretic mobility (Fig. 6A), indicative of the presence of O-linked glycans in the untreated core protein (in addition to the single chondroitin sulfate chain). BAG treatment of 293 cells expressing the NG2/D3 domain resulted in production of an NG2/D3 species with a small but reproducible shift in electrophoretic mobility compared to that of untreated NG2/D3. This suggests that there is at least some utilization of *O*-glycosylation sites in the D3 segment, although it is not possible from this limited analysis to specify how many sites are modified. In parallel, full length NG2 and NG2/D3 were tested for the presence of N-linked oligosaccharides by treatment with *N*-glycanase (Fig. 6B). Large shifts in electrophoretic mobility are seen in both cases, suggesting that D3 may contain a large percentage of the N-linked glycosylation sites present in NG2.

To evaluate the contribution of N- and O-linked oligosaccharides to NG2/D3 binding to galectin-3, we compared the binding capabilities of NG2/D3 prepared from 293 cells that were untreated, treated with BAG, and treated with tunicamycin, an inhibitor of N-linked glycosylation. BAG-treated NG2/D3 exhibits a pattern of binding to Gal-3F-GST beads, Gal-3C-GST beads, and GST beads that is indistinguishable from that seen with control NG2/D3 (Fig. 7A,B). In contrast, tunicamycin-treated NG2/D3 fails to bind to any of these beads (Fig. 7C), demonstrating that N-linked oligosaccharides within D3 are required for NG2 binding to galectin-3.

Since it is conceivable that *N*-glycosylation is important for correct folding of the D3 domain, we had to consider the possibility that galectin-3 fails to interact with tunicamycin-treated D3 due to a denatured or inactive conformation of the non-glycosylated D3 domain. Since we know that the NG2/D3 domain also contains a binding site(s) for the kringle domains of plasminogen [Goretzki et al., 2000], we were able to further evaluate the functionality of tunicamycin-treated D3 by testing its ability to interact with plasminogen. In contrast to galectin-3-coated

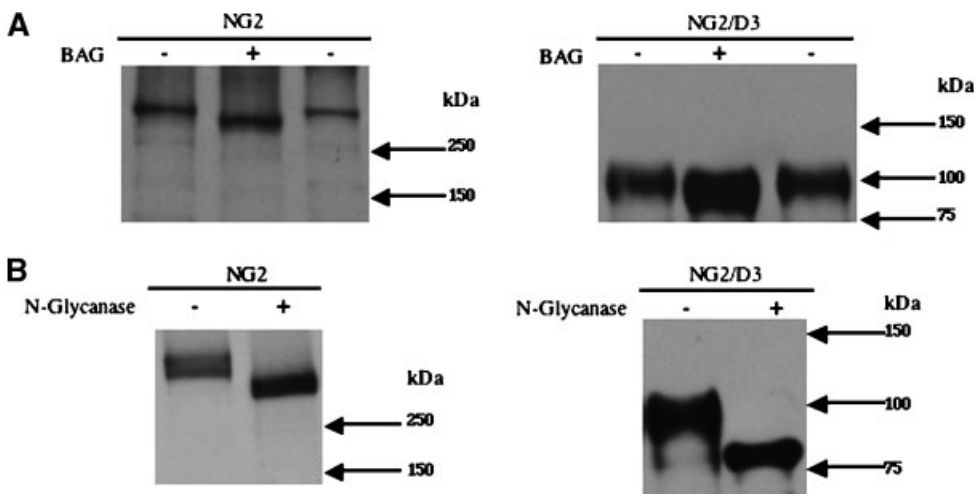

Fig. 6. O- and N-linked glycosylation of the NG2 core protein. **A**: The presence of O-linked glycans in full length NG2 was detected by BAG treatment of U251/NG2 cells. Detergent extracts of control and BAG-treated cells were treated with chondroitinase ABC and examined by immunoblotting with rabbit anti-NG2 antibody. Parallel analysis was performed by BAG treatment of 293 cells expressing recombinant NG2/D3, followed by purification of the recombinant protein. A clear shift in the electrophoretic mobility of full length NG2 is apparent following BAG treatment. A smaller, but consistent shift in mobility is seen with the NG2/D3 domain. These results suggest that some O-linked glycosylation does occur in the D3 portion of NG2. **B**: Treatment with *N*-glycanase was used to detect the presence of N-linked oligosaccharides in full length NG2 extracted from U251/NG2 cells and recombinant NG2/D3 purified from transfected 293 cells. In both cases a large shift in electrophoretic mobility is observed in comparing the *N*-glycanase treated NG2 or NG2/D3 with untreated controls. These results indicate the presence of a significant amount of *N*-glycosylation in NG2, much of which may occur within the D3 domain.

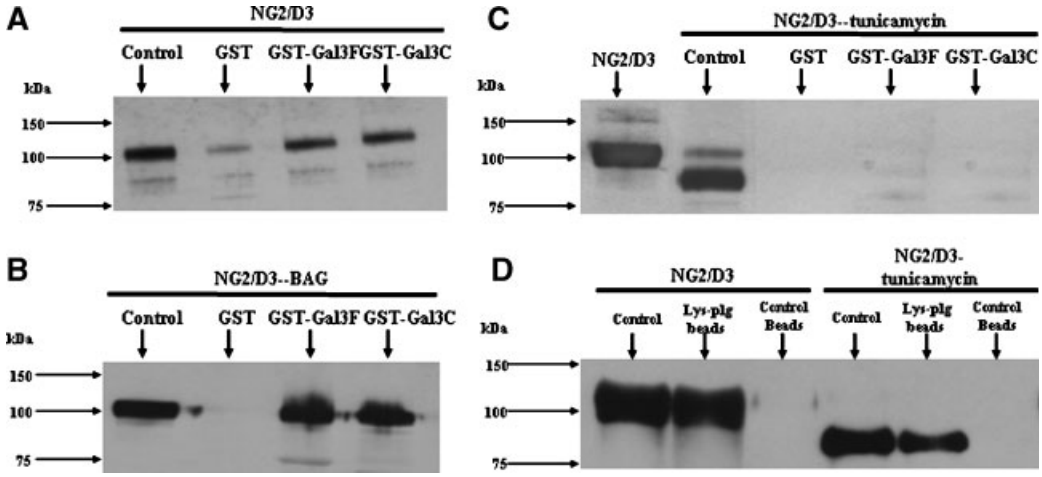

Fig. 7. N-linked glycosylation of NG2/D3 is vital for NG2/galectin-3 interaction. The relative functional importance of N- and O-linked glycosylation was tested by comparing galectin-3 binding to NG2/D3 prepared from control 293 cells (**A**), cells treated with the *O*-glycosylation inhibitor BAG (**B**), and cells treated with the *N*-glycosylation inhibitor tunicamycin (**C**). Beads coated with GST-Gal3F, GST-Gal3C, or GST alone were used to pull down the NG2/D3, NG2/D3-BAG, or NG2/D3-tunicamycin species. Bound material was detected by immunoblotting with a polyclonal rabbit anti-NG2 antibody. Loading controls for the NG2 species are shown in the left-hand lanes in each case. An additional untreated NG2/D3 control is shown in (C) to demonstrate the mobility shift produced by tunicamycin treatment. As shown in (B), BAG treatment does not change the ability of NG2/D3 to recognize either full-length galectin-3 or the CRD segment. In contrast, tunicamycin treatment of NG2/D3 abolishes its ability to bind to either of these galectin-3 species (C). In (**D**), the ability of D3 and tunicamycin-treated D3 to recognize a different ligand was tested via a pull-down assay using beads derivatized with lys-type plasminogen (lys-plg beads). Loading controls are shown for both D3 species (control) and underivatized beads (control beads) were used as a negative pull-down control. Both D3 and tunicamycin-treated D3 are pulled down by lys-type plasminogen-coated beads, but not by control beads, demonstrating that *N*-glycosylation of D3 is not required for its interaction with plasminogen.

beads (Fig. 7C), beads derivatized with lys-type plasminogen are effective in pulling down both D3 and tunicamycin-treated D3 (Fig. 7D). As before, control beads did not pull down either species. These results demonstrate that non-glycosylated NG2/D3 retains the ability to bind plasminogen, a strong indication that the D3 domain assumes a native conformation even when it is not modified by N-linked oligosaccharides.

DISCUSSION

The ability of a tumor to evade immune surveillance, resist apoptosis, expand in size, and metastasize to distant sites largely determines the degree of its malignancy. Malignant tumor cells are known to utilize a variety of strategies to enhance their proliferation, migration, and survival, often subverting normal cellular signaling mechanisms to serve their own purposes. Molecules that mediate these mechanisms can, therefore, serve as key targets for the development of anti-tumor drugs.

Based on earlier work by our group [Fukushi et al., 2004] and others, we believe that one type of molecular mechanism subverted by tumor cells may be the interaction between the NG2 proteoglycan and galectin-3. In developing vasculature, we have demonstrated that NG2 produced by microvascular pericytes can bind to galectin-3 on closely apposed endothelial cell surfaces. This interaction also involves the $\alpha 3\beta 1$ integrin, the resulting activation of which promotes endothelial cell motility and morphogenesis [Fukushi et al., 2004]. We further observed that A375 melanoma cells endogenously express all three components of this signaling complex. We hypothesized that this situation might lead to constitutive activation of the integrin, contributing to the aggressive phenotype of the melanoma cells. The co-expression of both NG2 and galectin-3 is not restricted to melanoma cells, since our current work shows a similar phenomenon in U87 glioblastoma cells (Fig. 1). Similar to the case of A375 cells, a physical interaction between NG2 and galectin-3 on the cell surface exists in U87 cells, as demonstrated by co-immunopatching and co-immunoprecipitation of the two proteins. The same type of physical interaction occurs when NG2 and galectin-3 are expressed

by transfection in U251 astrocytoma cells, which normally exhibit very low levels of both molecules (Fig. 2A,B). The potentially widespread occurrence of an NG2/galectin-3 interaction in tumor cells has prompted us to explore the nature of the interaction between the two proteins. Although we have not considered it in this study due to our focus on the molecular basis of NG2/galectin-3 interaction, both U87 and U251, like A375 cells, also express $\alpha 3\beta 1$ as their predominant $\beta 1$ integrin species [Makagiansar et al., 2004]. The participation of the integrin in the properties and consequences of the interaction will be an important topic of future investigation.

Members of the galectin family of mammalian lectin-like molecules are widely believed to make important contributions to the enhancement of cell proliferation, motility, and resistance to apoptosis [Liu and Rabinovich, 2005]. The large number of galectins, along with their extended array of binding partners, allows them to affect a wide range of cellular processes in a diverse collection of cell types. Galectin-3 is unique among the galectins due to the presence of a self-association domain in tandem with its carbohydrate recognition domain [Cooper, 2002; Leffler et al., 2004]. This combination allows galectin-3 to serve as a mediator of molecular multimerization, often a key factor in the amplification of signaling mechanisms. In this context, galectin-3 has been implicated not only in promoting cell proliferation and resistance to apoptosis, but also in mediating cell–cell and cell–matrix interactions [Inohara et al., 1998; Ochieng et al., 2004; Shekhar et al., 2004; Takenaka et al., 2004; Nakahara et al., 2005]. Galectin-3 has been widely studied for its ability to affect neoplastic progression by serving as a ligand for a variety of glycoconjugates expressed by tumor cells [Liu and Rabinovich, 2005].

The NG2 proteoglycan is also upregulated in several types of tumors, including melanomas, gliomas, chondrosarcomas, and lymphomas. Normally expressed by immature progenitors in a variety of tissues, NG2 is thought to contribute to the proliferation and migration of both progenitors cells and transformed cells [Stallcup, 2002]. The ability to potentiate signaling through well-characterized transmembrane signaling components such as integrins and growth factor receptors appears to be an important aspect of the proteoglycan's activities [Grako and Stallcup, 1995; Iida et al., 1995; Nishiyama et al., 1996b; Eisenmann et al., 1999; Grako et al., 1999; Fukushi et al., 2004; Makagiansar et al., 2004; Yang et al., 2004].

The GST-fusion protein pull-down assays presented here demonstrate a direct, carbohydrate-dependent interaction between NG2 and galectin-3. Furthermore, we identify the C-terminal CRD segment of galectin-3 as the mediator of binding to NG2. These results extend our earlier observation of an NG2/galectin-3 interaction on endothelial cells [Fukushi et al., 2004], and are consistent with the results of many other studies demonstrating the role of the galectin CRD in binding to glycoproteins [Liu and Rabinovich, 2005]. We have also been able to identify the portion of the NG2 ectodomain primarily responsible for galectin-3 binding. By using several recombinant fragments of the NG2 ectodomain [Tillet et al., 1997], we have demonstrated that the membrane-proximal D3 segment is comparable to the intact ectodomain in its ability to interact with galectin-3. By comparison, the central D2 domain exhibits a very low level of galectin-3 binding. At this point, we cannot rule out the possibility that the globular N-terminal D1 domain also contributes to galectin-3 binding, since the recombinant NG2/ECΔ3 fragment exhibits better binding to galectin-3 than D2 (although still lower than that of NG2/D3). However, our results show that the D3 domain is the primary domain responsible for NG2 binding to galectin-3.

An important result of our study is the finding that N-linked oligosaccharides in the D3 domain are required for recognition by galectin-3. Interestingly, *N*-glycosylation of D3 is not a requirement for at least one other functional property of this domain, namely its ability to mediate binding to plasminogen, an interaction that enhances u-PA-catalyzed activation of this zymogen to plasmin [Goretzki et al., 2000]. The multifunctional nature of the D3 domain, therefore, is dependent on distinct structural determinants present in this portion of the NG2 ectodomain, some carbohydrate in nature and some not. Earlier work in our group had demonstrated the existence of N-linked oligosaccharides in NG2, based on a shift in the electrophoretic mobility of the NG2 core protein derived from tunicamycin-treated cells [Stallcup et al., 1983]. Our current results show that many of these N-linked oligosaccharides are likely to be localized within the D3 domain, as

revealed by the significant shift in electrophoretic mobility of this species resulting from treatment with either *N*-glycanase (Fig. 6) or the *N*-glycosylation inhibitor tunicamycin (Fig. 7). The detection of N-linked glycosylation within NG2/D3 is consistent with the prediction of six potential asparagine linkage sites in this region of the core protein (analysis by NetNGlyc 1.0). Our results with the *O*-glycosylation inhibitor BAG demonstrate that *O*-glycosylation also occurs within the D3 domain. It is not possible to determine from these tests how many of the nine predicted O-linked glycosylation sites within NG2/D3 (analysis by NetOGlyc 3.1) are actually utilized. However, sites that are *O*-glycosylated, appear to have no impact on binding of NG2/D3 to galectin-3. The precise location of N- and O-linked glycosylation sites within NG2/D3 remains to be determined, either by mutational analysis or by mass spectrometry of proteolytic fragments.

U251 astrocytoma cells will be an important resource for ongoing studies of the functional consequences of NG2/galectin-3 interaction on the behavior of malignant human gliomas. These cells express the $\alpha 3\beta 1$ integrin [Makagiansar et al., 2004], but have little endogenous NG2 or galectin-3. Since we have generated both singly transfected U251/galectin-3 and U251/NG2 cells, as well as the doubly transfected U251/NG2/galectn-3 cells used in this report, we will be able to test the individual and synergistic effects of the two molecules on the cellular behavior of U251 cells. In addition, since we have partially localized the functional domains within galectin-3 and NG2 that mediate their interaction, we can explore the possibility of producing restricted protein fragments or peptides that are capable of disrupting the NG2/galectin-3 interaction. Such reagents will be of interest for their potential ability to reduce the aggressive properties of tumor cells.

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