Expression of NG2 Proteoglycan Causes Retention of Type VI Collagen on the Cell Surface

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NG2 is a membrane-associated chondroitin sulfate proteoglycan with a core protein of 300 kD. Previously it was shown immunochemically that the core protein of NG2 can bind type VI collagen (Stallcup, W., Dahlin, K., and P. Healy. 1990. *J. Cell Biol.* 111:3177–3188). We have extended our studies on the interaction of NG2 and type VI collagen by transfecting cells with the full-length rat NG2 cDNA. B28 rat neural cells and U251MG human glioma cells used for transfection do not synthesize NG2. Both cell lines secrete type VI collagen into tissue culture medium but do not anchor it at the cell surface. Upon transfection of these cells with the NG2 cDNA, NG2 was correctly localized to the cell surface. Furthermore, type VI collagen could now be detected on the surface of NG2-positive cells in a pattern that coincided with that of NG2. This ability of NG2 to anchor type VI collagen to the cell surface could be abolished by incubating the cells in the presence of anti-NG2 monoclonal antibodies. These findings indicate that NG2 functions as a cell surface receptor for type VI collagen and may play a role in modulating the assembly of pericellular matrix.

INTRODUCTION

The extracellular matrix (ECM) affects a wide range of cellular phenomena that includes cell adhesion, migration, proliferation, and gene expression (Hay, 1981; Bissell *et al.*, 1982). This matrix is composed of a variety of molecules, some of which exert their effects by interacting with cell surface receptors. The binding of a particular matrix molecule to its receptor is influenced not only by the inherent properties of the receptor but also by the interaction of the matrix molecule with other components of the matrix. Thus the cellular response to a matrix molecule need not be stereotyped but can be influenced by the composition of the matrix. Therefore it seems important to identify and investigate the factors that influence the composition and organization of the matrix.

It is now well established that integrins, which are heterodimeric membrane-associated glycoproteins, represent one major class of receptors for ECM molecules (Hynes, 1992). Integral membrane proteoglycans, an increasing number of which are being identified by cDNA cloning, comprise another group of receptors for ECM molecules (Hardingham and Fosang, 1992). For example, the syndecan family of

membrane-associated proteoglycans can bind such ECM molecules as collagen types I, III, V, fibronectin, and thrombospondin (Bernfield *et al.*, 1992). The binding appears to occur through the glycosaminoglycan chain of syndecan. However, the detailed nature of the interactions and the biological significance of the binding still remain unclear. CD44 is another newly defined class of integral membrane proteoglycans that includes various molecular forms with different ligand specificities and adhesive properties (Brown *et al.*, 1991; Stamenkovic *et al.*, 1991; Thomas *et al.*, 1992).

We have been studying a large chondroitin sulfate proteoglycan, NG2, which is expressed by dividing progenitor cells of glial and mesenchymal lineages (Stallcup and Beasley, 1987; Nishiyama *et al.*, 1991a). Structural analyses of NG2 have revealed that it is an integral membrane proteoglycan with a large, extracellular domain and a short cytoplasmic tail (Nishiyama *et al.*, 1991b). It has been shown that the core protein of NG2 interacts with type VI collagen (Stallcup *et al.*, 1990). In the developing rat embryo, NG2 has been found to be colocalized with type VI collagen on blood vessels and immature chondrogenic tissues (Stallcup *et al.*, 1990; Nishiyama *et al.*, 1991a).

Type VI collagen is widely distributed in connective tissues (Burgeson, 1988) in the form of thin, beaded filaments, distinct from other types of collagens (Van der Rest and Garrone, 1991). The structure of type VI collagen was elucidated by cDNA cloning of the chick (Bonaldo *et al.*, 1989, 1990; Koller *et al.*, 1989) and human (Chu *et al.*, 1989, 1990) molecules. It is composed of two small subunits, $\alpha 1$ and $\alpha 2$, of 140 kD and one large subunit, $\alpha 3$, of 250 kDa in size. This collagen species is unique among the collagens in that it has a high content of globular domain relative to the triple-helical collagenous domain. It has been shown to form large aggregates with extensive disulfide cross-linking. Although the functions of type VI collagen still remain largely unknown, it has been suggested to anchor interstitial collagen fibers to the cell surface (Bonaldo *et al.*, 1990).

In the present study we have investigated the effects of the NG2 proteoglycan on the behavior of type VI collagen by transfecting type VI collagen-producing cells with the full-length rat NG2 cDNA. We have found that the expression of NG2 causes retention of type VI collagen at the surface of cells that normally secrete type VI collagen. Thus NG2 can serve as a cell surface receptor for type VI collagen, providing one means of interaction between the cell surface and the ECM.

MATERIALS AND METHODS

Expression Constructs and Transfection

cDNA fragments encoding rat NG2 (Nishiyama *et al.*, 1991b) were ligated in pBluescript vector (Stratagene, La Jolla, CA) to obtain a construct that contained the entire coding region of the rat NG2 cDNA. The cDNA was excised by XbaI and partial HindIII digestion and ligated into the HindIII-XbaI site of the expression vector pRc/CMV (In Vitrogen, San Diego, CA). This vector contains the cytomegalovirus promoter to drive the transcription of the foreign DNA. The resulting plasmid, which contains 69 bp of 5'-untranslated region and 129 bp of 3'-untranslated region of the rat NG2 cDNA in addition to 6984 bp of the coding region, was designated as pCMVrNG2.

Supercoiled plasmid DNA was purified by CsCl equilibrium centrifugation and used for transfection. Transfection was performed by using Lipofectin reagent (Bethesda Research Laboratories, Grand Island, NY) and 8 μ g of plasmid DNA per 60 mm dish of cells.

B28, U251MG, and WI-38 VA13 cells were used for transfection. B28 is a rat neural cell line derived from a central nervous system tumor induced by ethylnitrosourea (Schubert *et al.*, 1974; Stallcup and Cohn, 1976). The U251MG line is a human astrocytoma cell line characterized by the presence of the marker, glial fibrillary acidic protein (Ponten and Westermark, 1978) and was provided to us by Dr. Eva Engvall (La Jolla Cancer Research Foundation). The WI-38 VA13 human fibroblast line was obtained from American Type Culture Collection (ATCC). NIH3T3 cells were obtained from Dr. Channing Der (University of North Carolina, Chapel Hill, NC). Cells were maintained in Dulbecco's modified Eagle's (DME) supplemented with 10% fetal calf serum (FCS) (Tissue Culture Biologicals, Tulare, CA).

For experiments in which NG2 was expressed transiently, transfected cells were analyzed for NG2 expression by immunofluorescence 3 d after transfection. To obtain stable transfectants that have integrated pCMVrNG2 or vector DNA, transfected cells were selected in the presence of 200 μ g/ml (in the case of B28 cells) or 400 μ g/ml (in

the case of U251MG cells) of neomycin (G418, Gibco Laboratories, Grand Island, NY). Resistant colonies were isolated and screened for the expression of NG2 by immunofluorescent staining using monoclonal anti-NG2 antibodies.

Immunofluorescence

Living cells were stained on tissue culture dishes by the indirect immunofluorescence method. Primary and secondary antibodies were diluted in *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered DME containing 2% calf serum. The same solution was used for the washes. The derivation of polyclonal and monoclonal anti-rat NG2 antibodies has been described (Levine and Stallcup, 1987; Stallcup *et al.*, 1990). Polyclonal (#279) and monoclonal (2C6 and 3C4) anti-human type VI collagen antibodies (Hessle and Engvall, 1984) were gifts from Dr. Eva Engvall (La Jolla Cancer Research Foundation). Monoclonal anti-rat lamin antibody was a gift from Dr. Don Newmeyer (La Jolla Cancer Research Foundation). Rabbit anti-human fibronectin antibody was obtained from Telios Pharmaceuticals (San Diego, CA). Rabbit anti-human laminin antibody was a gift from Dr. Eva Engvall. Monoclonal anti- $\beta 1$ integrin antibody AIB2 was a gift from Dr. C. Damsky (Hall *et al.*, 1990). Staining of living cells to detect cell surface antigens was performed as follows. Cells were incubated with the primary antibodies at room temperature for 30 min, washed four times, and then incubated with FITC- or TRITC-labeled secondary antibodies (Tago, Burlingame, CA) for 30 min at room temperature. Then the cells were washed three times with phosphate-buffered saline (PBS), fixed with 95% ethanol at -20°C for 5 min, dried, and mounted. Immunolabeled cells were examined with a Nikon Optiphot (Nikon, Melville, NY) microscope equipped for epifluorescence and phase contrast. Double labeling for NG2 and lamin was performed as follows. Living cells were immunolabeled with rabbit anti-NG2 antibody as described above using rhodamine-labeled anti-rabbit immunoglobulin antibody as the second antibody. After fixation with 95% ethanol at -20°C for 10 min, the cells were hydrated and rinsed in the washing buffer. Then they were incubated with monoclonal anti-lamin antibody for 30 min at 37°C followed by incubation with FITC-labeled anti-mouse immunoglobulin antibody. Double-labeled cells were examined with a Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) laser scanning confocal microscope. Confocal sections were made at 0.5 μ m throughout the height of the cell.

Metabolic and Surface Labeling of Cells and Immunoprecipitation

^{35}S -methionine-labeled cultures were used for precipitating secreted type VI collagen. Freshly confluent cultures of cells were labeled for 18 h with 100 $\mu\text{Ci}/\text{ml}$ of ^{35}S -methionine (TRANS ^{35}S -LABEL, ICN Biomedicals, Irvine, CA) in methionine-free DME supplemented with 1% dialyzed FCS. Conditioned medium was centrifuged once to remove cellular debris and incubated with rabbit anti-human type VI collagen antiserum followed by precipitation with protein A-Sepharose (Sigma, St Louis, MO) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF).

For immunoprecipitating NG2 and type VI collagen from the cell surface, cells were labeled with ^{125}I by the lactoperoxidase method (Hubbard and Cohn, 1972). Labeled cells were extracted with 1% NP40 in 10 mM tris(hydroxymethyl)aminomethane (Tris) HCl, pH 7.4, containing 1 mM PMSF. Insoluble material was removed after 10 min by centrifugation. Cleared extracts were incubated with a rabbit antibody against rat NG2 or rabbit anti-human type VI collagen antibody followed by incubation with protein A-Sepharose. All the precipitates were washed three times with PBS. In some cases the precipitates were treated with 0.02 units of chondroitinase ABC (ICN Biomedicals, Costa Mesa, CA) at 37°C for 1 h to remove chondroitin sulfate. The precipitates were boiled in electrophoresis sample buffer containing 5% 2-mercaptoethanol and 3% sodium dodecyl sulfate (SDS) and electrophoresed through 3–20% acrylamide gradient gels.

The gels were fixed and processed for autoradiography or fluorography using EN³HANCE (NEN, Boston, MA).

Antibody Blocking Experiments

To strip cells of NG2 and type VI collagen, cells were washed with PBS and treated with 0.05% trypsin and 0.53 mM EDTA (Gibco) at room temperature for 2 min. After removal of trypsin-EDTA solution, cells were washed with DME containing 10% FCS. Then they were suspended in the same medium and plated in the presence or absence of ascites containing mAbs against the rat NG2 core protein. In some cases, a mixture of five different anti-ectodomain mAbs were used at a dilution of 1:100. In other cases, individual mAbs were used at a dilution that gave bright immunofluorescent staining of NG2 on cells (i.e., 1:200 for D31, D4, and D120 mAbs; 1:100 for N109 mAb, and 1:25 for N3 mAb). The cells were stained for NG2 and type VI collagen by immunofluorescence 24 h later.

RESULTS

Transient Expression of Rat NG2

B28 rat neural cells and U251MG human glioma cells were transiently transfected with pCMVrNG2, containing the rat NG2 cDNA, or the vector pRc/CMV as control. Live cells were double-labeled by immunofluorescence for NG2 and type VI collagen 3 d after transfection. Cells in control-transfected cultures had no detectable levels of cell surface NG2. Cultures transfected with the NG2 cDNA contained cells that showed punctate staining with anti-NG2 monoclonal antibodies (Figure 1c). The pattern of NG2 staining on these cells was very similar to that on B49 cells that endogenously express NG2 (Figure 1a). The morphology of positively stained cells was not remarkably different from that of NG2-negative parent cells.

Labeling of the control-transfected cultures with rabbit anti-type VI collagen antibody revealed no detectable levels of type VI collagen on most of the cells. There was a small population of cells in these cultures that displayed a wavy, fibrillar pattern of type VI collagen as demonstrated previously (Stallcup *et al.*, 1990). In contrast, labeling of NG2-transfected cultures for type VI collagen showed strong immunoreactivity on the majority of NG2-positive cells (Figure 1d) while NG2-negative cells displayed no collagen staining or weak staining in a wavy fibrillar pattern as seen on some untransfected cells (Figure 1f). The punctate pattern of type VI collagen staining on NG2-positive cells coincided with the linear arrays of punctate staining of NG2. This coincidence of staining is similar to the pattern seen on B49 cells (Figure 1b) on which NG2 and type VI collagen have previously been shown to be colocalized (Stallcup *et al.*, 1990). The intensity of immunoreactivity for NG2 and type VI collagen was comparable in individual B28 and U251 MG transfectants, although U251MG cells exhibited a higher efficiency for transfection than B28 cells.

Figure 1. Immunofluorescent staining for NG2 and type VI collagen on B28 cells transiently transfected with the rat NG2 cDNA. Living cells were double-labeled with a mixture of anti-NG2 mAbs (1:100 dilution; a, c, and e) and rabbit anti-type VI collagen antibody (1:100 dilution; b, d, and f). a and b; B49 cells that endogenously express NG2. c through f; B28 cells transiently transfected with rat NG2 cDNA. Bar in f represents 20 μ m. Linear arrays of punctate staining for type VI collagen are seen to coincide with the pattern of NG2 staining on B49 cells (a and b) and NG2-positive transfected cells (c and d). Most NG2-negative cells have no type VI collagen, but the few that do display a weak staining in a wavy, fibrillar pattern (e and f).

Establishment of Cell Lines That Stably Express NG2

To further study the effects of NG2 on extracellular matrix organization, we isolated B28 and U251MG cell lines that stably expressed rat NG2. Neomycin-resistant colonies were screened by immunofluorescent staining for NG2. Two positive clones, B28NG11 and B28NG14, were isolated from B28 transfectants. Several NG2-positive clones were isolated from U251 MG transfectants. Of these U251NG35 and U251NG52 were chosen for further studies. While 100% of the cells in the U251NG52 line expressed surface NG2, only 60–80% of the cells in the B28 clones and U251NG35 were NG2-positive. Attempts to subclone the B28 clones to obtain a homogeneous population were unsuccessful, for all the subclones contained a certain percentage of cells that did not express NG2. It seems likely that NG2 expression is being lost over a period of time by a certain proportion of the cells in these populations. The reason for this is not clear to us at present. In parallel, we isolated neomycin-resistant clones from B28 and U251 MG cultures transfected with the pRc/CMV vector. These

lines were designated B28CMV and U251CMV, respectively, and used as controls in subsequent studies.

Colocalization of NG2 and Type VI Collagen on Stable Transfectants

Immunofluorescence. To study the effects of NG2 expression on the distribution of cell surface type VI collagen, NG2- and control-transfected cells were double-labeled with monoclonal anti-NG2 antibodies and rabbit anti-type VI collagen antibody. Figure 2A shows immunofluorescent staining for NG2 and type VI collagen on the surface of B28NG11 and B28CMV cells. As noted above, B28NG11 cultures contained a mixture of NG2-positive and negative cells. All the NG2-positive cells were labeled with anti-type VI collagen antibody while NG2-negative cells in B28NG11 and B28CMV had little detectable cell surface type VI collagen. A higher magnification of these cells in Figure 2B shows a high degree of colocalization of the linear arrays of punctate staining for NG2 and type VI collagen on the surface of B28NG11 cells (Panels a and b). The level of expression of NG2 and type VI collagen and the extent of colocalization of the two antigens were not significantly affected by cell density. Most B28CMV cells had no surface type VI collagen as seen in Figure 2B panel d. However, occasional B28CMV cells displayed a random, fibrillar pattern of type VI collagen in the absence of NG2 (Figure 2B panel f). This pattern was distinct from the punctate, linear array of type VI collagen on NG2-positive cells. To confirm that the labeling of NG2 and type VI collagen was localized at the cell surface and did not result from endocytosed antibodies, B28NG11 cells were double-labeled for NG2 and the nuclear intermediate filament protein lamin (Franke, 1987) and examined by confocal microscopy. Figure 3 shows a series of optical sections of a cell stained for lamin (a–d) and NG2 (e–h). The morphology of this cell was similar to that shown in Figure 2B, a and b (see drawing in Figure 3). In a section through the basal edge of the nucleus (a and e) NG2 was localized only at the periphery of the cell. In sections through the center of the nucleus (b and f, c, and g) NG2 was absent from the region immediately adjacent to the nucleus but was detected in a ring-like pattern confined to the outermost part of the cell. Above the nucleus where lamin staining was absent, NG2 could be detected over the central portion of the cell (d and h). This pattern of NG2 staining in relation to the nuclear lamin staining indicates that NG2 is indeed localized to the cell surface and not to the perinuclear region of the cytoplasm. These results and the findings obtained from cells transiently transfected with NG2 demonstrate that the expression of NG2 can cause anchoring of type VI collagen on the surface of cells that normally secrete but cannot retain the molecule at the cell surface.

To examine whether this effect of NG2 was specific

to type VI collagen, B28NG11 and B28CMV cells were double-labeled for NG2 and other extracellular matrix molecules (Figure 4). There was no difference in the distribution of fibronectin (Figure 4, a–f) or laminin (Figure 4, g–l) between control- and NG2-transfected cells. This indicates that NG2 specifically alters the distribution of type VI collagen without affecting other ECM molecules.

There was variability among different clonal cell lines in the levels of cell surface type VI collagen associated with NG2. For example, cells of the B28NG14 clone exhibited retention of type VI collagen on the surface of cells that expressed NG2 but not on NG2-negative cells. Upon close examination, however, the extent of colocalization of type VI collagen and NG2 on B28NG14 cells was lower than that on B28NG11 cells. Differences in the amount of NG2-associated type VI collagen could also be observed in the U251 clones. Although both U251NG35 and U251NG52 cells expressed high levels of NG2 at the cell surface, comparable to the B28 clones and the transiently transfected U251MG cells, only U251NG35 cells showed a significant level of type VI collagen at the cell surface. There was little type VI collagen on the surface of U251NG52 cells. The extent of colocalization of NG2 and type VI collagen in these stably selected U251MG cells was also less than that seen on the transiently transfected U251MG cells. These observations suggest that the anchoring of type VI collagen is affected by factors other than the expression of NG2 on the cell surface.

Integrins are another class of molecules that have been shown to bind collagens, including type VI collagen (Wayner and Carter, 1987; Aumailley *et al.*, 1989). To determine whether integrin expression contributes to or is affected by the NG2-type VI collagen interaction in NG2-transfected cells, the U251NG35 transfectants were compared with U251CMV control transfectants for staining with the A1B2 monoclonal antibody against the $\beta 1$ integrin subunit. Both cell types displayed diffuse, weak punctate staining over the entire surface of the cell. There was no detectable difference in the distribution of the $\beta 1$ subunit between the NG2⁺/type VI collagen⁺ cells and NG2[−]/type VI collagen[−] cells. This finding suggests that integrins do not contribute significantly to the formation of the NG2-type VI collagen complex, strengthening our hypothesis that NG2 can act independently as a receptor for type VI collagen.

Biochemical demonstration of NG2 and type VI collagen expressed by transfected cells. To confirm the expression of NG2 in transfected cell lines, cell surface NG2 was immunoprecipitated from ¹²⁵I-labeled detergent extracts made from cells of clones B28CMV and B28NG11. A heterodisperse component of 350 to 600 kD could be immunoprecipitated from NG2-transfected B28NG11 cells but not from control-transfected B28CMV cells (Figure 5A, B28.11, N; B28CMV, N). In the presence of chondroitinase ABC, this component

Figure 2. Immunofluorescent staining for NG2 and type VI collagen on B28 cells stably expressing NG2. (A) a–c (B28NG11): B28 cells transfected with NG2 cDNA. d–f (B28CMV): B28 cells transfected with pRc/CMV vector only. a and d: phase contrast. b, c and e, f: double-labeled with a mixture of anti-NG2 mAbs (b and e) and rabbit anti-type VI collagen antibody (c and f). Bar in a represents 100 μ m. (B) a and b (B28NG11): B28 cells transfected with NG2 cDNA. c–f (B28CMV): B28 cells transfected with pRc/CMV vector only. All cultures are double-labeled with monoclonal anti-NG2 antibody mixture (a, c, and e) and rabbit anti-type VI collagen antibody (b, d, and f). Bar in e represents 20 μ m. Type VI collagen is colocalized with NG2 on NG2-positive cells. Most NG2-negative cells have little or no surface type VI collagen, although a few show a wavy fibrillar pattern of collagen that is distinct from that on NG2-positive cells.

Figure 3. Immunofluorescent staining for nuclear lamin and NG2 in an NG2-transfected cell, B28NG11. A confocal microscope was used to obtain optical sections through 4 different planes of the cell (a and e, b and f, c and g, and d and h) as shown in the drawing at the top. a-d: stained with monoclonal anti-lamin antibody. e-h: stained with polyclonal anti-NG2 antibody. NU: nucleus. Bar in panel a represents 20 μ m. NG2 is localized to the outer edge and apical surface of the cell, suggesting that it is on the cell surface and not in the cytoplasm.

could be converted into a discrete band of 300 kDa (Figure 5A, B28.11, N*), which has an electrophoretic mobility identical to the NG2 core protein endogenously expressed by B49 cells (Figure 5A, B49, N*). A similar chondroitin sulfate proteoglycan could be immunoprecipitated from U251NG35 and U251NG52 cells but not from the vector-transfected U251CMV cells. These results indicate that NG2 in the transfected cells is correctly localized at the cell surface and can be posttranslationally modified by chondroitin sulfate in a manner similar to the endogenously expressed molecule.

In the foregoing experiments immunofluorescent staining of NG2-transfected cells revealed a difference in the pattern of expression of type VI collagen on the surface of NG2-positive and negative cells. To compare the distribution of type VI collagen biochemically, type VI collagen was immunoprecipitated from tissue culture supernatants and cell layers of NG2- and control-transfected cells.

Secreted type VI collagen was immunoprecipitated from the conditioned media of 35 S-methionine-labeled cells using rabbit anti-human type VI collagen antibody. As shown in Figure 5B, two bands of 130 to 140 kD, which correspond to the small subunits of type VI collagen, and a heterogeneous signal ranging from 200 to 260 kD corresponding to the large subunit could be detected at roughly comparable levels in the media from all the cell lines tested regardless of the level of NG2 expression.

To examine cell surface type VI collagen, 125 I-labeled extracts from B28NG11 and B28CMV cells were immunoprecipitated with anti-type VI collagen antibody.

A band of $\sim$ 140 kD, which corresponds to the small subunits of type VI collagen, could be immunoprecipitated from the extracts of B49 cells and NG2-transfected cells (Figure 5A, B49, C and B28.11, C) but not from vector-transfected cells (Figure 5A, B28CMV, C). Although the 260 kD subunit of type VI collagen is not evident in these experiments, previous studies have indicated that the large α 3 subunit is less heavily labeled by 125 I and can be difficult to detect by immunoprecipitation (Stallcup *et al.*, 1990). These findings indicate that upon transfection of cells with NG2, although a substantial amount of type VI collagen continues to be released into the medium, some of the type VI collagen remains associated with the cell surface. This substantiates the immunohistochemical differences we have noted between transfected and nontransfected cells.

To confirm that both the small and large subunits of type VI collagen were colocalized with NG2, WI-38 VA13 human fibroblasts were transiently transfected with the pCMVrNG2 and double-labeled with rabbit anti-NG2 antibody and monoclonal anti-human type VI antibodies 2C6 and 3C4 that recognize the large and the small subunits, respectively (Hessle and Engvall, 1984). Untransfected WI-38 VA13 fibroblasts did not express detectable cell surface NG2 or type VI collagen. Cells transiently transfected with NG2 expressed high levels of type VI collagen on the surface. The level of type VI collagen on these NG2-positive cells was much higher than that on transiently transfected U251MG cells. Both monoclonal anti-type VI collagen antibodies labeled the surface of NG2-positive WI-38 VA13 cells in a pattern similar to that of NG2 staining, indicating

Figure 4. Immunofluorescent staining for fibronectin and laminin on NG2-transfected cells. B28 cells transfected with NG2 (B28NG11, a–c and g–i) or pRc/CMV (B28CMV, d–f and j–l) were double-labeled with anti-NG2 mAb mixture (b, e, h, and k) and either rabbit anti-fibronectin (1:100 dilution, c and f) or rabbit anti-laminin (1:100 dilution, i and l). a, d, g, and j: phase contrast. Bar in a represents 100 μ m. There is no difference in the distribution of fibronectin or laminin on the surface of NG2-positive and NG2-negative cells.

that both subunits are utilized for the assembly of type VI collagen into the matrix on the surface of NG2-positive cells.

Monoclonal Anti-NG2 Antibodies Can Inhibit the Redistribution of Type VI Collagen Caused by the Expression of NG2

Anti-NG2 antibodies were tested for their effects on the redistribution of type VI collagen on the surface of NG2-transfected cells. NG2-positive cells were trypsinized to remove NG2 and type VI collagen from the cell surface and plated in the presence of a mixture of five monoclonal anti-NG2 antibodies, all of which recognize different epitopes on the extracellular domain of NG2. The cells were stained for NG2 and type VI collagen

24 h later. In the absence of anti-NG2 antibodies, extensive colocalization of NG2 and type VI collagen was observed (Figure 6, a and b). In the presence of a mixture of five anti-NG2 antibodies, type VI collagen could no longer be detected on the surface of cells that continued to express NG2 (Figure 6, e and f). When the cells were incubated with the individual mAbs that constituted the mixture, the inhibitory effect was incomplete. Type VI collagen could still be detected on the surface of NG2-positive cells even though the level was somewhat lower than control cultures in the case of some antibodies. An example of incubation with the D31 mAb is shown in Figure 6, i and j. No changes in the distribution of fibronectin could be seen after incubation with anti-NG2 antibodies (Figure 6, c, d, g, h, k, and l) indicating that

Figure 5. (A) Immunoprecipitation of NG2 and type VI collagen from ^{125}I -labeled NG2-transfected cells. Labeled extracts from B49 cells (B49), NG2-transfected B28NG11 cells (B28.11), and control-transfected B28CMV cells (B28CMV) were prepared and immunoprecipitated with a rabbit anti-type VI collagen antibody (C) or a rabbit anti-NG2 antibody (N, N*). N*: NG2 immunoprecipitates treated with chondroitinase ABC. C_p: Immunoprecipitated with preimmune serum of the anti-type VI collagen antibody. N_p: Immunoprecipitated with preimmune serum of the anti-NG2 antibody. Positions of molecular weight standards are shown on the left. Type VI collagen (→) is precipitated from extracts of NG2-positive B49 and B28NG11 cells but not from NG2-negative B28CMV cells. The 200 kD species that appears along with the 300 kD core protein (▶▶) in the chondroitinase-treated NG2 precipitates is likely to be a proteolytic fragment of NG2. (B) Immunoprecipitation of secreted type VI collagen from B28 cells transfected with NG2. B49

cells (lane 1), B28CMV cells transfected with pRc/CMV vector alone (lane 2), and B28NG11 and B28NG14 cells transfected with NG2 cDNA (lanes 3 and 4) were labeled with ^{35}S -methionine, and type VI collagen was immunoprecipitated from the conditioned media. The positions of molecular weight standards are shown on the left. Small (130–140 kD) and large (200–260 kD) subunits of type VI collagen are detected in the conditioned media from all the cell lines.

the ability of the antibodies to inhibit the anchoring of type VI collagen to the surface of NG2-expressing cells was an effect specific to type VI collagen.

DISCUSSION

Previously we have presented biochemical evidence that type VI collagen can interact with the core protein of NG2 and that NG2 and type VI collagen are co-localized to a high degree in cell lines that express both molecules (Stallcup *et al.*, 1990). These results suggested that an interaction between NG2 and type VI collagen occurs at the cell surface, but they did not allow us to assess the contribution of NG2 relative to other cell surface or matrix molecules in determining the organization of type VI collagen at the cell surface. Our present study extends this line of investigation by demonstrating that NG2 is in fact one of the most important receptors responsible

for anchoring type VI collagen to the cell surface. In this study we have utilized two cell lines, B28 and U251MG, which normally do not produce NG2 but synthesize type VI collagen and secrete it into the tissue culture medium. U251MG cells and the majority of B28 cells do not appear to deposit any type VI collagen on the cell surface or matrix, although a small percentage of B28 cells express a low level of pericellular type VI collagen in a fibrillar pattern that is distinctively different from the pattern seen on cells that express NG2. We have succeeded in expressing the full length rat NG2 cDNA in these two cell lines and have studied the effects of NG2 expression on the distribution of type VI collagen in the cells. Upon transfection of B28 and U251MG cells with the NG2 cDNA, type VI collagen can be localized to the surface of NG2-positive cells by immunofluorescence. The linear arrays of punctate type VI collagen staining coincide with the staining for NG2. This distribution of type VI collagen closely resembles that seen on cells which endogenously express both NG2 and type VI collagen at the surface. By confocal microscopy we were able to show unequivocally that the fluorescent labeling of NG2 in the transfected cells was located at the cell surface and not in the cytoplasm. Because other extracellular matrix molecules such as fibronectin and laminin are not affected by NG2 expression, the anchoring effect brought about by the expression of NG2 appears specific for type VI collagen. We were further able to show that the surface localization of type VI collagen on NG2-positive cells could be prevented by incubating the cells in the presence of anti-NG2 antibodies which recognize the ectodomain of the NG2 core protein. The finding that only the mixture of five monoclonal anti-NG2 antibodies and not the individual antibodies had the inhibitory effect suggests that none of the mAbs used recognize the site on NG2 directly involved in interacting with type VI collagen, but as a mixture they could physically interfere with the binding of type VI collagen to NG2. This series of results indicates that NG2 may function as a cell surface receptor for type VI collagen.

Several cell surface receptors are known to exist for type VI collagen. Wayner and Carter (1987) have identified four different receptor species for type VI collagen by using mAbs that could block the adhesion of mesenchymal cells to purified type VI collagen. Two of these receptors, ECMRI and II, were shown to be integrins that shared structurally common $\beta 1$ subunits. More recently, Aumailley *et al.* (1989) and Staatz *et al.* (1990) have clearly established the involvement of integrins in the attachment of cells to type VI collagen. Another receptor identified by Wayner and Carter (1987), ECMRIII, is a 90 kD species that is distinct from integrins and is not involved in cell attachment and spreading (Carter and Wayner, 1988). Recently, this receptor has been found to be identical to CD44 (Gallatin *et al.*, 1989).

Figure 6. Effects of anti-NG2 mAbs on the distribution of type VI collagen. B28NG11 cells were trypsinized and plated without antibodies (a–d), with a mixture of five anti-NG2 mAbs (1:100 dilution, e–h), or with D31 monoclonal anti-NG2 antibody (1:100 dilution, i–l). After 24 h of incubation, cells were double-labeled with anti-NG2 mAbs (a, e, and i) and rabbit anti-type VI collagen antibody (b, f, and j) or anti-NG2 mAb mix (c, g, and k) and rabbit anti-fibronectin (d, h, and l). Bar in a represents 100 μ m. The expression of type VI collagen on the surface of NG2-positive cells is abolished by incubation with the monoclonal anti-NG2 antibody mix but not with D31 mAb. The distribution of fibronectin is not altered by incubation with the anti-NG2 antibodies.

CD44 is a chondroitin sulfate membrane-bound receptor that was originally discovered as a lymphocyte homing receptor (Goldstein *et al.*, 1989; Stamenkovic *et al.*, 1989). These three previously described integral membrane receptors for type VI collagen are molecularly distinct from NG2. Thus NG2 appears to be a novel receptor for type VI collagen.

There has been evidence that cell surface proteoglycans and other cell surface receptors such as integrins exert a coordinated effect on matrix functions. An example is provided by Iida *et al.* (1992) who demonstrated that the adhesion of human melanoma cells to a fibronectin fragment requires both cell surface chondroitin sulfate proteoglycan and $\alpha 4\beta 1$ integrin. Minguell *et al.* (1992) have shown that a membrane-bound chondroitin

sulfate proteoglycan expressed by hematopoietic progenitor cells influences their ability to bind to fibronectin in an RGD-dependent manner. Such an interaction in turn stabilizes the expression of the proteoglycan on the cell surface. To determine whether NG2 could act in concert with other receptors for type VI collagen, particularly the integrins, we compared the distribution of integrins on control- and NG2-transfected cells. By using an anti- $\beta 1$ integrin antibody, A1B2, we were unable to detect any difference in the pattern of $\beta 1$ -integrin expression on the surface of NG2/type VI collagen-positive and negative cells. This suggests that NG2-type VI collagen interaction can occur independently of integrins, which implies the existence of a separate collagen anchoring/signaling pathway mediated through

the NG2 receptor. The possibility of a functional interaction between NG2 and the third collagen receptor, ECMRIII or CD44, still needs to be explored.

We have observed that transfectants of the two cell lines showed varying degrees of colocalization of NG2 and type VI collagen. For example, while U251MG cells transiently transfected with NG2 exhibited a high level of surface type VI collagen colocalized with NG2, stable NG2 transfectants of this cell line such as U251NG35 retained much lower levels of the collagen molecule at the cell surface, while U251NG52 cells retained almost no type VI collagen. In the case of B28 transfectants, clone B28NG11 exhibited a higher degree of the punctate co-location of NG2 and type VI collagen than clone B28NG14, on which some fibrillar collagen staining was apparent. These observations suggest that other mechanisms, in addition to the simple expression of NG2, participate in the organization of type VI collagen into the matrix of these cell lines. Molecules other than NG2 that interact directly with type VI collagen, stabilize the NG2-type VI collagen interaction, or affect signal transduction by interacting with or altering the cytoplasmic domain of NG2 may be involved in this process. In this regard it is of interest to note that in two NG2-positive cell lines that fail to anchor type VI collagen to the cell surface (U251NG52 and NIH3T3) we detected a mixture of the 300 kD NG2 core protein and a truncated 275 kD form. Immunoblots of these components using an antibody against the NG2 ectodomain and an antibody against the NG2 cytoplasmic domain (Nishiyama *et al.*, 1991b) indicated that the truncated species is NG2 lacking the cytoplasmic domain. The other NG2-positive cell lines, all of which contained detectable levels of type VI collagen at the cell surface, expressed only the 300 kD form of NG2. Whether the presence of the 275 kD form of NG2 interferes with the anchoring of type VI collagen to the cell surface is not clear at present.

The functional consequences of the binding of type VI collagen to NG2 at the cell surface still remain unclear. The interactions between collagens and ECM proteoglycans have long been of interest in the field of connective tissue research. Classically, proteoglycan-collagen complexes have been thought to be important in maintaining the stability of the ECM, based on the demonstration at the ultrastructural level of proteoglycans decorating collagen fibrils (Scott, 1988). Biochemically some proteoglycans such as decorin and fibromodulin, which belong to the family of leucine-rich molecules, have been shown to interact with collagens and alter the rate of collagen fibrillogenesis *in vitro* (Vogel *et al.*, 1984; Hedbom and Heinegard, 1989). Recently the core protein of decorin was shown to bind specifically to type VI collagen (Bidanset *et al.*, 1992). Type VI collagen also binds to other collagens including types I (Bonaldo *et al.*, 1990) and II (Bidanset *et al.*, 1992).

Thus type VI collagen-NG2 complex may serve as a link between the ECM and the cell.

The amount and composition of ECM that surrounds a cell has important effects on the proliferation and differentiation of the cell (Hamati *et al.*, 1989, for example). Assembly of type VI collagen into pericellular matrix is sensitive to transformation. Carter (1982) discovered that SV40-transformed WI-38 fibroblasts expressed much lower levels of both $\alpha 3$ and $\alpha 1$ and $\alpha 2$ subunits of type VI collagen in the matrix compared with the nontransformed parent cells. While $\alpha 3$ could be detected in the conditioned media of normal and transformed cells, $\alpha 1$ and $\alpha 2$ could be detected only in the conditioned media of nontransformed cells. Transformed cells did not contain any translatable mRNA for $\alpha 1$ and 2 (Trueb *et al.*, 1985). Schreier and others (1988) demonstrated that the inhibition of synthesis of $\alpha 1$ and $\alpha 2$ subunits of type VI collagen was a phenomenon common to transformation achieved by a variety of oncogenes including v-src, v-myc, and SV-40. These observations suggest that unbalanced synthesis of the subunits of type VI collagen associated with transformation may lead to a reduction in the amount that is incorporated into the matrix. Our findings presented here show that the level of NG2, a cell surface proteoglycan receptor for type VI collagen, may be another determinant of the extent of deposition of type VI collagen in the matrix. It will be important to compare the growth properties of NG2/type VI collagen-positive and negative cells.

In vivo, we have shown a colocalization of NG2 and type VI collagen on a small subpopulation of the developing cartilage cells (Stallcup *et al.*, 1990; Nishiyama *et al.*, 1991a). The interaction between the two molecules during development may have interesting consequences on the proliferative and differentiation potential of these cells. In glioma tissue, type VI collagen is increased in the vasculature and stroma (McComb *et al.*, 1987). Taken together with the finding by Schrappe and others (1991) that the expression of NG2 is upregulated in glioma cells and in the vasculature of glioblastoma, these observations lead to speculations about the possible role of the interaction in migration and invasion of neoplastic and developing cells. Further investigations are needed to test this possibility.

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