NG2 Proteoglycan and the Actin-binding Protein Fascin Define Separate Populations of Actin-containing Filopodia and Lamellipodia during Cell Spreading and Migration

Xiao-Hong Lin, Kathryn A. Grako, Michael A. Burg,
and William B. Stallcup*

La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037

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The transmembrane proteoglycan NG2 is able to interact both with components of the extracellular matrix and with the actin cytoskeleton. An examination of the distribution of NG2 during cell spreading suggests that NG2 can associate with two distinct types of actin-containing cytoskeletal structures, depending on the nature of the stimulus derived from the substratum. On fibronectin-coated dishes, cell surface NG2 associates exclusively with stress fibers developing within the cell. On poly- L-lysine-coated dishes, cell surface NG2 is associated with radial processes extending from the cell periphery. Spreading on fibronectin/poly-L-lysine mixtures, as well as on matrix components such as laminin, tenascin, and type VI collagen, produces cells with mosaic characteristics, i.e., NG2 is associated with both types of structures. NG2-positive radial processes are distinct from a second population of radial structures that contain fascin. NG2-positive extensions appear to be individual self-contained units (filopodia), whereas fascin is associated with actin ribs within sheets of membrane (lamellipodia). NG2- and fascin-positive structures are often localized to opposite poles of spreading cells, suggesting a possible role for the two classes of cellular extensions in the establishment of cell polarity during morphogenesis or migration. Time lapse imaging confirms the presence of lamellipodia on the leading edges of migrating cells, while numerous filopodia are present on trailing edges.

INTRODUCTION

NG2 is an integral membrane chondroitin sulfate proteoglycan with structural characteristics that make it unique among members of the proteoglycan family. Although many proteoglycans can be grouped into families based on structural similarities (syndecans, aggrecans, glypicans, etc.), NG2 does not contain structural motifs common to any of these known groupings (Nishiyama *et al.*, 1991). The singularity of its structure suggests that NG2 might also be unique in terms of its ability to participate in intermolecular interactions. Thus, a portion of our research has been focused on identification of ligands for NG2 as well as

identification of the domains of NG2 that mediate these interactions.

As a membrane-spanning molecule, NG2 has the potential to interact with both extracellular and cytoplasmic components and perhaps to participate in signaling between the extracellular and intracellular compartments of the cell. We have described several extracellular matrix components capable of serving as NG2-binding partners. The best characterized of these ligands is type VI collagen (Stallcup *et al.*, 1990; Nishiyama and Stallcup, 1993; Burg *et al.*, 1996), but NG2 can also interact with collagens type II and V and with laminin and tenascin (Burg *et al.*, 1996). We have also shown that NG2 potentiates the activity of the platelet-derived growth factor (PDGF)-AA/PDGF α receptor signaling pathway (Grako and Stallcup, 1995;

* Corresponding author.

Nishiyama *et al.*, 1996a,b). It remains to be shown whether NG2 affects this signaling system through direct interaction with the receptor or by sequestering and/or presentation of the growth factor, but in either case extracellular domains of the large NG2 core polypeptide (300 kDa) are likely to be involved.

Participation in any type of transmembrane signaling would require that NG2 also have intracellular binding partners. In this context, we have demonstrated that the proteoglycan is anchored to the actin cytoskeleton (Lin *et al.*, 1996). On cells with a very flattened morphology, NG2 is distributed on the cell surface in linear arrays which are precisely aligned with actin- and myosin-containing stress fibers in the cytoskeleton. Disruption of these microfilamentous stress fibers with cytochalasin D results in disruption of the orderly distribution of NG2 on the cell surface. In these respects NG2 appears similar to other transmembrane proteins we have examined, such as integrins, CD44, and L1, all of which are thought to be anchored in the actin cytoskeleton (Kalomiris and Bourguignon, 1988; Davis and Bennett, 1994; Clark and Brugge, 1995).

In addition to the ordered arrays of NG2 on the surface of flattened cells, we noted that NG2 was also present on long, delicate tendrils extending from the periphery of cells that had lost their flattened morphology. These cells were rounded, either in preparation for cell division or due to changes in the local adhesive environment (Lin *et al.*, 1996). The NG2-positive tendrils associated with such cells appear analogous to so-called retraction fibers previously observed in association with both dividing and migrating cells (Chen, 1981; Mitchison, 1992). We found that large numbers of rounded-up cells with NG2-positive tendrils or retraction fibers could be created artificially by disruption of microtubules with colchicine. In both the spontaneously occurring and colchicine-treated cases, these tendrils are actin positive and thus have cytoskeletal support. However, the tendrils do not appear to contain myosin, indicating that the composition of their cytoskeleton differs from that of stress fibers, which contain both actin and myosin. The cell surface composition of tendrils is also different from that of the cell surface as a whole. Of four cell surface markers examined, NG2 and $\alpha_5\beta_1$ integrin are associated with tendrils, whereas CD44 and L1 are not. This is indicative of differences in the way that these molecules are targeted and/or anchored to specific microdomains of the actin cytoskeleton. By exchanging cytoplasmic domains between NG2 and L1, we have shown that at least some of the specificity involved in this targeting/anchorage mechanism resides in the cytoplasmic domain of NG2.

In some cases NG2-positive tendrils can be seen not only on rounded cells, but also in association with the lamellae of flattened cells. Although they

are more complex in appearance, these projections from well-spread cells have cytoskeletal and cell surface properties that are identical to those observed for tendrils extending from rounded cells. The presence of NG2-positive projections from cells that appear to be responding to environmental cues by rounding up or spreading has led us to speculate that the association of NG2 with these structures could be important for regulating dynamic cellular processes such as motility and morphological development. A precedent for the involvement of proteoglycans in such phenomena can be seen in the case of the syndecans. Members of the syndecan family of proteoglycans are thought to be important for cotransduction of signals required for cell spreading on fibronectin and other matrix components (Elenius and Jalkanen, 1994; Lebakken and Rapraeger, 1996; Woods and Couchman, 1996). Similarly, evidence for the participation of NG2 in cell spreading has previously been described in the case of human melanoma cells (Harper and Reisfeld, 1983; 1987; Iida *et al.*, 1992; 1995). In our current work, we further examine the role of NG2 in the development of cell morphology by studying the distribution of the proteoglycan on cells that are attaching and spreading on different types of substrata. We show that cells spreading on poly-L-lysine-coated surfaces extend rather dramatic arrays of NG2-positive radial processes which are similar in most respects to the NG2-positive tendrils previously found to be associated with rounding cells. These actin-containing NG2-positive projections are distinct from another population of actin-positive radial structures that contain the actin-binding protein fascin (Yamashiro-Matsumura and Matsumura, 1986; Adams, 1995). Fascin-positive and NG2-positive structures occupy distinct domains of the spreading cell which can be distinguished morphologically as well as by differences in their molecular composition. Fascin-positive actin bundles are contained within sheets of membrane (lamellipodia) that can be recognized by the presence of myosin and focal adhesion plaques. NG2-positive extensions appear to be self-contained entities (filopodia) that lack both myosin and focal adhesion plaques. In extreme cases fascin-positive and NG2-positive extensions define opposite poles of polarized cells, suggesting separate roles for the two sets of radial structures in cell motility or in the development of cell morphology. Analysis of these polarized cells by time lapse differential interference contrast imaging shows that they are, in fact, in the process of migrating on the substratum. The lamellipodia observed in our immunofluorescence studies are strikingly evident on the leading edges of these migrating cells, while numerous filopodia are present on trailing edges.

MATERIALS AND METHODS

Cell Lines

The rat glioma cell lines B28 and B111 (Schubert *et al.*, 1974) were obtained from Dr. David Schubert (Salk Institute, La Jolla, CA). U251MG human astrocytoma cells (Ponten and Westermarck, 1978) were provided by Dr. Eva Engvall (The Burnham Institute, La Jolla, CA). A375M human melanoma cells (Fidler, 1986) were obtained from Dr. Erkki Ruoslahti (The Burnham Institute, La Jolla, CA). MG63 human osteosarcoma cells (Dedhar *et al.*, 1987) were provided by Dr. Michael Pierschbacher (The Burnham Institute). U373 human glioblastoma cells (Schrapp *et al.*, 1991) were obtained from Dr. Ralph Reisfeld (The Scripps Research Institute, La Jolla, CA). Stable transfectants of B28 and U251MG cells have been described previously (Nishiyama and Stallcup, 1993; Lin *et al.*, 1996). Stable transfectants of U373 cells were produced in a similar manner through the use of LipofectAMINE (Life Technologies, Gaithersburg, MD)-mediated transfection. The pcDNA1/amp eukaryotic expression vector (Invitrogen, San Diego, CA) containing full-length NG2 cDNA was used for these transfections (Nishiyama and Stallcup, 1993; Lin *et al.*, 1996). All cell lines were maintained in DMEM supplemented with 10% fetal calf serum (FCS, Tissue Culture Biologicals, Tulare, CA).

Antibodies and Immunofluorescence

The derivation of rabbit and monoclonal antibodies against the NG2 proteoglycan has been described previously (Stallcup *et al.*, 1990; Nishiyama *et al.*, 1991). An antibody against multiple B28 cell surface components was produced by immunizing a rabbit three times with whole B28 cells. The first and second immunizations were given subcutaneously with cells emulsified in complete Freund's adjuvant and incomplete Freund's adjuvant, respectively. The third immunization was done intravenously through the ear using cells resuspended in phosphate-buffered saline (PBS). Monoclonal antibody L1.1 recognizes an epitope in the extracellular domain of the human L1 neuronal cell adhesion molecule (Lin *et al.*, 1996). Monoclonal antibody against rat CD44 was purchased from PharMingen (San Diego, CA) and monoclonal antibodies against β -actin and vinculin were obtained from Sigma (St. Louis, MO). Monoclonal antibodies against human α_3 , α_4 , and α_5 integrin subunits were obtained from Life Technologies. Rabbit antibodies against actin and non-muscle myosin were obtained from Biomedical Technologies (Stoughton, MA). Rabbit antibodies against the $\alpha_5\beta_1$ integrin and phosphotyrosine were generously provided by Dr. Erkki Ruoslahti and Dr. Elena Pasquale, respectively (The Burnham Institute). Monoclonal antibody against fascin was a gift from Dr. F. Matsuura (Rutgers University, Piscataway, NJ). Fluorescein- and rhodamine-conjugated antibodies against rabbit and mouse immunoglobulins were purchased from Biosource International (Camarillo, CA).

Immunofluorescence staining of cell surface molecules was performed using live, unfixed cells. Primary and secondary antibodies were diluted in DMEM containing 2% bovine serum albumin (BSA) (DMEM/BSA). Incubation with primary antibodies was performed for 15 min at room temperature followed by three washes with DMEM/BSA. Incubations with secondary antibodies were also for 15 min at room temperature followed by three washes with DMEM/BSA. Cells were then given a final wash with PBS followed by fixation with 95% ethanol. For immunofluorescence double staining of two cell surface markers, cells were incubated simultaneously with both primary antibodies and then simultaneously with both secondary antibodies.

For immunofluorescence staining of intracellular molecules, cells were fixed either with absolute methanol for 2 min at -20°C (myosin, β -actin, and fascin) or at 4°C for 10 min with 4% paraformaldehyde in 0.1 M PIPES buffer, pH 6.8, containing 5 mM EGTA and 2 mM MgCl_2 (vinculin and phosphotyrosine). Fixed cells were washed three times with PBS, permeabilized for 5 min with

DMEM/FCS containing 0.1% Triton X-100, and blocked for 30 min with DMEM/FCS. Staining with primary and secondary antibodies was then performed as described above for cell surface molecules, except that the incubation times were extended to 30 min. In cases where cell surface staining was compared with intracellular staining, the cell surface staining of living cells was completed first followed by appropriate fixation and subsequent staining of the intracellular component.

Specimens were coverslipped in Immumount (Shandon, Pittsburgh, PA) and examined using a Nikon Optiphot microscope equipped with phase contrast as well as fluorescein and rhodamine optics. Photographs were taken with TMAX 400 black and white film (Eastman Kodak, Rochester, NY).

Cell Spreading

Dishes used for spreading experiments were 60-mm Falcon tissue culture dishes (Becton Dickinson, Oxnard, CA) coated with the desired substrate material. Fibronectin (Calbiochem, La Jolla, CA), laminin (Life Technologies), tenascin (Chemicon International, Temecula, CA), pepsin-extracted type VI collagen (a gift from Dr. R. Timpl, Max-Planck Institute, Martinsreid, Germany; see Odermatt *et al.*, 1983), poly-L-lysine (Sigma), or FCS was diluted at the desired concentrations into PBS and incubated overnight at room temperature in the tissue culture dishes. Before use, the dishes were washed twice with PBS and then blocked for 1 h with a solution of 2% BSA in PBS (PBS/BSA).

Cells used for spreading experiments were washed once with PBS and then treated for 3 min at 37°C with an enzyme-free cell dissociation buffer (Life Technologies) to induce detachment from the monolayer. Immunofluorescence staining confirmed that this procedure did not result in the loss of NG2 from the cell surface. Suspended cells were washed twice with DMEM/BSA and then plated into coated dishes in DMEM/BSA at a density of 200–400 cells/ cm^2 . Cell attachment and spreading were allowed to proceed at 37°C for times ranging from 15 min to 18 h. At the end of the desired time period, cells were analyzed by immunofluorescence for the distribution of various cell surface and cytoskeletal components. Immunofluorescence staining was performed at room temperature for abbreviated time periods to minimize further cell spreading during the staining period.

Time Lapse Imaging

B28NG2.6 cells were removed from monolayer culture using non-enzymatic cell dissociation buffer, washed twice with DMEM/BSA, and plated in DMEM/BSA at 500 cells/ cm^2 into glass bottom wells (MatTek, Ashland, MA) that had been coated overnight with 300 $\mu\text{g}/\text{ml}$ poly-L-lysine. Cells were observed with a Zeiss Axiovert 405M microscope equipped with a Plan-Neofluar 100 $\times$ objective (NA = 1.3) and Nomarski differential interference contrast optics (Carl Zeiss, Thornwood, NY). An enclosed chamber on the microscope stage was used to maintain the cells at 37°C and 5% CO_2 . Images of spreading cells were acquired at 30-s intervals using an Optronics ZVS-47E CCD video camera (Optronics Engineering, Goleta, CA) and a Uniblitz T-132 shutter (Vincent Associates, Rochester, NY). Image acquisition was controlled by Metamorph Image Analysis System software (Universal Imaging, Westchester, PA) run on a pentium PC. Individual frames were saved as .tif images, and the final composite figure was composed using Adobe Photoshop software.

RESULTS

As outlined in the INTRODUCTION, our primary goals in undertaking this research were twofold. First, because of our previous finding that NG2 is associated with two apparently distinct domains of the actin

cytoskeleton, i.e., stress fibers and retraction fibers (Lin *et al.*, 1996), we wanted to explore this phenomenon further by examining the dynamics of NG2 distribution as a function of cytoskeletal development during cell spreading. Second, because NG2 has previously been implicated as a mediator of cell attachment and spreading (Iida *et al.*, 1992, 1995; Harper and Reisfeld, 1983, 1987), we wanted to determine whether NG2 has a functional role in communication between the substratum and the cytoskeleton during the spreading process. To achieve both of these goals, we decided to begin our studies with the B28 rat glioma cell line which was transfected with NG2 cDNA (Nishiyama and Stallcup, 1993; Lin *et al.*, 1996). This would allow us not only to examine the distribution of NG2 during spreading, but also to compare the behavior of NG2-negative B28 parental cells with that of NG2-positive transfectants, designated B28NG2.6.

Cell Spreading on Fibronectin and Poly-L-Lysine

In our initial studies, we compared the spreading of B28NG2.6 cells on dishes coated with fibronectin (20 $\mu\text{g}/\text{ml}$) and poly-L-lysine (30 $\mu\text{g}/\text{ml}$). Assays were conducted in the presence of 2% BSA rather than serum to avoid introduction of additional adhesive proteins into the experiment. To allow visualization of both NG2 and the details of cell morphology, at the end of the experiment cells were double stained with antibodies against NG2 and against β -actin. β -actin staining proved effective not only for visualizing the developing actin cytoskeleton, but also for delineating the outermost boundaries of the spreading cell.

As shown in Figure 1, a and b, B28NG2.6 cells attach and spread rapidly on fibronectin-coated dishes. After 30 min the developing actin cytoskeleton is already apparent. At this point the distribution of NG2 on the cell surface lags somewhat behind the extension of the physical boundaries of the cell. NG2 is not present on

Figure 1. B28NG2.6 cells spreading on fibronectin and poly-L-lysine. As described in MATERIALS AND METHODS, spreading of B28NG2.6 cells on fibronectin-coated dishes (a–d) was compared with spreading on poly-L-lysine-coated dishes (e–h). Cells were double stained for NG2 (a, c, e, and g) and β -actin (b, d, f, and h). Pairs a–b and e–f were stained after 30 min, whereas pairs c–d and g–h were analyzed after 4 h. Bar, 10 μm .

the distal edges of the spreading cell (Figure 1a), even though these edges are clearly delineated by β -actin staining (Figure 1b). After 4 h, however, NG2 is spread over almost the entire cell surface (Figure 1c) and is well colocalized with the actin-containing stress fibers (Figure 1d), as we have reported previously (Lin *et al.*, 1996).

In contrast to this behavior on fibronectin, B28NG2.6 cells exhibit a different pattern of spreading on poly-L-lysine. After 30 min the cells are less flattened than on fibronectin and have a dense array of NG2-positive projections radiating from the central soma (Figure 1e). These structures are supported by a developing actin cytoskeleton, as indicated by their staining for β -actin (Figure 1f). After 4 h this pattern is somewhat changed. Although NG2-positive, actin-positive projections are still apparent (Figure 1, g and h), NG2 is restricted to the distal ends of the processes. An NG2-free zone is present between the NG2-positive soma and the NG2-positive tips of the processes. After an overnight incubation, B28NG2.6 cells on poly-L-lysine are virtually indistinguishable from those on fibronectin. Because B28 cells produce a large quantity of fibronectin (Nishiyama and Stallcup, 1993), we suspected that the time-dependent changes in morphology and NG2 distribution on poly-L-lysine might be due to deposition of fibronectin on the substratum by the B28NG2.6 cells. To test this possibility, we examined cell spreading on dishes coated first with poly-L-lysine and then with fibronectin. When we used a low concentration of fibronectin (1 μ g/ml) for these experiments, spreading of B28NG2.6 cells after 30 min (Figure 2, a and b) resembled the pattern seen after 4 h on poly-L-lysine alone (e.g., compare Figure 2a with Figure 1g). In both cases, NG2-positive radial projections are prominent on distal edges of the cells and an NG2-free intermediate zone is present between these processes and the central soma of the cell. When the coating mixture contained a high concentration of fibronectin (20 μ g/ml), NG2-positive projections were less prominent (Figure 2, c and d), although they could still be seen on cell edges. Because Figure 2d is overexposed to emphasize the presence of actin in the radial processes, it does not effectively illustrate the presence of stress fibers in the intermediate regions of these cells. However, indications of stress fiber formation can be seen in Figure 2c, where NG2 is beginning to appear in organized arrays external to the central mass of the cell (contrast c with a in this respect). Thus, plating B28NG2.6 cells on dishes coated with both fibronectin and poly-L-lysine produces a mosaic effect in which some NG2 is colocalized with the developing actin-positive stress fibers and some NG2 is associated with radial, actin-positive projections. These two types of structures have the appearance of being fundamentally different entities.

Figure 2. B28NG2.6 cells spreading on mixtures of poly-L-lysine and fibronectin. B28NG2.6 cells were double stained for NG2 (a and c) and β -actin (b and d) after spreading for 30 min on plates coated with 30 μ g/ml poly-L-lysine plus 1 μ g/ml fibronectin (a and b) or 30 μ g/ml poly-L-lysine plus 20 μ g/ml fibronectin (c and d). Bar, 10 μ m.

Cytoskeletal Characterization of Radial Projections

To investigate the nature of the NG2-positive, actin-positive projections, we tried to characterize them further with regard to the presence of other cytoskeletal components. Since the actin-binding protein fascin has been reported to be associated with radial actin bundles found in spreading muscle cells (Yamashiro-Matsumura and Matsumura, 1986; Adams, 1995), we compared the distribution of fascin and NG2 in spreading B28NG2.6 cells. Superficially, the appearance of fascin-positive and NG2-positive structures is similar in these cells. As with NG2, fascin-containing radial structures are not observed in cells spreading on fibronectin but are frequently seen in cells spreading on poly-L-lysine (Figure 3b). The colocalization of fascin and actin in these structures is readily apparent (Figure 3, a and b). However, when cells are double stained for NG2 and fascin, little if any overlap between the two markers can be seen. On cells in which NG2-positive and fascin-positive structures are arranged symmetrically around the cell, NG2-positive projections usually extend beyond the region occupied by the fascin-positive actin bundles (Figure 3, c and d), so that NG2- and fascin-stained elements occupy different regions of the spreading cell. In cases where the distribution of NG2 and fascin is asymmetrical, their patterns of localization once again appear to be almost mutually exclusive (Figure 3, e and f). In extreme cases the cells are highly polarized, with NG2-positive projections at one pole and fascin-positive structures at the other (Figure 3, g and h). These

findings suggest that NG2- and fascin-containing structures are distinct entities. This impression is reinforced by studies with stably transfected NG2-positive clones of two other cell lines, the human glioblastoma U373 and the human astrocytoma U251MG. On poly-L-lysine the NG2-positive clone U373NG2A extends NG2-positive radial processes but fails to exhibit fascin-positive actin bundles (Figure 4, a and b). In contrast, U251NG35 cells (Nishiyama and Stallcup, 1993) clearly exhibit fascin-positive structures when plated on poly-L-lysine, yet no NG2-positive projections are seen (Figure 4, c and d). Under these conditions, NG2 expression is restricted to the central mass of U251NG35 cells. These observations further establish the independent existence of the two populations of actin-containing radial structures.

Another difference between NG2- and fascin-positive elements can be seen in a comparison of their localization with the actin-binding protein myosin.

Whereas we have previously shown that NG2 is colocalized with myosin-containing stress fibers in static cells (Lin *et al.*, 1996), myosin is not present in areas of spreading B28NG2.6 cells that exhibit NG2-positive radial processes (Figure 5, a and b). In these cases myosin is restricted to a more central portion of the spreading cell occupied by the developing stress fibers. In contrast, myosin is present in areas of the cell which contain fascin-positive actin bundles (Figure 5, c and d), once again demonstrating a distinction between the two types of radial structures.

Finally, we have not been able to show that NG2-positive projections contain detectable levels of either vinculin or phosphotyrosine, two markers characteristic of focal adhesion plaques. Focal adhesion plaques are not found in B28NG2.6 cells spreading on poly-L-lysine alone, but on fibronectin-poly-L-lysine mixtures these plaques can be observed just internal to the NG2-positive radial processes (Figure 5, e-h). In

Figure 3. Comparison of NG2 and fascin in spreading B28NG2.6 cells. B28NG2.6 cells were analyzed for NG2 (c, e, and g) and fascin (d, f, and h) localization after 60 min of spreading on poly-L-lysine-coated dishes. a and b compare staining for actin and fascin, respectively. Bars, 10 μ m

highly asymmetric cells such as the example shown in Figure 3, g and h, focal adhesion plaques are concentrated in the pole of the cell occupied by the fascin-positive elements. Taken together these observations support the idea that NG2-positive and fascin-positive radial structures define separate microdomains of the architecture of spreading cells.

Compartmentalization of NG2 and Fascin-positive Elements

The foregoing observations suggest that NG2-positive and fascin-positive structures are associated with distinct and separate compartments of the cytoskeleton. In an attempt to make additional physical or structural distinctions between the cellular compartments occupied by these two types of components, we stained spreading B28NG2.6 cells with a rabbit antiserum raised against whole B28 cells. We hoped that this antiserum would recognize many different cell surface components and thus might allow visualization of all portions of the exposed cell membrane. Figure 6, a and b, shows that this antiserum, like anti-NG2, stains radial NG2-positive projections as individual structures without showing evidence of intervening membrane. In contrast, the NG2-negative intermediate region between the central soma and the NG2-positive processes is stained in a more homogeneous manner by the anti-B28 antibody (arrowheads, Figure 6b). This phenomenon is seen more clearly in polarized B28NG2.6 cells. The NG2-positive extensions at one pole are labeled as individual structures by the anti-B28 antibody, while there is an area of more homogeneous membrane staining at the opposite pole (Figure 6, c and d). This area of more homogeneous staining contains the fascin-positive actin bundles (Figure 6, e and f). Based on this information, we currently view the NG2-positive projections as individual self-contained units of membrane and cytoplasm (filopodia), whereas fascin is associated with actin ribs contained within sheets of membrane and cytoplasm (lamellipodia). In some portions of the following discussion, we will refer to NG2-positive projections as filopodia.

Time Lapse Video Analysis of Spreading Cells

In several of the foregoing examples, B28NG2.6 cells spreading on poly-L-lysine are clearly polarized, with NG2-positive projections at one pole and fascin-positive actin bundles at the other. Studies with the anti-B28 antibody suggest that this polarization is due to the presence of lamellipodia at one pole and filopodia at the other. This morphology is highly characteristic of cells in the process of migration on the substratum (Heath and Holifield, 1991). To verify this, we observed B28NG2.6 cells with differential interference contrast optics as they attached and spread on poly-L-lysine. Figure 7 shows such a cell in the process of

Figure 4. NG2 and fascin localization in spreading U373NG2A and U251NG35 cells. U373NG2A cells (a and b) and U251NG35 cells (c and d) were analyzed for NG2 (a and c) and fascin (b and d) localization after spreading for 30 min on poly-L-lysine-coated dishes. Bar, 10 μ m.

initially extending a fairly symmetrical array of filopodia (a) followed by extension of a lamella and associated lamellipodia at one pole (b–f). The migration of these polarized cells occurs in the direction of the developing lamellipodia, in this particular example toward the bottom of the photograph (small bits of debris at the top of a and b are left behind as the cell migrates). In Figure 7c black arrows mark the trailing edge of the cell, which continues to be characterized by large numbers of individual processes throughout the duration of the sequence. White arrows mark the leading edge of the cell. The portion of the leading edge containing the lamellipodia is clearly defined by numerous ribbed structures, corresponding to actin bundles. The smooth area behind the lamellipodia is the lamella. The resemblance between these differential interference contrast images and the fluorescence images of polarized B28NG2.6 cells (Figure 3, g and h, and Figure 6, c–f) is sufficiently striking as to leave

Figure 5. Cytoskeletal markers in spreading B28NG2.6 cells. B28NG2.6 cells spreading on poly-L-lysine-coated dishes (a–d) or dishes coated with poly-L-lysine plus 1 μ g/ml fibronectin (e–h) were stained after 1 h for the following markers: a and b, NG2 and myosin; c and d, fascin and myosin; e and f, NG2 and vinculin; and g and h, NG2 and phosphotyrosine. Bar, 10 μ m.

little doubt that fascin is associated with actin ribs in the leading edge of the cell, whereas NG2 is associated with projections from the trailing edge.

Cell Surface Characterization of NG2-positive Filopodia

To investigate membrane specialization in the NG2-positive filopodia on B28 cells, we have tested them for the presence of a few other well-defined cell surface proteins. Two of the three endogenously expressed proteins that we examined were not present on these filopodia. Both the neural cell adhesion molecule NCAM (Figure 8, a and b) and the integral membrane proteoglycan CD44 (Figure 8, c and d) are restricted to interior regions of the spreading cells. Only an antiserum against the $\alpha_5\beta_1$ integrin was found to stain NG2-positive filopodia (Figure 8, e and f). Since this antiserum recognizes both the α_5 and β_1 integrin subunits, it is likely that the staining seen here represents multiple integrin heterodimers containing the α_5 or β_1 subunits. In the case of the glioblastoma cell line U373NG2A, we were able to examine integrin

expression in more detail, since a better collection of subunit-specific monoclonal antibodies is available for use with human cells. We found that α_3 and α_5 integrins were expressed on NG2-positive radial processes, whereas α_4 integrins were restricted to the central cell soma (our unpublished observations). Thus, even among closely related molecules there is differential expression between filopodia and more interior regions of spreading cells.

To study the behavior of a protein that is not endogenously expressed by B28 cells but, like NG2, is expressed by virtue of cDNA transfection, we examined the spreading of B28 cells transfected with the neuronal cell adhesion molecule L1 (Moos *et al.*, 1988; Hlavin and Lemmon 1991; Lin *et al.*, 1996). We have previously shown that NG2 and L1 appear to have distinct mechanisms for interaction with the actin cytoskeleton (Lin *et al.*, 1996), and we can also distinguish the behavior of these molecules with regard to expression on filopodia. Unlike NG2, L1 is not present on actin-positive radial projections (Figure 9, a and b). Some insight into the basis for the difference in behav-

Figure 6. Compartmentalization of NG2-positive and fascin-positive structures in spreading B28NG2.6 cells. B28NG2.6 cells were analyzed after spreading for 30 min on poly-L-lysine-coated dishes. NG2 distribution (a and c) was compared with the staining obtained with a rabbit antibody against whole B28 cells (b and d). Fascin localization (e) was also compared with rabbit anti-B28 staining (f). Arrowheads in b illustrate areas stained by anti-B28 but not by anti-NG2. Bar, 10 μ m.

ior between NG2 and L1 can be gained from an examination of chimeric molecules containing portions of both of these molecules. NG2_E/L1_C contains the extracellular domain of NG2 attached to the transmembrane and cytoplasmic domains of L1. Conversely, L1_E/NG2_C contains the extracellular domain of L1 attached to the transmembrane and cytoplasmic domains of NG2 (Lin *et al.*, 1996). Whereas, NG2_E/L1_C is not expressed on actin-positive filopodia (Figure 9, c and d), the L1_E/NG2_C chimera is found on these projections (Figure 9, e and f). This indicates the specific involvement of the NG2 cytoplasmic domain in targeting NG2 to the filopodia.

Cell Spreading on Other Substrata

The ability of poly-L-lysine-coated surfaces to support a different type of cell spreading from that observed on fibronectin-coated surfaces led us to examine whether other more physiological substrates might support the extension of NG2-positive processes. Laminin, tenascin, and type VI collagen were all of interest to us, since NG2 has been shown to bind to

each of these extracellular matrix components (Burg *et al.*, 1996). Figure 10 illustrates the different patterns of NG2 distribution on B28NG2.6 cells spreading on dishes coated with these matrix molecules (20 μ g/ml overnight). Whereas NG2 distribution does not extend to cell edges in cells spreading on fibronectin (Figure 10, a and b; see also Figure 1), on laminin NG2 expression clearly outlines the borders of the spreading cell and defines a few processes extending from the cell periphery (c and d). On tenascin- and type VI collagen-coated surfaces (e-h), NG2 is again expressed on cell edges and on even more numerous processes extending from these edges. These NG2-positive filopodia are especially evident in association with the complex lamellae developed by B28NG2.6 cells on type VI collagen. Thus, cells spreading on laminin, tenascin, and type VI collagen exhibit some clear differences from cells spreading on fibronectin, but they also exhibit differences from cells spreading on poly-L-lysine. First, fascin staining of actin bundles is rarely seen in cells plated on any of the four extracellular matrix components that we examined (Figure 10c). Second,

Figure 7. Time lapse imaging of a spreading B28NG2.6 cell. The time course of spreading on poly-L-lysine was recorded for a single B28NG2.6 cell. Numbers in lower right corner of a–f indicate time in minutes after plating. Dark arrows in c indicate filopodia on trailing edge of the cell; white arrows indicate lamellipodia on leading edge. Direction of migration is downward (note that small bits of debris at top in a and b are left behind as the cell migrates). Bar, 5 μ m.

cells are much more flattened on the extracellular matrix components than on poly-L-lysine. In addition to the peripheral NG2-positive projections seen on laminin, tenascin, and type VI collagen, there are extensive areas in the more central regions of the cell where NG2 is colocalized with the developing stress fibers. In these respects cells spreading on laminin, tenascin, or type VI collagen resemble cells spreading on a mixture of poly-L-lysine and fibronectin in that a mosaic effect is created: some NG2 associates with stress fibers and some NG2 associates with filopodia.

Involvement of NG2 in Cell Spreading

Two types of information were evaluated in an attempt to determine whether NG2 plays a functional role in the extension of filopodia during cell spreading on poly-L-lysine. First, we asked whether NG2-positive projections invariably occur in cells spreading on poly-L-lysine. Although cell lines other than B28 were identified as being able to extend NG2-positive radial processes on poly-L-lysine, e.g., NG2-transfected U373 glioma cells (Figure 4, a and b), we already know from

the data shown in Figure 4, c and d, that NG2 is not found on radial processes in NG2-transfected U251 MG astrocytoma cells spreading on poly-L-lysine. Projections in these cells are of the fascin-containing variety. Enough additional examples of this behavior were seen during our survey of cell lines (e.g., B111, A375M, and MG63 cells) to convince us that NG2 expression alone does not ensure the presence of filopodia on poly-L-lysine.

The second type of functional analysis involved comparison of the behavior of NG2-positive and NG2-negative B28 cells on poly-L-lysine. Since NG2 cannot be used as a marker for filopodia in the NG2-negative B28 cells, we instead used the anti-B28, anti- β -actin, and anti-fascin antibodies to identify radial processes with appropriate characteristics. Figure 11 shows that the parental B28 cells are able to extend anti-B28-positive radial processes that are positive for β -actin (a and b) but negative for fascin (c and d). Thus, the presence of NG2 is not required for the formation of filopodia. Quantitative comparisons between NG2-positive and negative B28 cells on poly-L-lysine reveal

Figure 8. Localization of cell surface markers on spreading B28NG2.6 cells. B28NG2.6 cells spreading on poly-L-lysine were analyzed after 30 min for localization of NG2 (a, c, and e) and NCAM (b), CD44 (d), and $\alpha_5\beta_1$ integrin (f). Bar, 10 μ m.

no significant difference in their abilities to extend fascin-negative filopodia that can be stained with the rabbit anti-B28 antibody (Table 1). Roughly 60% of the cells in any given experiment are observed to extend filopodia, independent of the expression of NG2. A parallel study using NG2-positive and negative U373 cells gave the same results. In this case, filopodia were identified with anti-integrin antibodies in one set of experiments and with anti-B28 antibodies in a separate set of experiments. Similar results were obtained in both cases, suggesting that the results were not biased by the choice of marker. Thus, expression of NG2 does not appear to influence the ability of cells to extend filopodial processes on poly-L-lysine.

Although we do not yet understand the mechanisms responsible for formation of filopodial processes, we nevertheless have the impression that whenever an NG2-positive cell possesses the machinery necessary to extend filopodia, NG2 is preferentially targeted to these projections. Projections that are positive for the anti-B28 antibody, positive for integrins, and negative for fascin are almost invariably NG2 positive. In sup-

port of this idea, we have found that although not all NG2-positive cell lines extend filopodial processes on poly-L-lysine-coated surfaces (e.g., the U251NG35 cells shown in Figure 4, c and d), for almost all of these cell lines an appropriate substratum can be found which results in the appearance of NG2-positive radial projections. For a number of cell lines such a substratum can be created by coating dishes first with poly-L-lysine and then with FCS. On this surface, many cell types which do not extend NG2-positive filopodia on poly-L-lysine alone now exhibit NG2-positive projections on their distal edges (Figure 12). This is true not only for NG2-transfected cells, such as U251NG35 (a and b), but also for cell lines that express NG2 endogenously, such as B111 (c and d), A375M (e and f), and MG63 (g and h). The presence of α_5 integrin in the radial processes extended by MG63 cells (h) is consistent with our observation of integrins in NG2-positive filopodia seen under other plating conditions. Thus, although NG2 expression alone is apparently insufficient to drive the extension of filopodia on poly-L-lysine, when appropriate conditions are found which

Figure 9. Localization of L1 and L1/NG2 chimeras in spreading B28 cells. B28 cells transiently transfected with L1 (a and b), NG2_E/L1_C chimera (c and d), and L1_E/NG2_C chimera (e and f) were analyzed after spreading for 30 min on poly-L-lysine. Cells were stained for β -actin (a, c, and e) and either L1 (b and f) or NG2 (d). Bar, 10 μ m.

allow formation of these projections, NG2 preferentially associates with them.

DISCUSSION

Work in our laboratory has shown that the transmembrane proteoglycan NG2 is capable of interacting with both extracellular matrix components (Stallcup *et al.*, 1990; Nishiyama and Stallcup, 1993; Burg *et al.*, 1996) and the actin cytoskeleton (Lin *et al.*, 1996). This suggests the possibility that NG2 might mediate signaling between the extracellular matrix and the cytoskeleton that would influence cellular processes such as proliferation, morphogenesis, differentiation, and motility. We felt that examination of the behavior of NG2 during cell spreading on different substrata might provide additional clues about the participation of NG2 in some of these phenomena. The use of the NG2-negative cell line B28 and its NG2-transfected counterpart, B28NG2.6, has allowed us both to examine the localization of NG2 during cell spreading and to evaluate the functional importance of NG2 in this process.

Our results suggest that NG2 is associated with two distinct types of structures on spreading B28NG2.6 cells. The first of these are actin- and myosin-containing stress fibers which form increasingly ordered arrays within cells as they spread on many types of

extracellular matrix components. We have used fibronectin, which is very effective in promoting integrin-mediated attachment and spreading, as the prototype extracellular matrix molecule for demonstrating the development of cell surface NG2 arrays anchored to these stress fibers. In B28NG2.6 cells spreading on fibronectin, stress fibers can be seen within 30 min of the initial plating, with NG2 organization on the cell surface following closely behind. After several hours on fibronectin, the pattern of cytoskeletal stress fibers and their associated cell surface NG2 are indistinguishable from those seen in cells maintained in monolayer culture for extended periods of time (Lin *et al.*, 1996). In contrast to fibronectin, poly-L-lysine mediates cell attachment and spreading primarily on the basis of electrostatic mechanisms. In cells spreading on poly-L-lysine, NG2 is found on radial processes extending from the cell periphery. These projections contain actin, but unlike the cytoskeletal stress fibers, they do not contain myosin, and thus appear to represent a distinct domain of the actin cytoskeleton. The membrane composition of the radial projections also appears to be different from that of the cell membrane as a whole. Whereas NG2, integrins, CD44, NCAM, and L1 are all seen to be associated with stress fibers (Lin *et al.*, 1996), only NG2 and some integrin species are found on radial processes.

Figure 10. Spreading of B28NG2.6 cells on extracellular matrix components. Spreading of B28NG2.6 cells was analyzed after 45 min on fibronectin (a and b), laminin (c and d), tenascin (e and f), and type VI collagen (g and h). NG2 localization (b, d, f, and h) was compared with β -actin (a, e, and g) or fascin (c). Bar, 10 μ m.

Whereas spreading on fibronectin produces almost exclusively stress fiber-associated NG2 and spreading on poly-L-lysine produces mostly radial process-associated NG2, spreading of B28NG2.6 cells on some other types of extracellular matrix-derived substrates produces a mosaic of these two effects. Plating on tenascin, laminin, and type VI collagen results in the extension of numerous NG2-positive processes and in the formation of extensive areas occupied by stress fiber-associated NG2. This mosaic pattern resembles what we have observed with B28NG2.6 cells spreading on a mixed substrate containing both fibronectin and poly-L-lysine. We conclude that integrin-mediated adhesion of B28NG2.6 cells to fibronectin results in a situation in which radial projections do not form, and NG2 localization is restricted to stress fibers. In contrast, electrostatic adhesion to poly-L-lysine produces few cytoskeletal stress fibers in these cells, but instead leads to the formation of radial projections that are positive for NG2. Matrix components such as laminin, tenascin, and type VI collagen apparently promote enough integrin-mediated adhesion to generate

abundant stress fibers. In addition, however, they can promote nonintegrin-mediated adhesion which leads to formation of NG2-positive radial processes. In this respect, the mixed fibronectin/poly-L-lysine substratum mimics the behavior of these latter matrix components.

The NG2-positive projections extended by B28NG2.6 cells on poly-L-lysine appear to be a rather unique feature of the cellular architecture. Actin-positive radial structures containing the actin-binding protein fascin have been described previously in muscle cells spreading on thrombospondin-coated surfaces (Adams, 1995), but NG2-positive processes differ in several respects from fascin-positive elements. Fascin-positive structures are present in areas of the actin cytoskeleton that contain myosin, whereas NG2-positive projections are in myosin-deficient areas. Fascin-positive elements are present in areas of the cell that also contain focal adhesion plaques, whereas NG2-positive projections usually extend beyond this region. Fascin-positive actin bundles are contained within sheets of membrane resembling lamellipodia,

Figure 11. Extension of filopodia by B28 cells. Parental B28 cells were analyzed for extension of filopodia after spreading for 30 min on poly-L-lysine. Staining with rabbit anti-B28 (a and c) was compared with localization of β -actin (b) or fascin (d). Bar, 10 μ m.

whereas NG2-positive processes appear to be individual self-contained units, more like filopodia. Finally, in our system, fascin-positive structures have only been observed in cells spreading on poly-L-lysine, whereas NG2-positive processes have been observed on poly-L-lysine as well as on extracellular matrix components such as laminin, tenascin, and type VI collagen. Although we do not yet have a good understanding of how NG2-positive filopodia and fascin-positive actin ribs in lamellipodia differ in terms of their functional roles, the two types of radial structures nevertheless define two distinct regions within spreading cells. The fact that NG2-positive and fascin-positive elements are often found at opposite poles of spreading cells suggests that the two components could be involved in establishing polarity in differentiating or migrating cells. Time lapse video imaging confirms that B28NG2.6 cells undergo transient episodes of migration on poly-L-lysine-coated surfaces, probably in response to local discontinuities in the substratum (Lauffenburger and Horwitz, 1996). The migratory cells in these time lapse studies are morphologically polarized in the same manner observed in our immunofluorescence images. Structures identical to fascin-positive lamellipodia and NG2-positive filopodia are present on the leading and trailing edges, respectively, of the migrating cells.

There are numerous similarities between the NG2-positive filopodia extended by B28NG2.6 cells spreading on poly-L-lysine and the NG2-positive retraction fibers observed in these same cells when they round up in preparation for mitosis or in response to colchicine treatment (Lin *et al.*, 1996). In addition to being

morphologically very similar, both types of processes contain actin but lack myosin, indicating a similarity in cytoskeletal structure. Moreover, the spectrum of membrane proteins present in the two types of processes is similar. Both contain NG2 and some species of integrins, and both are deficient in L1, CD44, and N-CAM, which are restricted to the central cell mass. These observations have led to some uncertainty on our part as to whether NG2-positive processes are more appropriately designated as filopodia or as retraction fibers. By definition, filopodia are formed by extension of membrane and cytoskeleton beyond the edge of the cell into areas previously unoccupied by the cell. These projections are most often thought of in association with the exploratory behavior of the leading edge of a motile cell or cellular process (Lauffenburger and Horwitz, 1996). In contrast, retraction fibers are formed by retraction and condensation of membrane and cytoplasm around a residual cytoskeletal nucleus. Retraction fibers have been described in association with cells undergoing mitosis (Mitchison, 1992; Cramer and Mitchison, 1993) and with migrating cells (Chen, 1981; Sheetz, 1994). In the former case, retraction fibers are remnants of the previously flattened cell which remain attached to the substratum at points which represent the former outer boundaries of the extended cell. These remnants are thought to provide a scaffolding over which postmitotic daughter cells can rapidly reestablish a differentiated morphology. In the latter case, retraction fibers represent the trailing edge of the migrating cell and are thought to provide a specialized mechanism by which the trailing edge is released from the substratum to allow migra-

tion in the opposite direction. These trailing structures are thought to be less structurally rigid and more deformable than lamellipodial and filopodial structures at the leading edge of the cell (Schmidt *et al.*, 1993). Our findings that NG2-positive processes lack the actin-binding and cross-linking proteins myosin and fascin might be consistent with the idea of reduced rigidity of these structures.

By these criteria, the NG2-positive processes associated with B28NG2.6 cells in the process of rounding up would clearly appear to fall into the category of retraction fibers (Lin *et al.*, 1996). However, processes associated with cells freshly plated onto poly-L-lysine present a slightly more complex situation. Both our time lapse and immunofluorescence studies show that when round B28NG2.6 cells are plated onto poly-L-lysine, they respond almost immediately (within 30 s; see Figure 7) by extension of an array of NG2-positive processes. There is no indication that these processes are formed by retraction of material from previously existing cell edges or lamellae; indeed, lamellae develop secondarily to the array of processes. These initial projections would thus appear to fit the operational definition of filopodia. However, once cells begin to migrate on the substratum, it is apparent that the NG2-positive processes are not associated with the leading edge of the cell. In this sense they appear more similar to retraction fibers on the trailing edge of the cell. Additional work on the functional properties of these processes will be required before we can discuss these phenomena in terms of cellular mechanisms rather than semantics. For now, however, it appears that the distinction between these two type of cellular extensions may not be as sharp as previously supposed.

Our experiments indicate that, at least on poly-L-lysine-coated surfaces, NG2 does not play a direct role in the establishment of filopodial extensions. NG2-positive and negative B28 cells appear to have an equal capacity for extending this type of radial process. Moreover, some NG2-positive cell lines fail to extend filopodia on poly-L-lysine, showing that NG2 expression alone is not sufficient to drive this process. At present we have not performed experiments designed to examine B28NG2.6 cell morphology under conditions in which migration is directed or influenced by specific stimuli. Thus, it remains to be determined whether NG2 plays a more active role in the behavior of filopodia/retraction fibers (e.g., altered attachment to or release from the substratum) during migration in response to specific chemoattractants or substrata. The findings that NG2 can potentiate cell migration in response to PDGF (Grako and Stallcup, 1995) and is able to bind to extracellular matrix-derived substrates such as laminin, tenascin, and type VI collagen (Burg *et al.*, 1996) suggest that these will be important areas to explore. There does appear to be a

Table 1. Formation of filopodia by NG2-positive and negative cells

Cell line	% of Cells with RaB28 ⁺ fascin ⁻ processes		% of Cells with RaB28 ⁺ integrin ⁺ processes	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
B28 (NG2 ⁻)	56 ± 4	59 ± 8	—	—
B28NG2.6	55 ± 6	64 ± 4	—	—
U373 (NG2 ⁻)	66 ± 4	53 ± 5	57 ± 3	53 ± 7
U373NG2A	61 ± 7	55 ± 4	60 ± 5	47 ± 5

In each experiment, cells were allowed to attach and spread for 30 min on poly-L-lysine-coated dishes as described in MATERIALS AND METHODS. Cells were then double labeled with rabbit antibody against B28 cells (RaB28) and with monoclonal antibody against fascin. Three fields of at least 100 RaB28⁺ cells were examined and scored for the presence of filopodia that were positive for RaB28 staining and negative for fascin staining. The percentage of RaB28⁺ cells that extended RaB28⁺ fascin⁻ processes was calculated for each of the three fields, and an average percentage value ± SE was derived. The results of two independent experiments are shown. In the case of U373 cells, which never exhibit fascin⁺ elements under these conditions, we also tested an additional combination of antibodies. Cells were double labeled with the RaB28 antibody and a mixture of monoclonal antibodies against α_3 and α_5 integrin subunits, both of which are localized to filopodia (see text). The percentage of RaB28⁺ cells that extended processes labeled with both the RaB28 and integrin antibodies was calculated as described above. In no case did the NG2-positive cell line differ significantly from its NG2-negative partner in its ability to extend filopodia on poly-L-lysine.

rather unique relationship between filopodia and NG2. Although our understanding of the mechanisms involved in filopodial process formation is incomplete, our evidence suggests that NG2 preferentially associates with these specialized structures. This is seen with B28NG2.6 cells on a variety of substrates. Even more interestingly, it is seen with cell lines such as U251NG35, B111, A375M, and MG63 which fail to extend NG2-positive filopodia under conditions (plating on poly-L-lysine) which reproducibly elicit filopodial extension in B28NG2.6 cells. These other cell lines require a different substratum (poly-L-lysine and FCS) to extend filopodia, suggesting that the mechanistic requirements for process extension in these cells are somewhat different from those in B28NG2.6 cells. Two conclusions can be drawn from these observations. First, NG2 localization in filopodia is independent of whether the cells express NG2 endogenously (B111, A375M, MG63) or by virtue of cDNA transfection (U251NG35, U373NG2A, B28NG2.6). Second, NG2 localization in these projections is independent of the substratum required to elicit their extension (poly-L-lysine, poly-L-lysine + serum, extracellular matrix molecules). The preferential localization of NG2 in filopodia whenever these elements are able to form and whenever NG2 is present indicates the existence

Figure 12. Cell spreading on mixtures of poly-L-lysine and FCS. Dishes were coated overnight with 30 $\mu\text{g}/\text{ml}$ poly-L-lysine followed by a 2-h incubation with 2% FCS. Cells were allowed to spread for 45 min in medium containing 2% FCS. U251NG35 cells: a, NG2; b, β -actin; B111 cells: c, NG2; d, β -actin; A375M cells: e, NG2; f, β -actin; MG63 cells: g, NG2; h, $\alpha 5$ integrin. Bar, 10 μm .

of a specific mechanism for targeting and/or anchoring the proteoglycan in these structures. Our experiments with other cell surface molecules and with chimeric derivatives of NG2 and L1 show that at least a portion of the specificity for this localization resides in the NG2 cytoplasmic domain. Future research will identify key motifs in the NG2 cytoplasmic domain which control this process. Additional studies will be required to identify the cytoplasmic ligand(s) responsible for targeting and anchorage of NG2 in filopodia.

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