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Cytoskeletal Reorganization Induced by Engagement of the NG2 Proteoglycan Leads to Cell Spreading and Migration

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Cells expressing the NG2 proteoglycan can attach, spread, and migrate on surfaces coated with NG2 mAbs, demonstrating that engagement of NG2 can trigger the cytoskeletal rearrangements necessary for changes in cell morphology and motility. Engagement of different epitopes of the proteoglycan results in distinct forms of actin reorganization. On mAb D120, the cells contain radial actin spikes characteristic of filopodial extension, whereas on mAb N143, the cells contain cortical actin bundles characteristic of lamellipodia. Cells that express NG2 variants lacking the transmembrane and cytoplasmic domains are unable to spread or migrate on NG2 mAb-coated surfaces, indicating that these portions of the molecule are essential for NG2-mediated signal transduction. Cells expressing an NG2 variant lacking the C-terminal half of the cytoplasmic domain can still spread normally on mAbs D120 and N143, suggesting that the membrane-proximal cytoplasmic segment is responsible for this process. In contrast, this variant migrates poorly on mAb D120 and exhibits abnormal arrays of radial actin filaments decorated with fascin during spreading on this mAb. The C-terminal portion of the NG2 cytoplasmic domain, therefore, may be involved in regulating molecular events that are crucial for cell motility.

INTRODUCTION

The NG2 chondroitin sulfate proteoglycan is an integral membrane protein with an extensive extracellular domain of 2195 amino acids and a much smaller cytoplasmic domain of 76 amino acids. As a membrane-spanning molecule, NG2 is in a position to mediate communication between the extracellular and intracellular compartments of the cell. We have presented evidence that the ectodomain of NG2 may interact with several types of extracellular matrix (ECM) ligands (Burg *et al.*, 1996), the best studied of which is type VI collagen (Stallcup *et al.*, 1990; Nishiyama and Stallcup, 1993; Burg *et al.*, 1996, 1997; Tillet *et al.*, 1997). These studies show that the central nonglobular segment of the NG2 ectodomain contains a binding site for type VI collagen, which allows the proteoglycan to serve as an efficient cell surface receptor for this ECM component. NG2-positive cells, therefore, may be able to interact with the matrix via the NG2/type VI collagen complex.

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Our previous results also suggest that NG2 can interact with the actin cytoskeleton, because NG2 is codistributed with actin in two types of situations. In well-spread cells, NG2 is organized on the cell surface into linear arrays that colocalize with cytoskeletal stress fibers, suggesting that NG2 might use stress fibers as a means of anchorage (Lin *et al.*, 1996a). In cells that are rounding up or migrating, NG2 is present in actin-positive retraction fibers, suggesting a possible role for the proteoglycan in release of specific microdomains of the cell from the substratum (Lin *et al.*, 1996b).

If NG2 mediates interactions with both the ECM and the actin cytoskeleton, the possibility exists that engagement of the NG2 ectodomain by extracellular ligands may initiate signaling events that lead to reorganization of the cytoskeleton. These changes in cytoskeletal architecture might then influence cell shape and motility. For example, we have shown that expression of NG2 on the cell surface leads to enhanced cell motility in response to type VI collagen (Burg *et al.*, 1997). Although other cell surface receptors also appear to influence this response, an NG2-specific component of migration can still be recognized. Anti-NG2 antibodies are able to inhibit the NG2-specific portion of the migratory response, and a similar portion of the response is lost in cells carrying NG2 deletion mutants missing the type VI collagen–binding site. These results suggest that the NG2-type VI

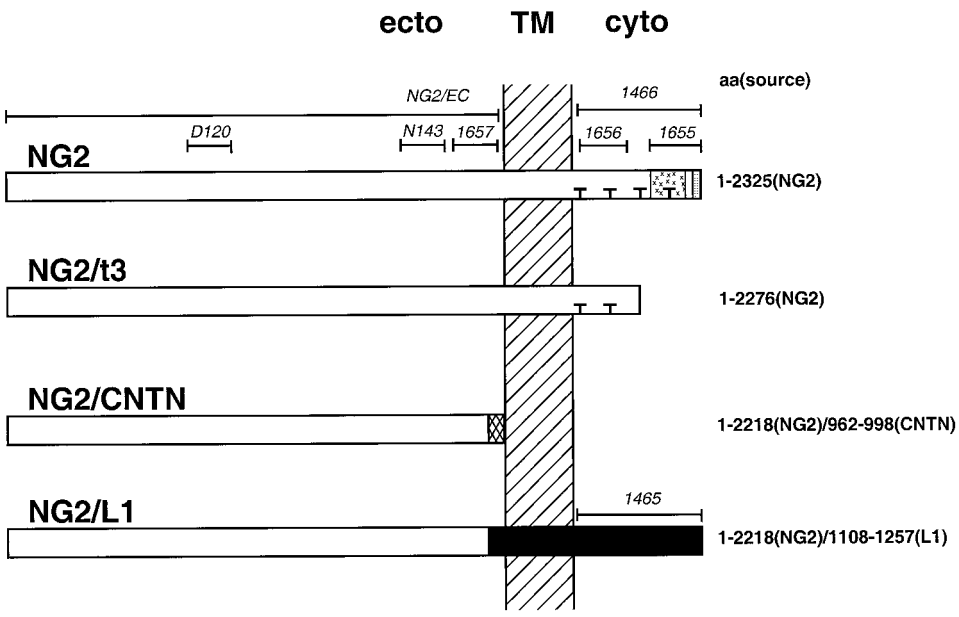

Figure 1. NG2 constructs. The NG2 constructs used for transfection are shown schematically with respect to their association with the cell membrane (diagonally hatched segment). Ecto, TM, and cyto refer to the extracellular, transmembrane, and cytoplasmic portions of these molecules, respectively. Segments corresponding to NG2 are represented by open bars, and segments corresponding to contactin (CNTN) or L1 are represented by cross-hatched and solid bars, respectively. Within the NG2 cytoplasmic domain, boldface Ts represent threonine residues that are candidates for phosphorylation. The stippled area at the extreme C terminus represents the PDZ-binding domain, whereas the area marked with Xs is the proline-rich region. Amino acid residues and the sources of these amino acids are shown at right. Brackets show the approximate location of antibody-binding sites (antibody names are in italics).

collagen interaction triggers signaling events that lead to increased cell motility.

To investigate the potential role of NG2 as a signal-transducing molecule, we have now examined the ability of NG2 to affect the organization of the actin cytoskeleton. The results indicate that engagement of individual extracellular epitopes of NG2 by immobilized mAbs is able to initiate specific rearrangements of the actin cytoskeleton that lead to cell spreading and migration. Furthermore, participation of discrete portions of the NG2 cytoplasmic domain is required for these events to occur, suggesting that NG2 serves directly as a transducer of signaling between the substratum and the cytoskeleton.

MATERIALS AND METHODS

Cell Lines

Cell lines used in this study include the B28 rat glioma (Schubert *et al.*, 1974), the U251MG human astrocytoma (Ponten and Westermark, 1978), the U373 human glioblastoma (Schrappe *et al.*, 1991), rat-1 fibroblasts (Botchan *et al.*, 1976), and rat mesangial cells (a gift from Dr. John Harper, LifeCell, Houston, TX). These cells were maintained in DMEM supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, CA).

Antibodies

Rabbit antibodies and mouse mAbs against rat NG2 have been described previously (Nishiyama *et al.*, 1991, 1995; Tillet *et al.*, 1997), as has a rabbit antibody against the L1 cytoplasmic domain (Prince *et al.*, 1991). mAbs against the human β1 integrin subunit (GIBCO-BRL, Gaithersburg, MD), rat CD44 (PharMingen, La Jolla, CA), fascin (DAKO, Carpenteria, CA), β-actin (Sigma, St. Louis, MO), and vinculin (Sigma) were obtained commercially. Rhodamine-labeled phalloidin was obtained from Molecular Probes (Eugene, OR). Unlabeled affinity-purified goat antibody against mouse immunoglobulins was purchased from Biosource International (Camarillo, CA), as were fluorescein- and rhodamine-labeled goat antibodies against rabbit and mouse immunoglobulins. Rabbit antisera against whole B28 and U251 cells were obtained by initial immunization with cells emulsified in complete Freund's adjuvant, followed by multiple boosts with live cells in suspension.

cDNA Constructs and Transfections

We have previously described the use of Lipofectin and LipofectAmine (GIBCO-BRL) for heterologous expression of wild-type and mutant forms of NG2 with the pcDNAI/amp expression vector (Nishiyama and Stallcup, 1993; Lin *et al.*, 1996a; Burg *et al.*, 1997). Figure 1 shows the panel of NG2 constructs stably transfected into NG2-negative cell lines for use in the current studies. In addition to the wild-type NG2, we have used a truncated mutant (NG2/t3) lacking the C-terminal half of the cytoplasmic domain. This mutant was prepared by creation of a stop codon resulting in the termination of the polypeptide after amino acid residue Glu-2276 (Nishiyama *et al.*, 1991). We have also used two chimeric molecules containing almost the entire ectodomain of NG2 (terminating after amino acid residue Leu-2218) but lacking the NG2 transmembrane and cytoplasmic domains. In the case of the NG2/contactin chimera (NG2/CNTN), this segment of the NG2 polypeptide is replaced by the glycosylphosphatidylinositol (GPI) linkage region of human contactin (Berglund and Ranscht, 1994; Dahlin-Huppe *et al.*, 1997). In the case of the NG2/L1 chimera, the same segment is replaced by the membrane-spanning and cytoplasmic domains of the human L1 neuronal cell adhesion molecule (Hlavin and Lemmon, 1991; Dahlin-Huppe *et al.*, 1997). In both cases, a segment of the NG2 cDNA coding for the transmembrane and cytoplasmic domains was excised from the pcDNA/NG2 plasmid and replaced by a PCR product coding for the desired segment of either contactin or L1.

Cell-spreading Assays

Surfaces used for cell-spreading assays were prepared by incubating 35-mm Falcon (Lincoln Park, NJ) Petri dishes overnight at 4°C with 1.5 ml of 0.05 M Tris-HCl, pH 9.5, containing 1.5 μg/ml affinity-purified goat antibody against mouse immunoglobulins. The next day, these plates were washed once with PBS and then blocked for 4 h at room temperature with a solution of 1% heat-

inactivated BSA in PBS (PBS/BSA). The coated dishes were then incubated for another night at 4°C with PBS/BSA containing mAbs (10 μg/ml) against a variety of cell surface components. After these mAb-coated dishes were once again washed with PBS and blocked for 4 h with PBS/BSA, they were ready for use in the spreading assay. Poly L-lysine (PLL)–coated dishes were prepared by overnight incubation of 35-mm Falcon tissue culture dishes with 1 ml of water containing 30 μg/ml PLL (Sigma). These dishes were washed once with PBS before use.

Cells to be used in spreading assays were serum starved overnight in unsupplemented DMEM. Before the assay, they were harvested with enzyme-free cell dissociation buffer (GIBCO-BRL), collected by centrifugation, and resuspended in DMEM containing 1% heat-inactivated BSA (DMEM/BSA). Cells were left in suspension for 1 h, with occasional agitation, before being used for spreading assays.

Spreading assays were initiated by the addition of 2×10^4 cells to coated dishes containing 1 ml of DMEM/BSA. Spreading was then allowed to proceed at 37°C for the desired period of time. In most cases, several replicate plates were used for each set of conditions, so that multiple time points could be taken and several different antigens examined by immunofluorescence. Assays were terminated by fixation of the cells with either paraformaldehyde or methanol, depending on which antibodies were being used for immunofluorescence analysis of the spreading (see Immunofluorescence)

In some cases, the extent of cell spreading was evaluated quantitatively through use of the Image 1/Metamorph imaging system (version 2) (Universal Imaging, West Chester, PA). For this purpose, spreading cells were fixed as described above and stained for immunofluorescence with polyspecific rabbit antibodies against multiple cell surface determinants (see Antibodies). Fluorescent images were captured using a Zeiss (Thornwood, NY) Axiovert 405 M microscope interfaced with the Metamorph system and were stored as TIFF files. For each set of experimental conditions, surface areas (in arbitrary units) were obtained for roughly 100 cells.

Cell-migration Assays

The motility of NG2 transfectants on a variety of coated substrata was quantified by evaluating the ability of individual cells to migrate away from cell aggregates adhering to the substrata (Xu *et al.*, 1998). Cell aggregates were prepared in the following manner. Cells from three subconfluent 100-mm plates were harvested with nonenzymatic cell dissociation buffer, replated in DMEM containing 10% FCS at near confluence in a single 100-mm Petri dish, and allowed to recover overnight. Cells were then harvested by pipetting and pelleted by centrifugation. Pellets were triturated gently by pipetting with a Pasteur pipet, so that the resulting suspension contained numerous aggregates of various sizes. Aggregates were "size fractionated" by allowing them to settle in a 15-ml conical centrifuge tube. Intermediate sized aggregates (200–400 μm) were saved for further use. Larger aggregates were separated and retriturated, and smaller aggregates were separated, recentrifuged, and then retriturated. After two or three rounds of selection, a good quantity of intermediate sized aggregates was obtained. These were plated in DMEM/FCS on a 60-mm Petri dish that had been previously blocked with PBS/BSA to minimize cell attachment. Aggregates were allowed to recover and compact in these dishes for 6–8 h, after which they were washed in DMEM/BSA and replated in this same medium in a fresh PBS/BSA-blocked Petri dish. After an overnight incubation under these serum-free conditions, aggregates were ready for plating onto coated surfaces.

Coated dishes were prepared in exactly the same manner described for cell spreading, except that coating was restricted to a circle 1 cm in diameter in the center of the dish. This minimized the number of aggregates that had to be prepared for each condition and ensured that all aggregates would be easily visible in the center of the microscope field. Aggregates were plated on these circles in DMEM/BSA, returned to the 37°C incubator, and monitored by microscopy for 48 h. Replicate dishes were used for each set of experimental conditions so that aggregates could be fixed with 2% paraformaldehyde at various time points and saved for photography (using Kodak [Rochester, NY] TMAX 100 film) and quantitation. Quantitation was accomplished by counting the number of cells that migrated away from the central aggregate mass. At least 10 separate aggregates were used for quantitation in each case.

Immunofluorescence

Fixed cells from the spreading assays were stained with a variety of reagents. For staining with rhodamine-labeled phalloidin, anti-vinculin mAb, and polyspecific rabbit antisera against B28 or U251 cells, fixation was done for 10 min at room temperature with 2% paraformaldehyde. For staining with mAbs against β-actin and fascin, cells were fixed for 2 min at −20°C with 100% methanol. In both cases, the fixed cells were washed with PBS and then incubated for 30 min with DMEM/FCS. Cells were then incubated for 30 min at room temperature with the primary antibody (or phalloidin) in DMEM/FCS containing 0.1% Triton X-100 to facilitate permeabilization. After three washes, cells were incubated for another 30 min with the appropriate rhodamine-labeled second antibodies. After three final washes, cells were postfixed in 95% ethanol, air dried, and coverslipped in Immu-mount (Shandon, Pittsburgh, PA).

In a few cases, unfixed living cells were stained with a rabbit antibody against NG2. The same protocol was once again followed, except for the omission of Triton X-100 from the initial antibody incubation. All specimens were examined using a Nikon (Garden City, NY) Optiphot microscope equipped for phase contrast and epifluorescence. Photographs were taken with Kodak TMAX 400 film.

Immunoblotting

Cells used for preparation of immunoblotting samples were pelleted in 1.5-ml Eppendorf tubes and extracted for 10 min on ice with 100 μl of PBS containing 1% NP40 and 100 μg/ml soybean trypsin inhibitor. After removal of insoluble material by centrifugation, the supernatant was treated for 1 h at room temperature with 0.02 U of chondroitinase ABC (ICN Biomedical, Costa Mesa, CA). This extract was then mixed with 100 μl of 2× SDS-PAGE sample buffer and boiled. Samples of the extract were then fractionated by SDS-PAGE on 3–20% gradient gels and transferred by electroblotting onto Immobilon P membranes (Millipore, Bedford, MA). Blocking and probing of these membranes were performed as previously described (Nishiyama *et al.*, 1995; Grako *et al.*, 1999). Immunoreactive bands were visualized using an ECL chemiluminescence kit (Amersham Life Science, Buckinghamshire, England).

Immunoprecipitation

Immunoprecipitation of detergent-extracted material from ^{125}I-labeled cells was performed as described previously (Nishiyama *et al.*, 1991; Dahlin-Huppe *et al.*, 1997). In some experiments, the ^{125}I-labeled cells were treated with phosphoinositol-specific phospholipase C (Oxford Glycosystems, Abingdon Oxon, United Kingdom) before detergent extraction and immunoprecipitation. After preparation of the immunoprecipitates, half of each sample was treated with 0.02 U of chondroitinase ABC for 1 h at room temperature. Samples were then boiled in SDS-PAGE sample buffer and fractionated by SDS-PAGE on 3–20% gels. Gels were dried and analyzed by autoradiography using Kodak X-OMAT AR film.

RESULTS

Biochemical Characterization of Mutant NG2 Molecules

Mutant NG2 molecules used in these studies were characterized by immunoprecipitation and immunoblotting with a

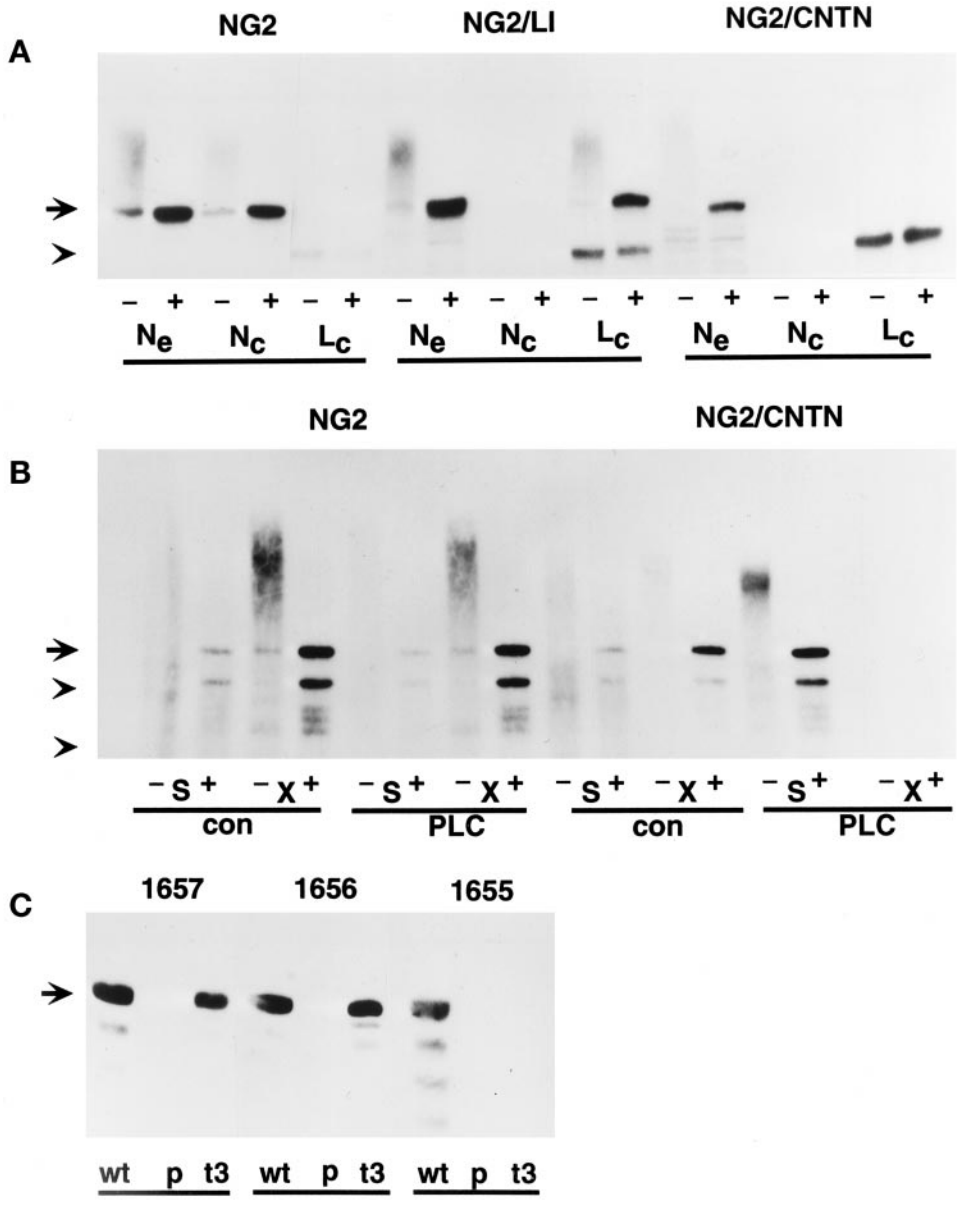

Figure 2. Characterization of transfected molecules. U251 cells transfected with wild-type NG2 (U251NG2.51), the NG2/CNTN chimera (U251NG2/CNTN.40), the NG2/L1 chimera (U251NG2/L1.7), and the truncated NG2/t3 mutant (U251NG2/t3.4) were characterized by the following immunochemical criteria. (A) NP40 extracts of ^{125}I-labeled U251NG2.51, U251NG2/CNTN.40, and U251NG2/L1.7 cells were used for immunoprecipitation studies with the rabbit antibodies NG2/EC (N_e), 1466 (N_c), and 1465 (L_c). The locations of the epitopes recognized by these antisera are shown in Figure 1. Half of each immunoprecipitate was treated with chondroitinase ABC to remove chondroitin sulfate (+), whereas the other half was left untreated (−). Samples were subjected to SDS-PAGE analysis on 3–20% gradient gels. The position of the 300-kDa NG2 core protein is marked in the left margin by an arrow. The arrowhead marks the position of a nonspecifically precipitated 200-kDa component. Molecules containing the NG2 ectodomain are precipitated by NG2/EC in all three cases. In contrast, the 1466 antibody precipitates only the wild-type NG2, which contains the NG2 cytoplasmic domain, and 1465 precipitates only the NG2/L1 chimera, which contains the L1 cytoplasmic domain. (B) ^{125}I-labeled U251NG2.51 (NG2) and U251NG2/CNTN.40 (NG2/CNTN) cells were suspended in 0.2 ml of PBS and divided into two aliquots. One aliquot was treated for 10 min at 37°C with 5 U/ml PI-PLC (PLC), and the other was incubated under similar conditions without the enzyme (con). After these incubations, centrifugation was used to separate supernatants and cell pellets. Both the supernatants (S) and NP40 extracts of the cell pellets (X) were used for immunoprecipitation with the NG2/EC antibody. Half of each immunoprecipitate was treated with chondroitinase ABC (+), and the other half was left untreated (−). Samples were then analyzed by SDS-PAGE on 3–20% gradient gels. The autoradiograms of these gels show that PI-PLC treatment of the U251NG2/CNTN.40 cells causes quantitative release of the NG2/CNTN chimera into the supernatant. In contrast, wild-type NG2 remains associated with the cell surface during PI-PLC treatment. The arrow at left indicates the position of the 300-kDa NG2 core protein. Arrowheads mark the positions of 200- and 116-kDa molecular mass standards. (C) NP40 extracts of U251NG2.51 (wt), U251NG2/t3.4 (t3), and parental U251 cells (p) were treated with chondroitinase ABC, electrophoresed on 3–20% SDS-PAGE gels, and electrophoretically transferred to Immobilon P membranes. After blocking, the membranes were probed with rabbit antibodies specific for defined segments of the membrane-proximal NG2 ectodomain (1657) and cytoplasmic domain (1655 and 1656; see Figure 1). Both wild-type NG2 and the NG2/t3 mutant are recognized by the 1657 and 1656 antisera, but only wild-type NG2 is recognized by the C-terminal 1655 antibody. Parental cells do not contain immunoreactive NG2 species. The arrow at left indicates the position of the 300-kDa NG2 core protein.

panel of antibodies. The approximate location of NG2 epitopes recognized by these reagents is shown in Figure 1. Figure 2A shows that wild-type NG2 and both the NG2/L1 and NG2/CNTN chimeras can be immunoprecipitated by an antiserum against the NG2 ectodomain (NG2/EC). As expected, however, only wild-type NG2 is immunoprecipitated by an antiserum against the NG2 cytoplasmic domain (1466), because both chimeric molecules lack this segment of the proteoglycan. An antiserum against the L1 cytoplasmic domain (1465) is able to immunoprecipitate the NG2/L1 chimera but does not recognize wild-type NG2 or NG2/CNTN.

The NG2/CNTN chimera is further characterized by its release from the cell surface upon cleavage of its GPI linkage with phosphoinositol-specific phospholipase C (PI-PLC). Figure 2B shows that treatment of NG2/CNTN transfectants with PI-PLC results in release of the chimeric molecule into the tissue culture supernatant. Under control conditions, NG2/CNTN remains associated with the cell surface and can be extracted with detergent. In contrast, wild-type NG2 remains associated with the cell surface under control conditions as well as after exposure to PI-PLC.

Immunoblotting with a series of anti-peptide antibodies was used to confirm the deletion of the C-terminal portion of the cytoplasmic domain in the NG2/t3 mutant (Figure 2C). Antibodies against the membrane-proximal portions of the NG2 ectodomain (1657) and cytoplasmic domain (1656) are able to recognize both wild-type NG2 and the NG2/t3 mutant extracted from U251 transfectants. However, only the wild-type molecule can be recognized by an antibody against the C-terminal portion of the cytoplasmic domain (1655). Extracts from parental U251 cells are not reactive with any of these antisera.

Levels of NG2 Expression in Stable Transfectants

Quantitative immunoblotting with a rabbit antibody against the NG2 ectodomain was used to compare the level of NG2 expression in all of the stable transfectants generated for these studies. Densitometry was used to establish the relative amounts of NG2 present in each of these cell lines (Table 1). We used the amount of NG2 present in rat-1 fibroblasts as a baseline (100%) for these comparisons. In general, the transfected cells express higher levels of NG2 than the endogenous expressors (rat-1 and mesangial cells). This is especially true of the U251 transfectants, which have two to three times as much proteoglycan as rat-1 cells.

Table 1. Comparison of NG2 expression

Cells	NG2 expression (%)
Rat-1	100
Mesangial	87
B28NG2.6	155
B28NG2.8	172
B28NG2/CNTN.M	121
B28NG2/CNTN.D	88
B28NG2/t3.6	148
B28NG2/t3.8	167
U251NG2.51	265
U251NG2.35	281
U251NG2/CNTN.40	187
U251NG2/CNTN.10	203
U251NG2/L1.7	273
U251NG2/L1.3	302
U251NG2/t3.4	288
U251NG2/t3.18	244

NP40 extracts of 5×10^5 cells were treated with chondroitinase ABC, fractionated by SDS-PAGE, and analyzed by immunoblotting with affinity-purified rabbit antibody against NG2 (similar to the examples shown in Figure 2C). After developing the immunoblots with an ECL kit, we used densitometry to compare quantitatively the intensities of the NG2 bands from all of the cell lines. The quantity of NG2 found in rat-1 fibroblasts was arbitrarily assigned a value of 100%, and all other values are expressed in relation to this standard.

Engagement of NG2 Initiates Cell Spreading

Although it will clearly be important to elucidate the role of NG2 in cellular interactions with physiological substrata, our initial studies have focused mainly on substrates coated with mAbs against various cell surface components, including NG2. These substrates allow us to investigate cellular responses that result directly from engagement of the individual surface molecules recognized by the immobilized antibodies.

Figure 3 shows the results of experiments in which NG2 transfectants of the U251 human astrocytoma were allowed to attach and spread for 1 h on several types of surfaces. They were then fixed with paraformaldehyde and stained with a polyspecific antiserum against multiple cell surface components of U251 cells to visualize the full extent of cell morphology. Wild-type NG2 transfectants (NG2.51) are able to attach and spread on dishes coated with PLL (a), with a mAb against the β1 integrin subunit (mAb β1; b), and with a mAb against NG2 (mAb D120; c). The D120 mAb recognizes an epitope in the central D2 subdomain of the NG2 ectodomain (Figure 1), the same general region that is responsible for type VI collagen binding (Tillet *et al.*, 1997). This result shows that engagement of the NG2 ectodomain can initiate signal transduction events that lead to cytoskeletal rearrangement and cell spreading. Figure 3, j–l, shows that spreading of the wild-type NG2 transfectants on each of the three surfaces is blocked by the addition of cytochalasin D, demonstrating the importance of the actin cytoskeleton in the spreading mechanism.

If NG2 is responsible for transmitting a signal across the cell membrane that leads to cytoskeletal reorganization and cell spreading, then we might expect the membrane-spanning and cytoplasmic domains of the molecule to be essential for this process to occur. Alternatively, if NG2 associates with another transmembrane molecule, such as an integrin, which is responsible for signal transmission across the membrane, then the membrane-spanning and cytoplasmic domains of NG2 might not be required for the cell spreading observed in Figure 3. To test the involvement of these two domains in cell spreading, we used U251 cells expressing chimeric molecules containing the extracellular domain of NG2 coupled to the GPI membrane linkage region of contactin (NG2/CNTN) or to the transmembrane and cytoplasmic domains of L1 (NG2/L1). These molecules, therefore, lack both the NG2 transmembrane and cytoplasmic domains. U251 cells expressing NG2/CNTN and NG2/L1 are able to attach and spread normally on PLL and mAb β1–coated dishes (Figure 3, d, e, g, and h). These cells are also able to attach to mAb D120–coated dishes but are unable to spread to a significant degree on this surface (Figure 3, f and i).

Although the NG2/CNTN transfectants express only ~70% as much immunoreactive material as the wild-type transfectants, the NG2/L1 transfectants express as much or more material than the wild-type transfectants (Table 1). Therefore, it seems likely that the absence of the NG2 intracellular domains, and not the relative levels of NG2 expression, explains the failure of the two types of chimeric transfectants to spread on NG2 mAbs. These experiments

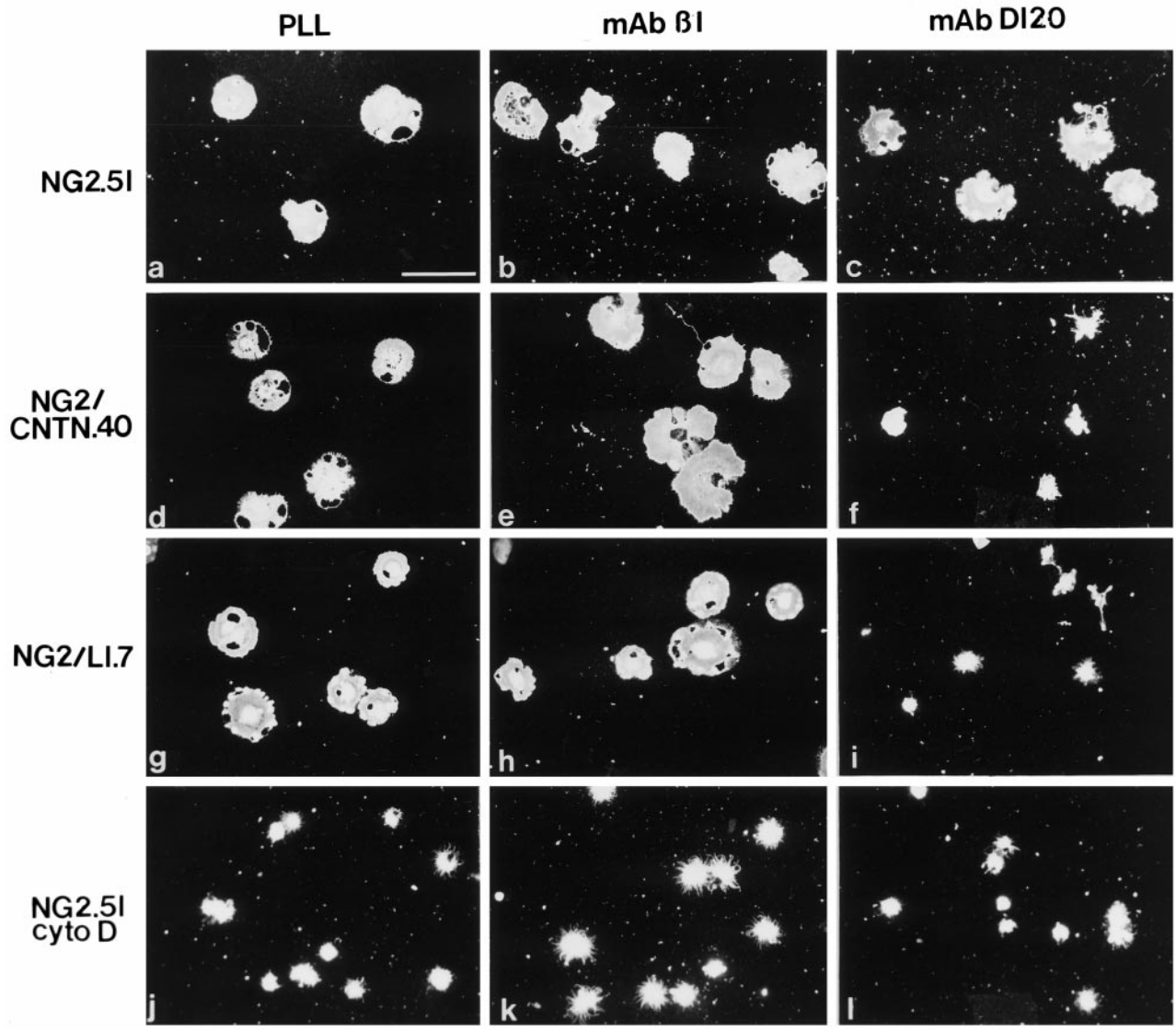

Figure 3. Spreading of U251 transfectants. Spreading of wild-type transfectants (U251NG2.51, a–c) and chimeric transfectants (U251NG2/CNTN.40, d–f, and U251NG2/L1.7, g–i) was compared on surfaces coated with PLL, mAb β1, and mAb D120. Spreading was stopped after 1 h by fixation with 2% paraformaldehyde, and cells were stained with a polyspecific antiserum raised against whole U251 cells. Each of the three cell populations exhibited good spreading on PLL and mAb β1, but only the wild-type transfectants spread effectively on mAb D120. Similar results were obtained with independently derived clones of each cell type (U251NG2.35, U251NG2/CNTN.10, and U251NG2/L1.3). In j–l, cytochalasin D (10^{-6} M) was used to inhibit spreading of the wild-type transfectants on each of the three surfaces. Bar in a, 50 μm.

demonstrate the involvement of the NG2 intracellular domains in triggering cytoskeletal rearrangement and reinforce the concept that NG2 itself is responsible for signal transmission across the plasma membrane.

The spreading behavior illustrated in Figure 3 was not restricted to NG2-transfected U251 cells but was also observed with NG2 transfectants of the U373 human glioblastoma and the B28 rat glioma. Spreading on NG2 mAb-coated surfaces was also obtained with cell lines such as rat-1 fibroblasts and mesangial cells that are endogenous expressors of NG2. Spreading results for the rat-1 fibroblasts and B28 transfectants were quantified with the Metamorph imaging system to measure surface areas of individual cells. Along with the rat-1 cells, two independent clones of B28 transfectants expressing wild-type NG2 were compared with two clones expressing the NG2/CNTN chimera for their ability to spread on surfaces coated with PLL, NG2 mAb N143, and NG2 mAb D120. The extent of spreading was visualized after 1 h by fixing the cells and then staining them with a polyspecific rabbit antibody against B28 cells. Table 2 shows that the rat-1 cells did not spread to a significant extent on PLL-coated dishes but that the surface areas of all four B28 cell types were similar after spreading on this surface. Thus, the wild-type and chimeric B28 transfectants

Table 2. Quantitation of B28 cell spreading

	Surface		
Cell type	PLL	mAb N143	mAb D120
Rat-1	2.51 ± 0.20	5.96 ± 0.27	5.62 ± 0.29
NG2.6	9.02 ± 0.42	7.23 ± 0.32	7.05 ± 0.30
NG2.8	8.49 ± 0.32	5.88 ± 0.33	5.36 ± 0.28
NG2/CNTN.M	7.78 ± 0.37	3.11 ± 0.15[a]	2.71 ± 0.12[a]
NG2/CNTN.D	7.77 ± 0.31	2.67 ± 0.11[a]	2.29 ± 0.09[a]

Cell spreading and quantitation of cell surface areas were performed on surfaces coated with PLL, mAb N143, and mAb D120, as described in MATERIALS AND METHODS. Values for the average cell surface areas, along with SEM, are given in arbitrary units derived from the Metamorph analysis.

[a] Cases in which surface areas of NG2/CNTN transfectants are significantly different from the corresponding values for wild-type NG2 transfectants and rat-1 cells on the same surface (confidence level of $p < 0.0001$ in a nonpaired t test).

had comparable abilities to spread on this control surface. On dishes coated with the two NG2 mAbs, the rat-1 cells and the two B28 clones expressing wild-type NG2 exhibited much larger surface areas than the two clones expressing the NG2/CNTN chimera. Although the NG2/CNTN transfectants express only approximately two-thirds as much NG2-immunoreactive material as the wild-type NG2 transfectants, they express roughly the same amount of material as the rat-1 fibroblasts (Table 1). Thus, the spreading deficiency observed for the chimeric B28 transfectants is likely to be due to their lack of the intracellular NG2 domains rather than to the quantity of NG2 expressed by these cells. These quantitative spreading data once again emphasize the role of the intracellular domains of the proteoglycan in NG2-mediated cell spreading. They also show that the endogenous level of proteoglycan in natural NG2 expressors is sufficient to trigger spreading in response to NG2 engagement.

To determine if specific portions of the NG2 cytoplasmic domain are required for cell spreading on NG2 mAbs, we transfected cells with a truncated form of NG2 (NG2/t3) that is missing the C-terminal half of the cytoplasmic domain. U251 cells expressing NG2/t3 were compared with wild-type transfectants for their ability to spread on PLL, mAb D120, mAb N143, and mAb β1. Cells were stained with polyspecific antisera as before, and the extent of spreading was quantitatively evaluated using the Metamorph system. We were unable to detect significant differences between the ability of wild-type NG2 and NG2/t3 transfectants to spread on any of these surfaces. The similarity in spreading behavior of these two cell populations on mAb D120 and mAb N143 indicates that the membrane-proximal half of the NG2 cytoplasmic domain is capable of supporting a major component of the signal transduction to the cytoskeleton that occurs upon engagement of NG2. This involvement of the membrane-proximal segment in cytoskeletal interaction is also seen when the distribution of NG2 variants on the cell surface is examined. As we have reported previously, wild-type NG2 on the surface of B28 cells is found in highly ordered arrays that are coaligned with cytoskeletal stress fibers (Lin *et al.*, 1996a). The NG2/t3 variant exhibits this same ordered distribution on the surface of B28 cells (Figure 4). In contrast, the NG2/CNTN chimera has a very disorganized distribution on the cell surface, similar to what we have observed for contactin itself and for the L1/contactin chimera (Dahlin-Huppe *et al.*, 1997). These results suggest that NG2 relies on the membrane-proximal portion of the cytoplasmic domain for anchorage to stress fibers.

Cell Migration in Response to NG2 Engagement

U251 cells transfected with the various NG2 constructs were compared for their ability to migrate on surfaces coated with PLL, mAb β1, mAb D120, and mAb N143 (Figure 5). Cells expressing all forms of NG2 were found to migrate very well on mAb β1 (Figure 5, a, d, and g) and extremely poorly on PLL (Figure 5, c, f, and i). Wild-type NG2 transfectants also exhibited good migration on mAb D120 (Figure 5b), whereas the corresponding migration of cells expressing either the NG2/

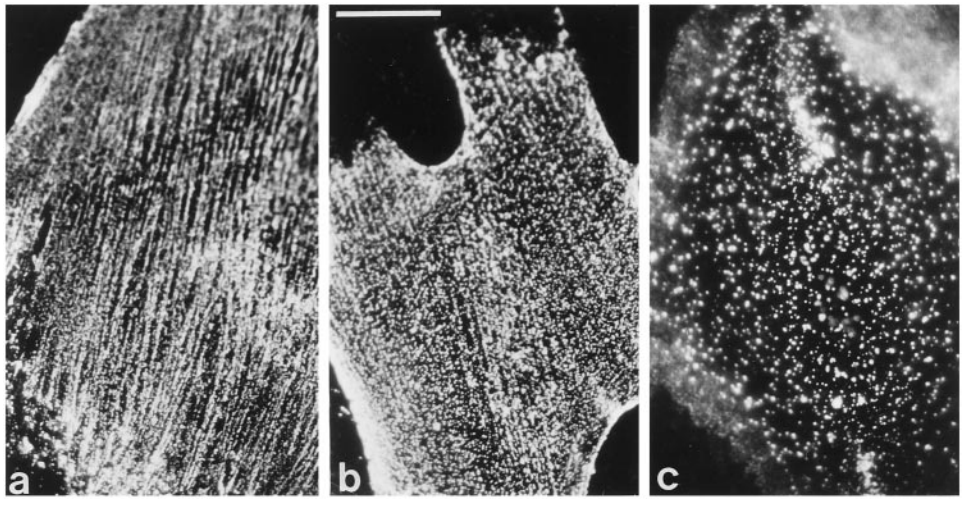

Figure 4. Cell surface distribution of NG2. Immunofluorescence staining with rabbit antibody against NG2 was used to compare the distribution of wild-type NG2 and NG2 variants on the surface of B28 cells. Cells were allowed to grow overnight on tissue culture dishes coated with PLL to achieve maximal spreading. Immunostaining was carried out at room temperature with living cells. (a) Wild-type NG2 (B28NG2.6). (b) Truncated NG2 (B28NG2/t3.6). (c) Chimeric NG2 (B28NG2/CNTN. M). Bar in b, 10 μm.

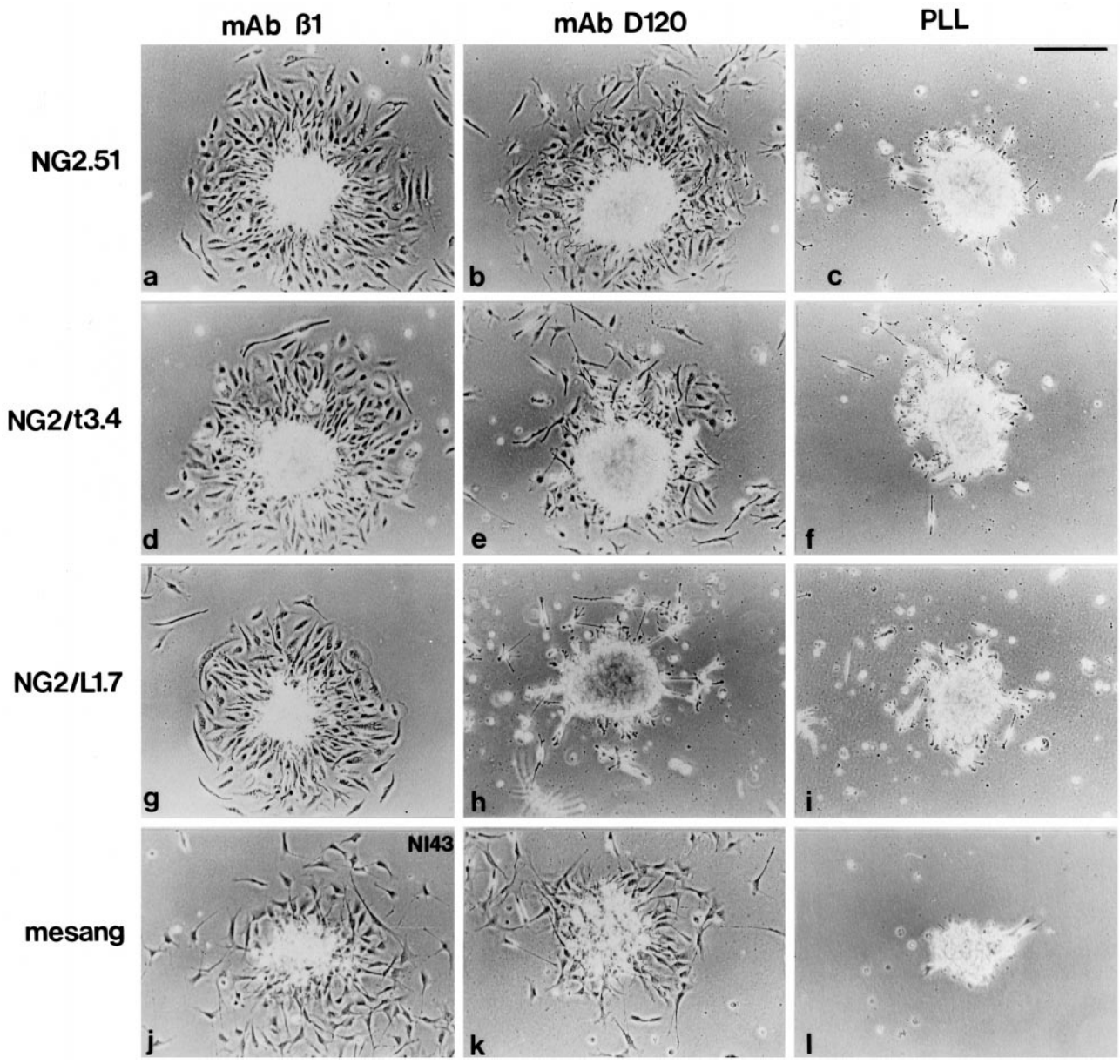

Figure 5. Migration of U251 transfectants. Aggregates of U251NG2.51 (a–c), U251NG2/t3.4 (d–f), U251NG2/L1.7 (g–i), and rat mesangial cells (mesang, j–l) were plated on surfaces coated with mAb β1, mAb D120, mAb N143, or PLL. Cultures were photographed after 16 h to allow assessment of cell migration out of the aggregates. All three U251 cell populations exhibit good migration on mAb β1 and very poor migration on PLL. Differences in migration can be seen on mAb D120, where wild-type transfectants migrate very well (b), NG2/L1 transfectants migrate poorly (h), and NG2/t3 transfectants exhibit an intermediate level of migration (e). Mesangial cells migrate well on NG2 mAbs N143 (j) and D120 (k) but very poorly on PLL (l). Bar in c, 200 μm.

CNTN or NG2/L1 chimeras was poor (Figure 5h), as might be expected from the lack of ability of these cells to spread on this surface. Rat mesangial cells also exhibited good migration on NG2 mAb-coated surfaces and poor migration on PLL, showing that endogenous levels of NG2 expression are sufficient to mediate NG2-dependent motility (Figure 5, j–l).

Interestingly, even though the spreading behavior of wild-type and NG2/t3 transfectants was found to be very similar on mAb D120, cells expressing the truncated NG2/t3 mutant appeared to migrate less efficiently than the wild-type transfectants on this surface (Figure 5e). A quantitative assessment of all the U251 cell migration data verifies the significance of the difference between the wild-type and NG2/t3 transfectants on mAb D120 (Figure 6). Two independent clones of each cell type were used to establish the reproducibility of this finding. In contrast, no difference in

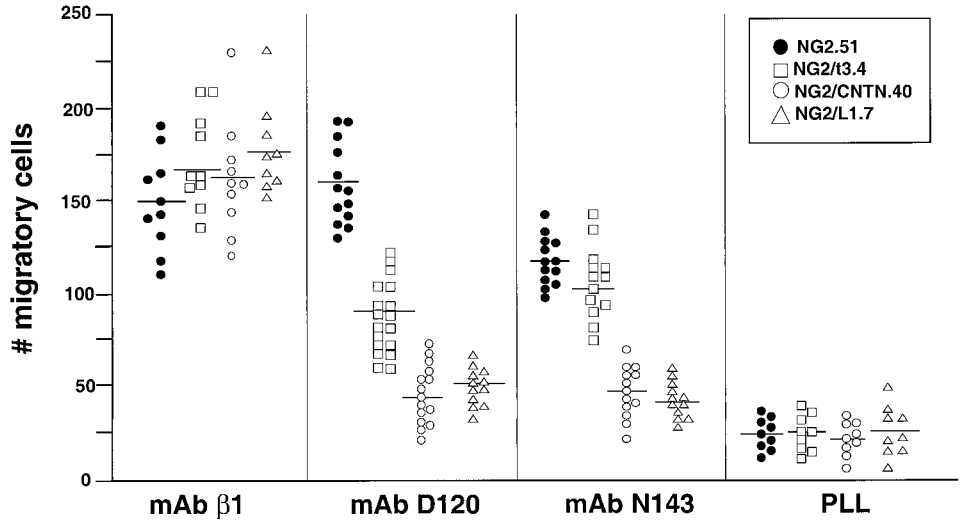

Figure 6. Quantitation of migration of U251 transfectants. Experiments similar to those shown in Figure 5 were conducted using two independent U251 clones of the following cell types: wild type (U251NG2.51 and U251NG2.35), NG2/t3 mutant (U251NG2/t3.4 and U251NG2/t3.18), NG2/CNTN chimera (U251NG2/CNTN.40 and U251NG2/CNTN.10), and NG2/L1 chimera (U251NG2/L1.7 and U251NG2/L1.3). Experiments were carried out for 24 h rather than 16 h (as in Figure 5) to obtain larger numbers of migratory cells for counting. The distribution of data points is shown for only one of the two clones of each cell type, but the behavior of the two clones was extremely similar in all cases (not significantly different from each other). Horizontal lines show the mean value for migration in each set of points. Statistical analysis of the data by a nonpaired *t* test showed that, on mAb D120–coated surfaces, the migration of the NG2/t3-transfected cells was significantly different from the migration of both wild-type NG2 transfectants and chimeric transfectants ($p < 0.0001$). On mAb N143–coated surfaces, the migration of both NG2 and NG2/t3 transfectants was significantly different from the migration of the chimeric transfectants ($p < 0.0001$), but the behavior of NG2- and NG2/t3-expressing cells could not be statistically distinguished.

the migration of these two cell types could be found on mAb N143–coated surfaces.

Cytoskeletal Rearrangements Mediated by Engagement of NG2

Some of the cytoskeletal rearrangements that occur during cell spreading can be observed by examining the details of actin polymerization. We have used rhodamine-labeled phalloidin to study the distribution of filamentous actin in NG2-positive U251 cells spreading on various surfaces. As shown in Figure 7, two types of actin distribution are seen 20 min after the initiation of spreading. On PLL-coated surfaces, the cells exhibit radial actin spikes (Figure 7a), whereas on mAb β1, the actin is found in cortical bundles (Figure 7d). Double staining of these cells with a polyspecific antiserum that labels the entire membrane surface of the U251 cells allows us to see that the radial actin spikes in Figure 7a are found in thin, filopodia-like membrane protrusions (Figure 7e), whereas the cortical actin bundles in Figure 7d are located at the outer edges of extensive lamellipodia-like sheets of membrane (Figure 7h). Interestingly, the engagement of NG2 can lead to the formation of either of these types of actin arrangements, depending on the NG2 mAb used to coat the substratum. Spreading on mAb D120 induces extension of radial actin spikes associated with filopodia (Figure 7, b and f), whereas spreading on mAb N143 leads to the formation of cortical actin bundles within sheets of membrane (Figure 7, c and g). In contrast to the central location of the D120 epitope in domain 2, the epitope recognized by mAb N143 is located in the membrane-proximal domain 3 of the NG2 ectodomain (Figure 1). Thus, engagement of different epitopes of NG2 can lead to short-term differences in the details of actin assembly. After longer periods (60 min), these distinctions were lost, as cells on each of the four surfaces became well spread and contained extensive arrays of stress fibers (Figure 7, i–l). Staining of these cells with the polyspecific anti-U251 cell antiserum gave the same type of results shown in Figure 3. In the case of well-spread cells on mAb β1–coated plates, small focal adhesion plaques could be observed after staining with anti-vinculin antibodies. Focal adhesions were not observed on the other surfaces.

In contrast to the wild-type NG2 transfectants, U251 transfectants expressing the NG2/CNTN and NG2/L1 chimeric molecules failed to exhibit significant numbers of either filopodia or lamellipodia on the NG2 mAbs, consistent with their failure to spread effectively on these surfaces. On the other hand, U251 cells expressing the NG2/t3 mutation were indistinguishable from wild-type NG2 transfectants in terms of their short-term responses to mAb D120– and mAb N143–coated surfaces. Surfaces coated with mAb D120 once again elicited formation of radial actin spikes in the NG2/t3 transfectants, whereas mAb N143 induced formation of cortical actin bundles.

Although a contribution of the C-terminal portion of the NG2 cytoplasmic domain to the reorganization of the actin cytoskeleton is not seen in these phalloidin-stained specimens, the effect of this cytoplasmic segment on cell spreading can be observed by examination of a different aspect of cytoskeletal organization. A number of actin-binding proteins are known to be associated with filamentous actin under different conditions. We used antibodies against β-actin and the actin-binding protein fascin (Yamashiro-Matsumura and Matsumura, 1986; Lin *et al.*, 1996b) to examine actin organization during spreading of U251 cells transfected with wild-type NG2 and with NG2/t3. After spreading on PLL-coated surfaces for 40 min, both transfectants exhibit prominent radial actin spikes decorated with the actin-binding protein fascin (Figure 8, a–d). A different pattern is seen on mAb β1, where neither of the transfectants

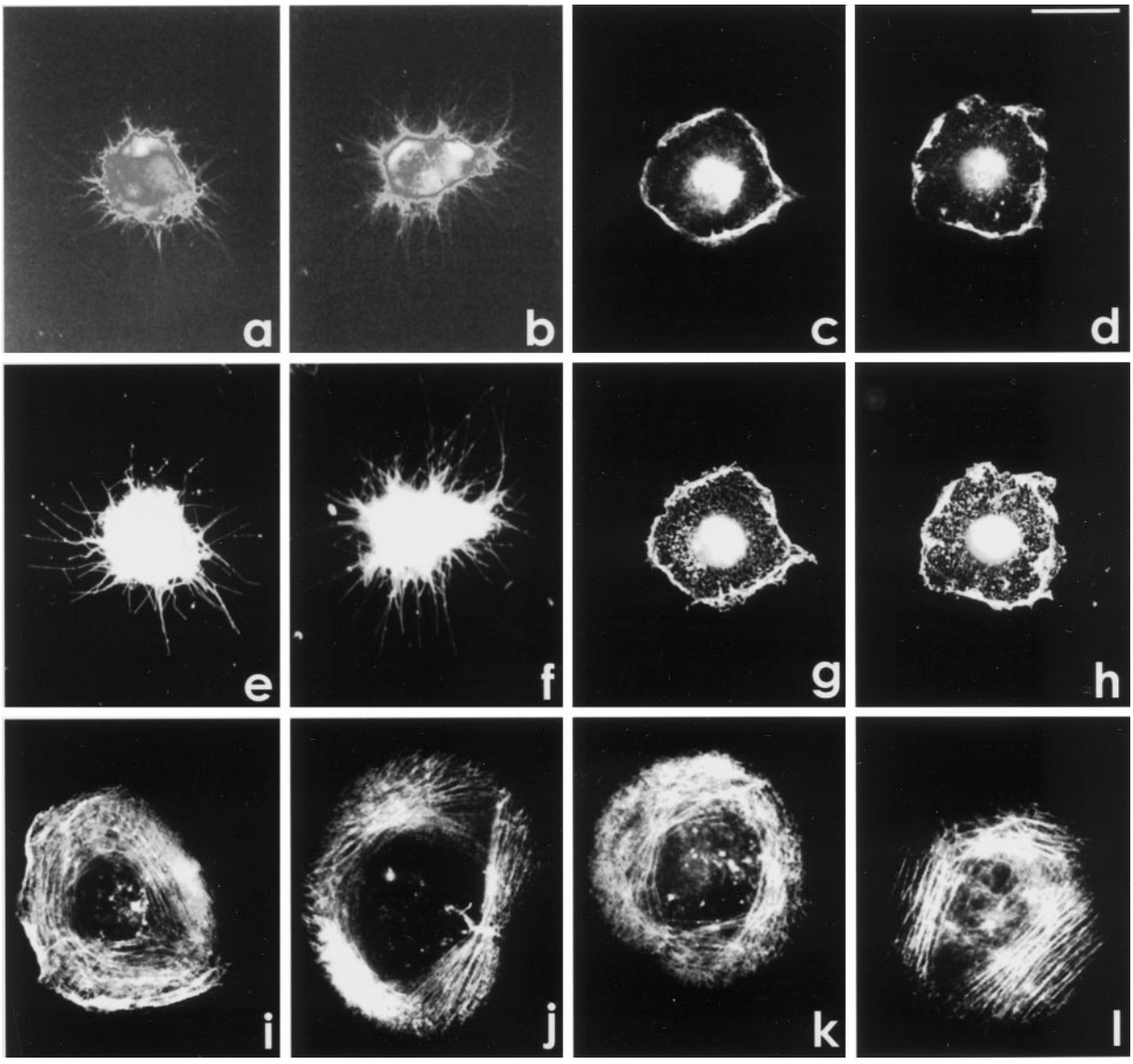

Figure 7. Organization of actin in spreading U251 transfectants. Wild-type NG2 transfectants (U251NG2.51) were allowed to spread for 20 (a–h) or 60 (i–l) min on surfaces coated with PLL (a, e, and i), mAb D120 (b, f, and j), mAb N143 (c, g, and k), or mAb β1 (d, h, and l). Cells were then fixed with 2% paraformaldehyde and double immunostained with a combination of rhodamine-labeled phalloidin (to allow visualization of filamentous actin in a–d) and a polyspecific rabbit antiserum against U251 cells (to allow visualization of the entire cell surface in e–h). After 20 min, radial actin spikes associated with thin filopodia-like protrusions were seen on PLL and mAb D120, whereas cortical actin bundles embedded in sheets of spreading membrane were seen on mAb N143 and mAb β1. Panels a and e, b and f, c and g, and d and h are pairs of images from the double staining. After 60 min, stress fibers were apparent in all cases (single labeling with rhodamine-labeled phalloidin, i–l). Bar in d, 10 μm.

exhibit these radial actin spikes (Figure 8, e–h). Instead, both the actin and fascin staining are more amorphous in nature, with some hints of stress fiber formation. (Although the β-actin antibody is superior to rhodamine-labeled phalloidin in highlighting the radial actin spikes, it is substantially inferior in labeling stress fibers. Phalloidin labeling of these cells would reveal the same sort of stress fibers shown in Figure 7.) On the NG2 mAb D120, only the wild-type NG2 transfectants exhibit this amorphous distribution of actin and fascin, whereas the NG2/t3 transfectants contain prominent actin spikes decorated with fascin (Figure 8, i–l). The mAb D120–coated surface, therefore, allows discrimination between the behavior of the wild-type and NG2/t3 transfectants. In contrast, on the NG2 mAb N143, both transfectants exhibit the same amorphous distribution of actin and fascin observed on mAb β1. It should be noted that fascin staining is not associated with the actin spikes found in the filopodia that form at the earlier time points (20 min) exam-

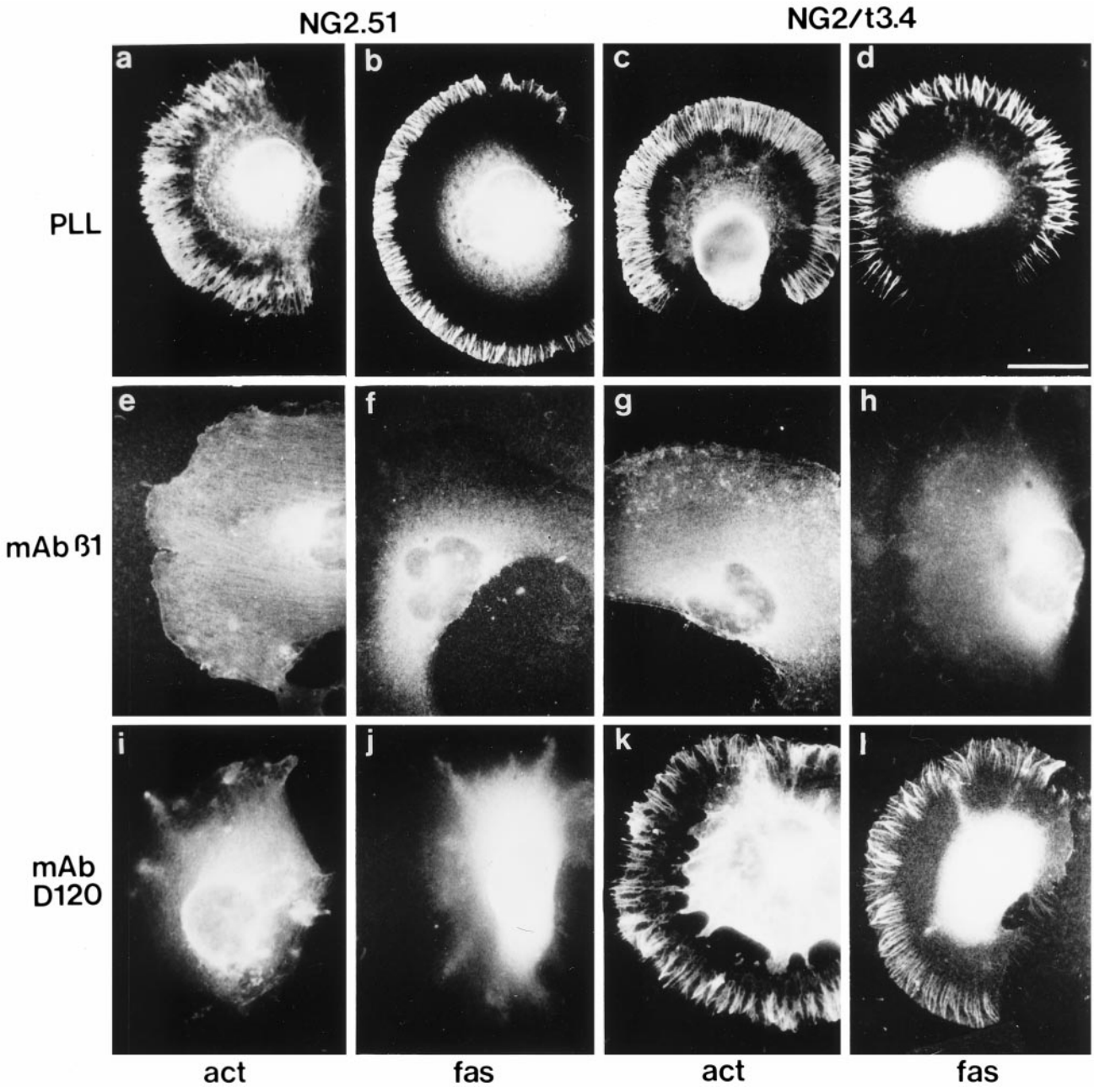

Figure 8. Distribution of the actin-binding protein fascin during spreading of U251 transfectants. U251NG2.51 (a, b, e, f, i, and j) and U251NG2/t3.4 (c, d, g, h, k, and l) cells were allowed to spread for 40 min on PLL–, mAb β1–, and mAb D120–coated surfaces. Cells were then fixed with methanol and stained with mAbs against either β-actin (act; a, e, i, c, g, and k) or fascin (fas; b, f, j, d, h, and l). On PLL, both cell types contain radial actin spikes decorated with fascin (a–d), whereas on mAb β1, these radial spikes are absent (e–h; note some evidence of stress fiber formation in panel e, although the β-actin antibody is substantially inferior to phalloidin in labeling these structures). On mAb D120 (i–l), radial actin spikes decorated with fascin are seen only in the case of the NG2/t3.4 transfectants. Bar in d, 10 μm.

ined in Figure 7. Thus, these fascin-positive actin filaments seen after 40 min represent a later stage in the organization of polymerized actin.

A quantitative evaluation of the tendency of various transfectants to exhibit fascin-positive actin filaments is presented in Figure 9. The percentage of cells containing fascin-positive filaments is shown for parental U251 cells and for two clones each of U251 cells expressing wild-type NG2 and the NG2/t3 mutant. This summary emphasizes the difference between the localization of fascin seen in the wild-type and mutant transfectants when they spread on mAb D120. The other surfaces tested, including a second NG2 mAb (N143),

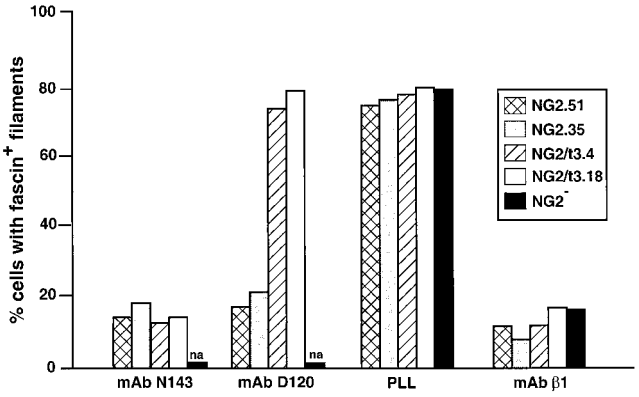

Figure 9. Quantitation of formation of fascin-positive actin filaments. Experiments like that shown in Figure 8 were performed with two independent clones of each of the following U251 transfectants: wild-type NG2 (U251NG2.51 and U251NG2.35), NG2/t3 (U251NG2/t3.4 and U251NG2/t3.18), and parental U251 cells ($NG2^-$). After examining at least 200 cells of each type upon spreading on four different surfaces, we determined the percentage of cells in each population that exhibited fascin-positive radial actin filaments. In the case of parental U251 cells plated on mAbs D120 and N143, no value was obtained because the cells are nonadherent (na) on this surface.

do not distinguish between the wild-type and mutant transfectants. Similar results were obtained with the corresponding B28 transfectants, illustrating the generality of this phenomenon. Thus, even though extensive cell spreading on mAb D120 is seen with the NG2/t3 transfectants, the detailed cytoskeletal rearrangements that occur in these mutants are different from those seen in wild-type NG2 transfectants.

DISCUSSION

Participation of the transmembrane proteoglycan NG2 in signaling events has been previously suggested by experiments with human melanoma cells (Iida *et al.*, 1995). These studies implicated NG2 and the $\alpha 4\beta 1$ integrin as coreceptors for mediating cell spreading on fibronectin. A role for NG2 in signal transduction is also supported by two previous observations made in our laboratory. First, heterologous expression of NG2 in glioma cell lines that are normally NG2 negative leads to an enhancement of cell migration in response to type VI collagen (Burg *et al.*, 1997). Expression of mutant forms of NG2 that lack the collagen-binding domain (Burg *et al.*, 1997; Tillet *et al.*, 1997) does not produce this enhanced response to type VI collagen, thus allowing us to define a component of cell motility that is dependent on the interaction of NG2 with the collagen. The specificity of NG2/type VI collagen binding was previously established in several types of in vitro assays (Stallcup *et al.*, 1990; Nishiyama and Stallcup, 1993; Burg *et al.*, 1996), suggesting that NG2 could provide a mechanism for cellular interaction with the ECM. The motility studies show that this interaction is functionally important and indicate that binding of type VI collagen to NG2 triggers signaling mechanisms that lead to enhanced cell migration.

Second, a possible linkage between NG2 and the actin cytoskeleton was suggested by studies that demonstrated codistribution of cell surface NG2 with actin-rich structures in the cytoplasm, namely stress fibers and retraction fibers (Lin *et al.*, 1996a,b). Significantly, treatment of cells with certain mAbs against NG2 resulted in reduced detergent solubility of the proteoglycan (Lin *et al.*, 1996a). This finding indicated that engagement of key NG2 epitopes might modulate the strength or the nature of NG2 interaction with the actin cytoskeleton, once again suggestive of a signal-transducing role for NG2.

The apparent effect of NG2 mAbs on the NG2-cytoskeleton interaction suggested a means by which we might be able to determine the effects of NG2 engagement on cell behavior. By using surfaces coated with the mAbs, we could create substrata capable of specifically engaging the proteoglycan without directly engaging other types of cell surface receptors. Physiological substrates, such as collagens and other ECM components, interact with multiple types of cell surface receptors, making it difficult to distinguish the NG2-dependent components of the cellular response. In contrast, mAbs recognize individual cell surface molecules. Therefore, the specific contributions of NG2, integrins, and other components to cell behavior can be examined after engagement by the appropriate antibody-coated surfaces.

Our experiments show that NG2-transfected cells attach, spread, and migrate on surfaces coated with anti-NG2 mAbs. Moreover, the magnitude of these responses is comparable to that of responses seen on surfaces coated with mAbs against the $\beta 1$ integrin subunit. This suggests that engagement of the NG2 ectodomain by the substratum triggers signaling events that lead to changes in cell morphology (spreading) and enhanced cell motility. These events do not occur to a significant extent in cells transfected with NG2 mutants (NG2/CNTN and NG2/L1) lacking the transmembrane and cytoplasmic domains of the proteoglycan. We conclude from this finding that NG2 itself provides the means of transducing signals across the cell membrane. That is, engagement of the NG2 ectodomain causes a conformational change that is transmitted to the transmembrane and/or cytoplasmic domains, resulting in changes in the interaction of these intracellular domains with the cytoplasmic machinery.

The patterns of actin reorganization that are observed during the early phases of cell spreading on surfaces coated with anti-NG2 mAbs may provide important clues about the signaling pathways that are activated by the engagement of NG2. Cells spreading on NG2 mAb D120 exhibit radial actin spikes characteristic of the formation of filopodia, whereas cells spreading on mAb N143 assemble cortical actin bundles associated with lamellipodia or membrane ruffles. Thus, the engagement of different NG2 epitopes can lead to differences in the details of actin organization. The epitope recognized by the D120 mAb is located near the junction of NG2 domains 1 and 2 (Nishiyama *et al.*, 1991), roughly in the vicinity of the type VI collagen–binding site (Burg *et al.*, 1997; Tillet *et al.*, 1997). The N143 epitope, on the other hand, is located in the membrane-proximal domain 3 of the NG2 ectodomain. Engagement of these two distinct epitopes, therefore, might trigger different conformational changes in the NG2 ectodomain, resulting in apparent differences in the activation of cytoplasmic signaling pathways by the NG2

cytoplasmic domain. It is interesting to speculate that extracellular matrix ligands for NG2 might also trigger different types of cytoskeletal rearrangements, depending on which portion of the NG2 ectodomain serves as the site of interaction. Clearly, it will be important for us to determine whether interaction of NG2 with physiological ligands such as type VI collagen results in responses similar to those reported here.

The formation of filopodia is a process believed to be mediated through the activation of cdc42, a member of the rho family of small GTPases (Nobes and Hall, 1995). In contrast, the formation of lamellipodia is controlled by activation of rac, another rho GTPase family member (Ridley *et al.*, 1992). Activation of cdc42 or rac can also lead to subsequent activation of rho itself, an event that leads to formation of stress fibers (Ridley and Hall, 1992; Mackay and Hall, 1998). This may account for the observation of stress fiber formation after an extended period of spreading on either mAb D120 or mAb N143. Because the involvement of cdc42 and/or rac in cellular signaling phenomena can be assessed by assaying for the activation of specific downstream kinases (Manser *et al.*, 1993, 1994; Martin *et al.*, 1995; Yang and Cerione, 1997; Bourdoulous *et al.*, 1998; Price *et al.*, 1998) or through the use of dominant negative mutants of the two GTPases (Ridley *et al.*, 1992; Nobes and Hall, 1995; Bourdoulous *et al.*, 1998; Price *et al.*, 1998), we can plan future experiments to test more directly the hypothesis that these two molecules are differentially activated upon engagement of distinct epitopes of NG2.

Because NG2/t3 transfectants missing the C-terminal half of the cytoplasmic domain are virtually indistinguishable from wild-type NG2 transfectants in terms of their ability to 1) spread on NG2 mAbs and 2) extend filopodia and lamellipodia on mAbs D120 and N143, respectively, we can conclude that the membrane-proximal portion of the NG2 cytoplasmic domain plays an important role in mediating these phenomena. A role for the C-terminal half of the NG2 cytoplasmic domain is not apparent in the cell-spreading experiments, but it begins to manifest itself in the cell-migration studies (Figures 5 and 6). Compared with wild-type NG2 transfectants, the NG2/t3 transfectants exhibit a significantly reduced ability to migrate on mAb D120–coated surfaces. A structural correlate for these differences in migration may be found in the fascin-staining patterns seen during the spreading of these two types of transfectants on mAb D120. Fascin is amorphously distributed in wild-type transfectants spreading on this surface, much like the pattern seen in cells spreading on mAb β1 (Figure 8). In contrast, fascin is associated with radial actin filaments in NG2/t3 transfectants spreading on mAb D120, similar to the pattern seen in cells spreading on PLL. The formation of fascin-positive actin filaments, therefore, appears to correlate with a poorly motile cellular phenotype, because poor migration is observed with all types of cells on PLL and with NG2/t3 transfectants on mAb D120. This correlation is further extended by consideration of cell behavior on mAb N143–coated surfaces. Wild-type and NG2/t3 transfectants are equally motile on mAb N143 (Figure 6), and both transfectants exhibit the amorphous fascin distribution pattern during spreading on this surface (Figure 9).

One conclusion from the results with poorly motile cells might be that fascin associates with actin filaments that are stabilized in a manner that is incompatible with efficient cell motility. However, this is the opposite of what might be expected on the basis of previous reports that the presence of fascin-containing microspikes correlates with increased cell motility (Adams, 1997; Yamashiro *et al.*, 1998). Although the reasons for this discrepancy are not currently understood, one possibility is that fascin may associate with more than one form of actin-containing structure (Adams, 1997). Because of differences in the details of their biochemical composition, the fascin-positive structures we have observed may differ in some critical functional manner from those reported by other workers. Another intriguing possibility is that, although the nonmotile cells in our study are able to assemble fascin-positive actin bundles and extend membrane protrusions, they may be unable to coordinate the assembly of these structures in a polarized manner. These cells would be poorly motile as a result of the fact that the membrane protrusions are extended symmetrically rather than with the type of polarity required for effective migration. This type of phenomenon has been observed in cells transfected with a kinase-inactive version of the p21-activated kinase Pak-1 (Sells *et al.*, 1999). In this latter scenario, NG2 might play a role in directing the polarized extension of filopodia or lamellipodia.

The phosphorylation of fascin may be an important factor in determining some of the differences in cell behavior that we have observed. PKC-mediated phosphorylation at serine 39 reduces the actin-bundling activity of fascin, resulting in its disappearance from actin microspikes (Yamakita *et al.*, 1996; Ono *et al.*, 1997). Because PKC activity is influenced to some extent by the rho family GTPases, as a result of their effects on phosphatidylinositol 4,5-bisphosphate metabolism (Stossel, 1993; Chong *et al.*, 1994; Hartwig *et al.*, 1995), engagement of NG2 may alter the level of fascin phosphorylation, resulting in changes in the dynamics of the actin-fascin association. To understand the NG2-fascin relationship, therefore, we will need to correlate the behavior of wild-type and mutant NG2 transfectants with the ability of these NG2 species to modulate fascin phosphorylation.

Because cell motility depends on regulation of the kinetics of actin polymerization, depolymerization, and cross-linking by a variety of actin-binding molecules in addition to fascin, it seems likely that NG2 might also influence some of these other types of cytoskeletal interactions through its apparent ability to induce activation of the cdc42 and rac GTPases. Pak-1, the direct downstream target of both cdc42 and rac, is known to be a potent regulator of cell morphology and cell motility (Sells *et al.*, 1999). Activation of Pak-1 via either cdc42 or rac, therefore, provides a means by which NG2 could stimulate migration. An important mechanism in this regard may be the regulation of myosin light chain phosphorylation and dephosphorylation, processes that determine the contractile properties of the actin/myosin complex (Burridge *et al.*, 1997). Along with rho kinase (Amano *et al.*, 1996; Kimura *et al.*, 1996), Pak-1 is a key regulator of the state of myosin light chain phosphorylation (Sanders *et al.*, 1999; Sells *et al.*, 1999). Thus, it will also be important for us to determine the ability of both wild-type and mutant forms of NG2 to alter myosin light chain phosphorylation during NG2-mediated spreading and migration.

Another topic of interest will be the identification of NG2 cytoplasmic motifs responsible for the spreading and migration phenomena we have observed. The NG2 cytoplasmic domain contains several structural features of interest (Figure 1; see also Nishiyama *et al.*, 1991). The four C-terminal residues (QYWV) represent a potential PDZ-binding motif (Ponting *et al.*, 1997; Songyang *et al.*, 1997) that might play a role in the anchorage of NG2 to cytoplasmic scaffolding proteins (Saras and Heldin, 1996). There is also a proline-rich segment in the C-terminal half of the cytoplasmic domain. Although the spacing of these prolines does not precisely match the PXXP motif expected for molecules that bind to SH3 domains (Ren *et al.*, 1993; Feng *et al.*, 1994; Yu *et al.*, 1994), we have preliminary evidence for the ability of NG2 to interact with the SH3 domains present in some types of cytoplasmic adapter molecules (our unpublished results). Finally, there are three threonine residues (Thr-2255, Thr-2264, and Thr-2277) in consensus sequences that could be substrates for phosphorylation by PKC and a fourth (Thr-2313) that could be phosphorylated by a proline-dependent kinase (Kemp and Pearson, 1990). The NG2/t3 mutant was created by terminating the NG2 protein at residue 2276, thereby eliminating the putative PDZ-binding domain, the proline-rich segment, and two of the potential phosphorylation sites. The functional role of the C-terminal portion of the cytoplasmic domain, deduced from the cell-migration and fascin-localization results, may therefore depend on one or more of these structural features. The importance of the membrane-proximal segment of the cytoplasmic domain, observed in the studies of cell spreading and actin rearrangement, may depend on the phosphorylation state of the first two threonine residues or on some other as-yet-unrecognized feature. Additional NG2 cytoplasmic mutants will be needed to assess these possibilities.

Mutations in the transmembrane domain may also prove instructive. The membrane-spanning domain of the syndecan family proteoglycans is thought to be important for mediating intermolecular interactions (Rapraeger and Ott, 1998). This conclusion is based partly on the observation that syndecan-1–mediated cell spreading still occurs in the absence of the syndecan-1 cytoplasmic domain (Lebakken and Rapraeger, 1996). Because in many respects syndecans represent the prototype for understanding membrane-spanning proteoglycans, functional comparisons between the mechanisms of action of NG2 and syndecan family members are likely to be valuable.

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