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Interaction of the NG2 Proteoglycan With the Actin Cytoskeleton

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Abstract The NG2 chondroitin sulfate proteoglycan is a membrane-spanning molecule expressed by immature precursor cells in a variety of developing tissues. In tightly adherent cell lines with a flattened morphology, NG2 is organized on the cell surface in linear arrays that are highly co-localized with actin and myosin-containing stress fibers in the cytoskeleton. In contrast, microtubules and intermediate filaments in the cytoskeleton exhibit completely different patterns of organization, suggesting that NG2 may use microfilamentous stress fibers as a means of cytoskeletal anchorage. Consistent with this is the observation that cytochalasin D disrupts the organization of both stress fibers in the cytoskeleton and NG2 on the cell surface. Very similar linear cell surface arrays are also seen with three other cell surface molecules thought to interact with the actin cytoskeleton: the $\alpha_5\beta_1$ integrin, the CD44 proteoglycan, and the L1 neuronal cell adhesion molecule. Since the cytoplasmic domains of these four molecules are dissimilar, it seems possible that cytoskeletal anchorage in each case may occur via different mechanisms. One indication of such differences can be seen in colchicine-treated cells which have lost their flattened morphology but still retain long actin-positive tendrils as remnants of the actin cytoskeleton. NG2 and $\alpha_5\beta_1$ are associated with these tendrils while CD44 and L1 are not, suggesting that at least two subclasses of cell surface molecules exist which can interact with different subdomains of the actin cytoskeleton.

Key words: integral membrane proteoglycan, actin cytoskeleton, extracellular matrix, transmembrane signalling

NG2 is a large chondroitin sulfate proteoglycan whose structure and expression pattern make it unique among the growing family of proteoglycans. First, NG2 is one of the few chondroitin sulfate proteoglycans whose core protein is a membrane spanning polypeptide. Second, the core protein can be expressed on the cell surface either with or without glycosaminoglycan chains, placing NG2 in the category of part-time proteoglycans [Stallcup et al., 1983]. Third, neither the extracellular nor cytoplasmic domains of NG2 contain structural motifs with obvious similarities to those found in other proteoglycans [Nishiyama et al., 1991a]. Fourth, NG2 expression in a variety of developing tissues occurs on immature progenitor cells and is down-regulated when these progenitors undergo terminal differentiation [Stallcup and Beasley, 1987; Levine and Stallcup, 1987; Levine et al., 1993; Stallcup and Nishiyama, 1993; Nishiyama et al., 1991b; Grako and Stallcup, 1995; Nishiyama et al., 1996 a, b]. Finally, NG2 expression is up-regulated in several types of malignancies, including melanomas, glioblastomas, chondrosarcomas, and myeloid leukemia blasts [Schrappe et al., 1991; Schlingemann et al., 1990; Leger et al., 1994; Smith et al., 1996]. Our research has been concerned with elucidation of the functional roles played by NG2 in both developmental and pathological situations and with identification of the relevant intermolecular interactions responsible for these functions.

As a membrane-spanning molecule, NG2 has the potential to interact with both extracellular and cytoplasmic components and perhaps to participate in the exchange of information between the extracellular and intracellular compartments. We have described several extracellular NG2 binding partners that are components of the extracellular matrix. The best characterized of these interactions is that between NG2 and type VI collagen. NG2 serves as an effective cell surface receptor for type VI collagen, thus providing one means of cell anchorage to the extracellular matrix [Stallcup et al., 1990; Nishiyama and Stallcup, 1993]. Direct binding studies us-

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ing purified NG2 and individual extracellular matrix proteins show that in addition to binding type VI collagen, NG2 can also interact effectively with collagens type II and V, and with laminin and tenascin [Burg et al., 1996]. Due to the large size of the extracellular portion of NG2, it is possible that distinct binding domains exist for each of these extracellular matrix components. There is also evidence to suggest that NG2 interacts with and potentates the activity of the PDGF-AA/PDGF α receptor signalling pathway [Grako and Stallcup, 1995; Nishiyama et al., 1996a, b]. Experiments are underway to determine whether the effect of NG2 on this signalling system is mediated by direct interaction with the growth factor receptor or whether the proteoglycan participates in sequestering and/or presentation of the growth factor.

Participation in any type of transmembrane signalling would require that NG2 also have intracellular binding partners. Except for the presence of three threonine residues which appear to be capable of serving as sites of phorphorylation by protein kinase C, the sequence of the NG2 cytoplasmic domain provides few clues about the identity of potential intracellular ligands. Nevertheless, we have pursued the topic of NG2 cytoplasmic interactions as a means of investigating the possible role of the proteoglycan in transmembrane communication. In this manuscript we present evidence that NG2 is capable of interacting with the actin cytoskeleton. We also show that while this interaction has some similarities to that which occurs between integrins and the actin cytoskeleton, it appears to be distinct from that seen with other cell surface molecules. The data indicate that the cytoplasmic domain of NG2 is largely responsible for the specificity of anchorage of the proteoglycan to microfilamentous stress fibers.

METHODS

Cell Lines

The rat neural cell lines B28, B49, and B111 [Schubert et al., 1974] were obtained from Dr. David Schubert (Salk Institute, La Jolla, CA). Rat-1 fibroblasts were originally described by Steimer and Klagsbrun [1981]. All cell lines were mycoplasma-free and were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS - Tissue Culture Biologicals, Tulare, CA). Cells were routinely grown on untreated Falcon tissue culture dishes, although for some experiments dishes were coated overnight with a solution of 30 μg/ml poly-L-lysine (Sigma, St. Louis, MO).

Antibodies and Immunofluorescence

The derivation of rabbit and monoclonal antibodies against the NG2 proteoglycan has been previously described [Stallcup et al., 1990; Nishiyama et al., 1991a]. Monoclonal antibody L1.1 recognizes an epitope in the extracellular domain of the human L1 neuronal cell adhesion molecule. The L1.1 hybridoma was derived by fusing P3x63Ag8.653 myeloma cells [Kearney et al., 1979] with spleen cells taken from a Balb/c mouse immunized with Balb/c 3T3 fibroblasts expressing transfected human L1 (see section on cDNA construction and transfection). Further details of the derivation of this antibody will be presented elsewhere [Dahlin-Huppe et al., in preparation]. Monoclonal antibody against rat CD44 was purchased from Pharmingen. Rabbit antibodies against the $\alpha_5\beta_1$ integrin were generously provided by Dr. Erkki Ruoslahti (The Burnham Institute, La Jolla, CA). Rabbit antibodies against vimentin were gifts from Dr. Jon Singer (University of California, San Diego, CA). Rabbit antibody against non-muscle myosin was purchased from Biomedical Technologies Inc. (Stoughton, MA). Monoclonal antibodies against vinculin and β-tubulin were obtained from Sigma (St Louis, MO). Rhodamine-labelled phalloidin was obtained from Molecular Probes Inc. (Eugene, OR). Fluorescein and rhodamine-conjugated goat 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% FCS (DMEM/FCS). Incubations with primary antibodies were performed for 30 min at room temperature, followed by three washes with DMEM/FCS. Incubations with secondary antibodies were also for 30 min at room temperature followed by three washes with DMEM/FCS. 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 abso-

lute methanol for 2 min at −20°C (myosin, tubulin, and vimentin) or at 4°C for 10 min with 4% paraformaldehyde in 0.1M PIPES buffer, pH 6.8, containing 5mM EGTA and 2 mM $MgCl_2$ (vinculin and phalloidin). 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. 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, Pittsburg, 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 Co., Rochester, NY).

Polyacrylamide Gel Electrophoresis, Immunoprecipitation, and Immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 3–20% gradient slab gels according to the buffer system of Laemmli [1970]. Samples were dissolved by boiling for 2 min in buffer containing 3% SDS and 5% 2-mercaptoethanol.

For analysis of NG2 release from the cell surface, B49 cells were surface labelled [Hubbard and Cohn, 1972] with ^{125}I (New England Nuclear) and then suspended in DMEM/FCS culture medium for 1–2 h at either room temperature or 37°C. Immunoprecipitation of ^{125}I-labelled NG2 released into the tissue culture medium was accomplished by incubating samples of the culture supernatant with rabbit antibody against NG2 for 2 h, followed by an additional 2 h incubation with proteinA-sepharose (Sigma). Protein A-sepharose pellets were washed three times with PBS containing 0.2% NP40 and 0.02% SDS, treated for 1 h at room temperature with 0.01 units of chondroitinase ABC (ICN Biomedicals, Costa Mesa, CA) to remove chondroitin sulfate chains from the NG2 core protein, and analyzed by SDS-PAGE. Labelled components were identified by autoradiography using XAR-5 film (Eastman Kodak).

For immunoblotting of NG2, the contents of a 60 mm dish of B49 cells were washed two times with PBS and extracted for 10 min in the cold with 30 μl of PBS containing 1% NP40 and 0.1 mg/ml soybean trypsin inhibitor (Sigma). Insoluble material was removed by centrifugation in an Eppendorf microfuge (Brinkman Instruments), and supernatants were treated with 0.05 units of chondroitinase ABC for 1 h at room temperature. After the addition of 30 μl of 2X SDS-PAGE sample buffer, samples were boiled and run on SDS-PAGE gradient gels. Protein bands were transferred electrophoretically to Immobilon P membranes (Millipore, Inc., Bedford, MA) in 10 mM CAPS buffer, pH 11.0, containing 10% methanol, and the membranes were blocked overnight in Tris buffered saline, pH 7.4, containing 10% bovine serum albumin and 5% calf serum. NG2 core protein was identified on the membranes by incubation with rabbit anti NG2 antibody, followed by extensive washing with Tris-saline and a second incubation with peroxidase-labelled goat antibodies against rabbit immunoglobulin (BioRad). After final washings, bands were visualized using an ECL chemiluminescence kit (Amersham) and Kodak XAR-5 film.

cDNA Construction and Transfection

cDNA containing the complete nucleotide coding sequence for rat NG2 [Nishiyama et al., 1991a; Nishiyama and Stallcup, 1993] was inserted into the multiple cloning site of the eukaryotic expression vector pcDNAI/amp (Invitrogen, San Diego, CA). Likewise, cDNA containing the full coding sequence for human L1 was also incorporated into pcDNAI/amp. The L1 cDNA used in our work was cloned independently in our lab and conforms to the sequence reported by Hlavin and Lemmon [1991]. A complete description of our L1 cDNA cloning will be published elsewhere [Dahlin-Huppe et al., in preparation; see Harper et al., 1991].

In addition two chimeric cDNAs were constructed. The first of these, designated $L1_E/NG2_C$, contains the L1 ectodomain coding sequence (minus the sequence for the fifth fibronectin type III repeat) attached to the sequence coding for the NG2 transmembrane and cytoplasmic domains. The second, designated $NG2_E/L1_C$, contains the NG2 ectodomain sequence (minus a portion of the sequence coding for 185 amino acids proximal to the plasma membrane) linked to the L1 transmembrane and cytoplasmic domain sequence. In each case a segment of cDNA coding for the cytoplasmic domain, trans-

membrane domain, and a small portion of the membrane-proximal ectodomain of one molecule was excised and replaced by a PCR product coding for the transmembrane and cytoplasmic domains of the other molecule. Thus the $L1_E$/$NG2_C$ chimera consists of L1 amino acids 1–1005 linked to NG2 amino acids 2209–2325. Conversely, the $NG2_E$/$L1_C$ chimera consists of NG2 amino acids 1–2040 linked to L1 amino acids 1105–1257. The sequence of both chimeric cDNA constructs was confirmed by the dideoxy chain termination method [Sanger et al., 1977]. The authenticity of the chimeric proteins produced by these cDNA's was also verified by their ability to be expressed on the cell surface and recognized by the appropriate antibodies.

Transfection of B28 cells with each of the four expression plasmids was accomplished using LipofectAmine (Gibco/BRL) according to the manufacturer's recommendations. For transient transfections, B28 transfectants were removed from culture dishes by trypsinization 48 h after transfection. They were replated onto poly-L-lysine coated dishes and allowed to recover overnight before being analyzed by immunofluorescence staining. For establishment of stably transfected B28 clones, expression plasmids were co-transfected with the pSV2neo plasmid, and potential transfectants were selected for resistance to 400 μg/ml G418 (Gibco/BRL). G418-resistant colonies were expanded into 35mm tissue culture plates and analyzed by immunofluorescence for expression of the relevant cell surface molecule. Cells from positive colonies were then cloned by limiting dilution into 96 well plates (Linbro). These clones were re-tested by immunofluorescence for both homogeneity and level of expression.

RESULTS

Cell Surface Distribution of NG2

In our previous work [Stallcup et al., 1990; Nishiyama and Stallcup, 1993], it was apparent that on some cell lines NG2 distribution on the cell surface occurs in a highly organized fashion. Our current results show that on cell lines such as B49 which do not spread extensively (Fig. 1a), this pattern of organization is not as obvious as it is on extremely flattened cell lines such as B111 and Rat-1 (Fig. 1c,e). On the latter cells the organization of cell surface NG2 into linear arrays is quite striking. These linear arrays are extremely well co-localized with cytoskeletal stress fibers, which we have identified by staining with an antibody against non-muscle myosin (Fig. 1b,d,f). Figure 2a and b shows that a similar pattern of co-localization is seen when B111 cell surface staining for NG2 is compared to staining of filamentous actin with phalloidin. In contrast, tubulin and the intermediate filament protein vimentin have cytoskeletal distributions that are completely different from the cell surface distribution of NG2 (Fig. 2c–f). These results suggest that the distribution of NG2 on the cell surface might depend on cytoskeletal anchorage via microfilamentous stress fibers rather than via microtubules or intermediate filaments. It is worth noting that NG2 is present on the cell surface wherever the stress fibers extend. It is not restricted, for example, to the specialized cell surface subdomains known as focal adhesions, identified by staining with antibodies against vinculin (Fig. 2g,h). It is important to note that when cells are fixed with 2% paraformaldehyde prior to labelling for NG2, the same ordered pattern of expression on the cell surface is observed. We have only included images of live cell staining in the figures, because the quality of immunostaining in fixed preparations is significantly inferior in terms of intensity and background signal.

Disruption of Cell Surface NG2 Distribution

If the cell surface distribution of NG2 depends on anchorage to the actin-containing microfilaments, it is possible that disruption of the microfilaments might cause a change in the localization of NG2. Treatment of B111 cells with 2×10^{-6} M cytochalasin D causes depolymerization of microfilaments, resulting in a loss of organized stress fibers that can be detected by staining with anti-myosin antibodies (Fig. 3f) or phalloidin (not shown). In parallel to this disruption of stress fibers, NG2 no longer appears in distinct linear arrays, but instead aggregates into patches on the cell surface (Fig. 3e). Figure 3g and h shows that although cytochalasin D treatment drastically affects NG2 distribution, as expected it has little effect on microtubule structure. In contrast, treatment of B111 cells with 2×10^{-5} M colchicine results in depolymerization of microtubules (Fig. 3l) without obvious effects on microfilaments (Fig. 3j) or NG2 (Fig 3i,k). These findings reinforce the impression that NG2 may be anchored to microfilamentous stress fibers. Similar results were obtained with cytochalasin D-treated B49 cells, although the effect

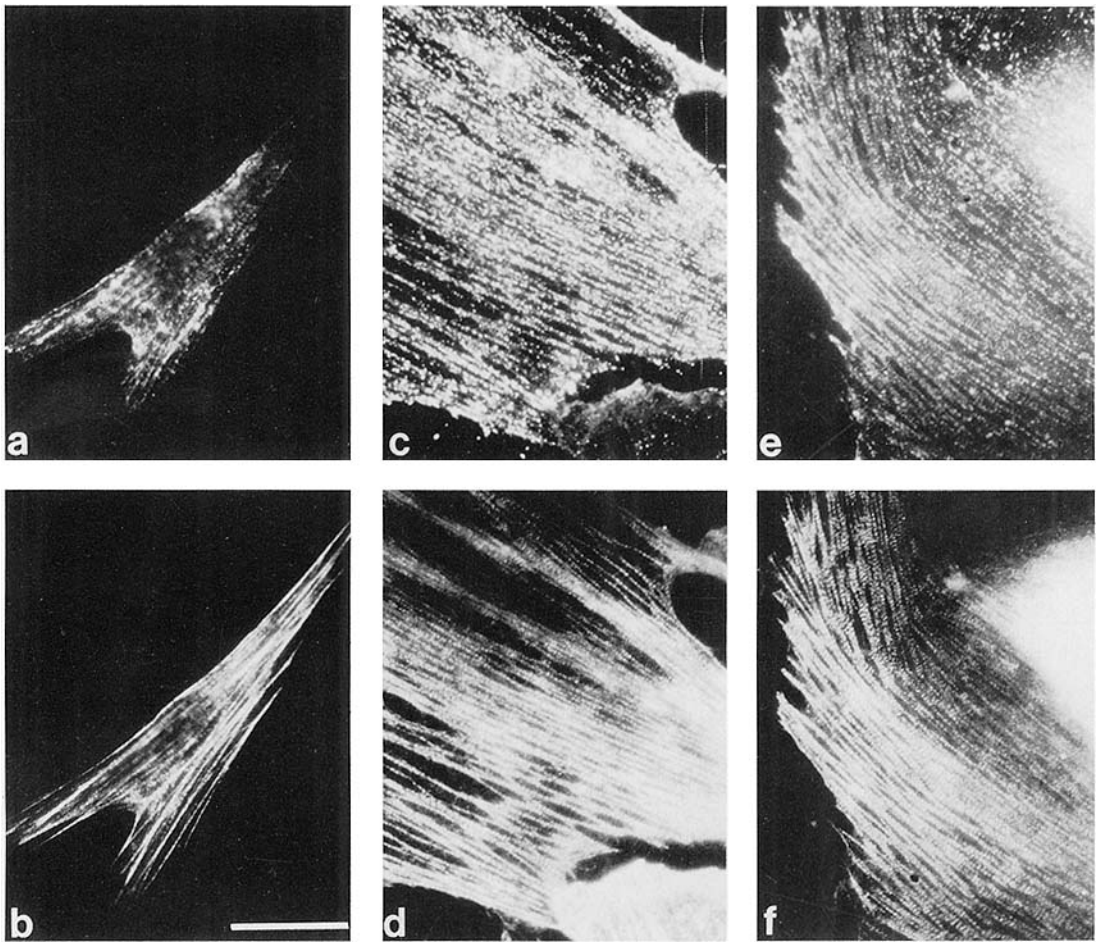

Fig. 1. Co-localization of NG2 with cytoskeletal stress fibers. B49, B111, and Rat-1 cells grown on poly L-lysine coated dishes were double stained for NG2 (**a,c,e**) and myosin (**b,d,f**). The organization of NG2 into linear arrays that co-localize with myosin-labelled stress fibers is somewhat less apparent in B49 cells (a and b) than in the more flattened B111 (c and d) and Rat-1 cells (e and f). Bar in b = 10 μm.

was visually less striking. This is due to the less flattened morphology of B49 cells and to their tendency to lose their attachment to the dish during cytochalasin D treatment.

An additional piece of information in this regard is the finding that cytochalasin treatment results in an increase in release of NG2 from the cell surface. Figure 4 shows that substantially more NG2 can be immunoprecipitated from the tissue culture supernatant of B49 cells treated for 1 or 2 h with cytochalasin D than from the supernatant of untreated cells. The increase in cytochalasin D-induced release of NG2 at 37°C vs. room temperature correlates with the increased effects of cytochalasin D on microfilament structure at the higher temperature. We have previously shown that the spontaneously released form of NG2 found in the supernatant is a proteolytically-generated, truncated molecule missing the transmembrane and cytoplasmic domains of the core protein [Nishiyama et al., 1995]. Western blotting with antibodies against the NG2 ectodomain and against the cytoplasmic domain confirm that the NG2 species released in larger quantities in response to cytochalasin D treatment is indistinguishable from the spontaneously released NG2 found under control conditions: i.e., it lacks both the transmembrane and cytoplasmic portions of the core protein (not shown).

Antibody Enhanced Interaction of NG2 With the Cytoskeleton

Iida et al. [1995] have demonstrated that a monoclonal antibody against the human homolog of NG2 appears to trigger a signalling event involved in the spreading of melanoma cells on fibronectin. In our current studies we tested

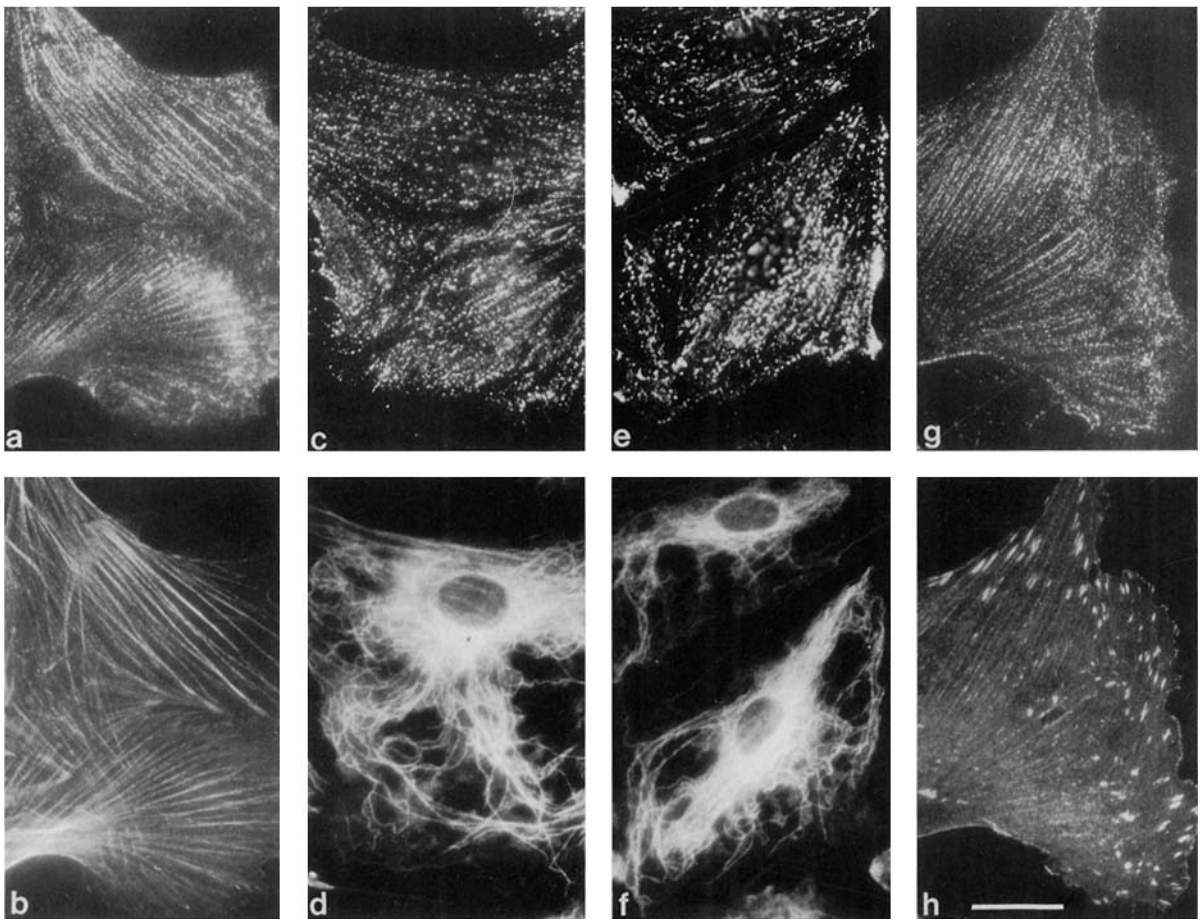

Fig. 2. Comparison of NG2 distribution with cytoskeletal structures. Using B111 cells grown on poly L-lysine coated dishes, the cell surface distribution of NG2 (**a,c,e,g**) was compared with the cytoskeletal distribution of filamentous actin (phalloidin staining, **b**), tubulin (**d**), vimentin (**f**), and vinculin (**h**). Only actin-positive stress fibers mirror the cell surface organization of NG2. Bar in h = 10 μm.

several anti-NG2 monoclonal antibodies [see Stallcup et al., 1990; Nishiyama et al., 1991a] for their ability to initiate signalling events which would lead to alterations in cytoskeletal architecture and/or cell morphology. Although no obvious effects on these cellular characteristics were observed, we did detect dramatic changes in the detergent extractability of NG2 in response to treatment with certain combinations of monoclonal antibodies. While treatment of NG2-positive cells with some combinations of these antibodies had no obvious effect on the detergent extractability of the proteoglycan, other combinations resulted in a greatly increased resistance to extraction of NG2 with 1% NP40. Figure 5A illustrates the decrease in the quantity of NG2 that can be extracted by NP40 from B49 cells treated with a cocktail of monoclonal antibodies N3/D4/D31. By comparison, treatment with a cocktail of monoclonal antibodies N3/N11/D31 causes no appreciable change in the amount of NG2 that can be extracted with NP40. The N3/D4/N11 cocktail has an effect similar to that seen with N3/D4/D31. Release studies such as those shown in Figure 4 do not reveal an increased loss of NG2 from the cell surface during treatment with the N3/D4/D31 cocktail (not shown). Instead, the bulk of the NG2 in this case appears to be more tightly associated with the cell, and can only be extracted with SDS-containing buffers. When the insoluble cell pellets from NP40 extractions are re-extracted with SDS-PAGE sample buffer containing 3% SDS, a large quantity of NG2 can be obtained from the N3/D4/D31 treated cells, while little remains to be removed from control cells or cells treated with the N3/N11/D31 cocktail (Fig. 5B). Treatment with the N3/D4/D31 cocktail does not appear to

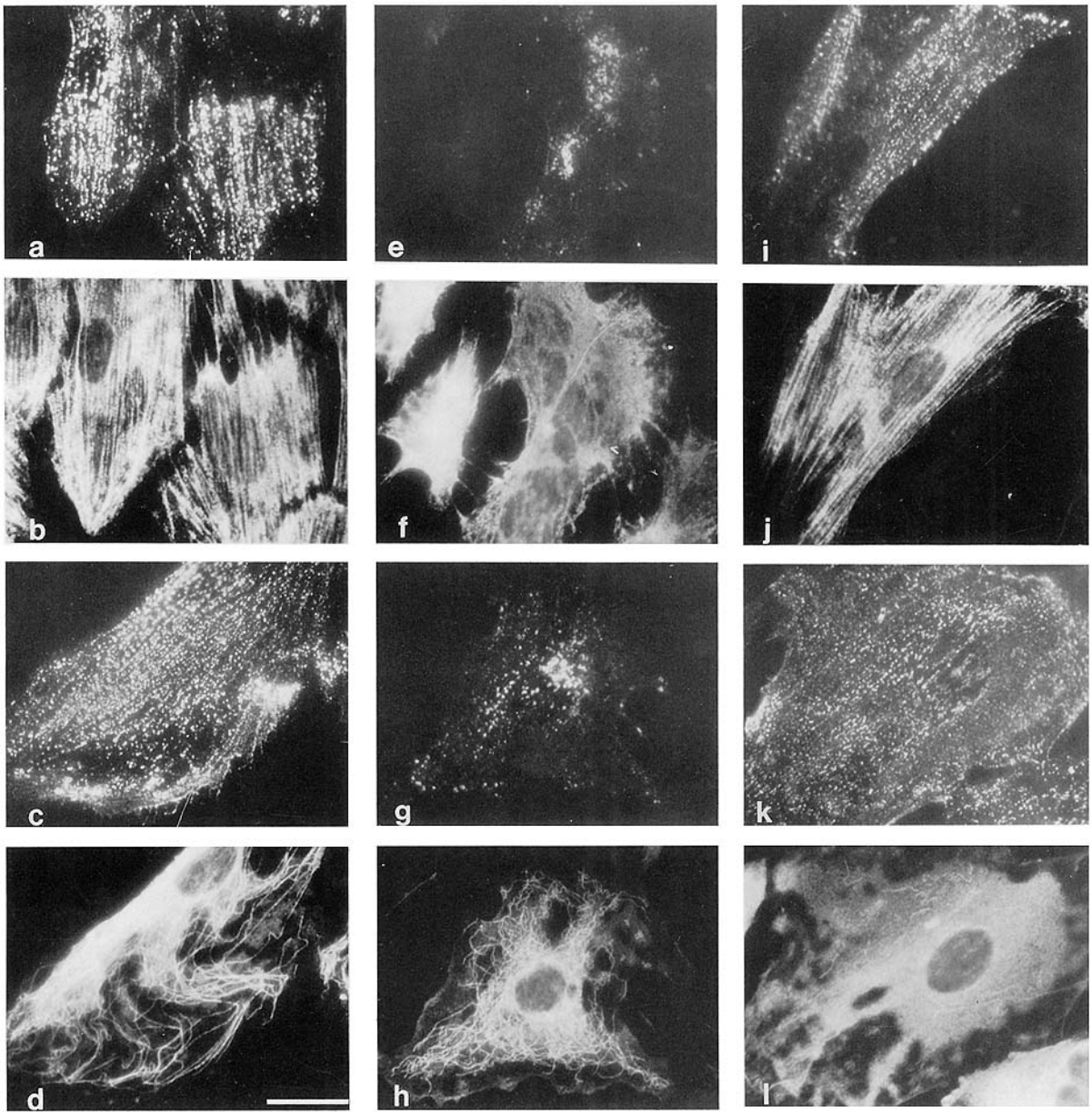

Fig. 3. Effects of cytoskeletal disruption on NG2 distribution. The distribution of NG2 on B111 cells was examined after treatment with 2 x 10^{-6}M cytochalasin D or 2 x 10^{-5}M colchicine for 30 min at 37°C. B111 cells were grown on poly L-lysine coated dishes. **a,b**: NG2 and myosin in untreated cells. **e,f**: NG2 and myosin after cytochalasin D treatment. **i,j**: NG2 and myosin after colchicine treatment. **c,d**: NG2 and tubulin in untreated cells. **g,h**: NG2 and tubulin after cytochalasin treatment. **k,l**: NG2 and tubulin after colchicine treatment. Treatment of cells with colchicine causes depolymerization of microtubules but has no effect on NG2 distribution. In contrast, cytochalasin D treatment causes depolymerization of microfilaments and results in disruption of cell surface NG2 organization. Bar in d = 10 μm.

produce a general decrease in the detergent solubility of components other than NG2, since CD44, type VI collagen, and laminin can be extracted with NP40 in normal fashion from antibody-treated cells (not shown). These results are consistent with the idea that treatment with the N3/D4/D31 cocktail may induce a conformational change in NG2 that results in a tighter association of the proteoglycan with the cytoskeleton.

Cell Surface Distribution of Transfected NG2

To determine whether NG2-positive cells have a specialized cytoskeleton that allows anchorage of the proteoglycan to stress fibers, we used cDNA transfection to introduce NG2 into a cell

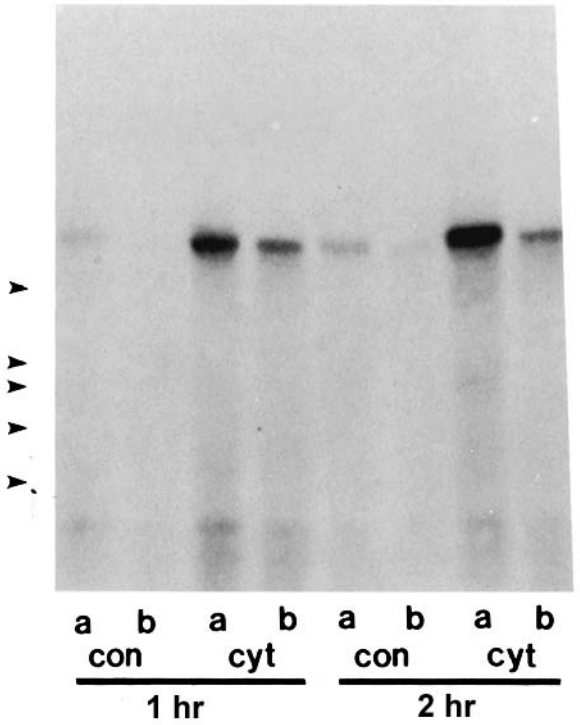

Fig. 4. Cytochalasin D-induced release of NG2. B49 cells were surface-labelled with ^{125}I and returned to culture medium for 1 or 2 h at either 37°C (**a**) or room temperature (**b**). Some cells were treated with 2 x 10^{-6}M cytochalasin D (cyt) while others were untreated (con). NG2 was immunoprecipitated from culture supernatant, stripped of chondroitin sulfate chains, and analyzed by SDS-PAGE. Substantially more NG2 is released from cytochalasin D-treated cells than from control cells. Arrows at left show positions of molecular weight markers at 200, 116, 92, 68, and 45 kDa.

line (B28) that is normally negative for NG2 [see also Nishiyama and Stallcup, 1993]. In these transfectants NG2 is once again found to be organized into linear cell surface arrays that are co-localized with cytoskeletal stress fibers (Fig. 6a,b). Thus NG2 anchorage to the cytoskeleton apparently occurs via interaction with a molecule or molecules common to both NG2-positive and negative cells. This NG2 localization pattern in B28 cells can be disrupted by treatment with cytochalasin D in the same manner that we previously observed with cell lines that express NG2 naturally (not shown).

In order to further investigate the nature of the interaction of NG2 anchorage to stress fibers, we also used B28 cells to compare the cell surface localization of three other cell surface molecules that have been reported to interact with the actin cytoskeleton. Two of these molecules, the $\alpha_5\beta_1$ integrin and the CD44 proteoglycan, are endogenously expressed by B28 cells, thus allowing us to compare NG2 distribution with that of naturally occurring cell surface molecules. The third molecule, the L1 neuronal cell adhesion molecule, is introduced into B28 cells by transfection, so that we can compare NG2 distribution with that of another transfected molecule. Figure 6c–f shows that $\alpha_5\beta_1$ and CD44 are organized on the B28 cell surface in much the same manner as NG2. Similarly, L1 is also present in linear arrays that co-localize with cytoskeletal stress fibers (Fig. 6g,h). These finding are consistent with the idea that each of these four molecules is anchored in the cytoskeleton via interactions with actin-containing stress fibers.

Interestingly, the cytoplasmic domains of these four molecules have no readily apparent similarities, raising the possibility that their respective interactions with the actin cytoskeleton may occur via different mechanisms and/or different linker molecules. Consistent with this hypothesis, we have been able to demonstrate differences in the cell surface distribution of these molecules on B28 cells that have been treated with colchicine. B28 cells are somewhat more susceptible to morphological changes in response to colchicine treatment than were the B111 cells examined in Figure 3. Disruption of microtubules in B28 cells with 2 x 10^{-5}M colchicine causes them to begin to retract cytoplasm and lose their flattened morphology. As shown in Figure 7a, these cells nevertheless retain colchicine-resistant tendrils that are positive for NG2. As noted earlier, cells that are fixed with 2% paraformaldehyde prior to immunostaining also exhibit this pattern of NG2 localization. NG2-positive tendrils appear to contain remnants of the actin cytoskeleton since they can be stained with phalloidin (Fig. 7b) and with actin antibodies (not shown). However, unlike the stress fibers in untreated cells, the tendrils are negative for myosin, which is restricted to the cell soma (Fig. 7c,d). Thus the NG2-positive, phalloidin-positive tendrils seem to represent a subdomain of the actin cytoskeleton which does not bind myosin. Figure 7e and f shows that the $\alpha_5\beta_1$ integrin is also well-represented on these tendrils and is distributed in a manner similar to that seen with NG2. In contrast, neither CD44 nor L1 is present on the $\alpha_5\beta_1$-positive tendrils. CD44 and L1 both are restricted to the soma of colchicine-treated B28 cells (Fig. 7g–j). Thus we can define two subclasses of cell surface

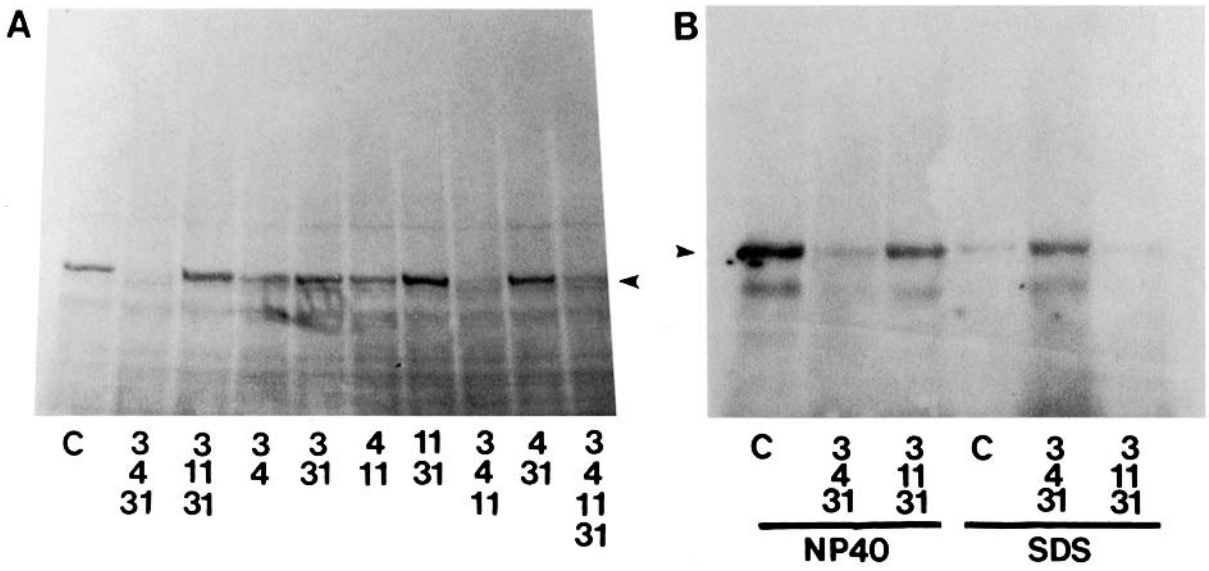

Fig. 5. Antibody-induced detergent resistance of NG2. **A**: B49 cells were treated for 3 h with various combinations of the anti-NG2 monoclonal antibodies N3, N11, D4, and D31. Control cells (C) were not treated with antibodies. Following the antibody incubation, cells were extracted with 1% NP40, and the NG2 content of the extracts was analyzed by inmunoblotting with rabbit anti-NG2. Substantially smaller amounts of NG2 were extracted from cells treated with the N3/D4/D31, N3,D4,N11, and N3/D4/N11/D31 cocktails than from cells treated with other combinations. **B**: B49 cells were treated for 3 h with antibody cocktails N3/D4/D31 and N3/N11/D31. Control cells (C) were not treated. Cells were extracted with 1% NP40. Insoluble pellets from these extractions were re-extracted with SDS-PAGE sample buffer containing 3% SDS and 5% 2-mercaptoethanol. Both NP40 extracts (NP40) and SDS re-extracts (SDS) were analyzed for NG2 content by immunoblotting with rabbit anti-NG2. A substantial amount of NG2 can be extracted by SDS from NP40-insoluble material derived from N3/D4/D31-treated cells. In contrast SDS extracts little additional NG2 from NP40-insoluble pellets derived from control cells or cells treated with the N3/N11/D31 cocktail. Arrowheads in A and B mark position of the 300 kDa NG2 core protein.

molecules that appear to interact differentially with the actin cytoskeleton.

In order to investigate the role of the cytoplasmic domain in determining the mode of interaction of cell surface molecules with the actin cytoskeleton, two chimeric cDNA's were constructed. The chimeric proteins encoded by these cDNA constructs link the extracellular domain of L1 to the transmembrane/cytoplasmic portion of NG2 ($L1_E/NG2_C$), and conversely, the extracellular domain of NG2 to the transmembrane/cytoplasmic portion of L1 ($NG2_E/L1_C$). Not surprisingly, when expressed in B28 cells, both of these chimeric molecules are organized on the cell surface in linear arrays that are coincident with cytoskeletal stress fibers (Fig. 8a–d). However, after colchicine treatment the distribution of the two chimeras becomes distinguishable. The $L1_E/NG2_C$ chimera, like full-length NG2, is able to associate with the $\alpha_5\beta_1$-positive tendrils, while the $NG2_E/L1_C$ chimera, like full-length L1, is restricted to the cell soma (Fig. 8e–h). These results indicate that the cytoplasmic domains of NG2 and L1 are primarily responsible for determining the disparate ability of the two molecules to associate with the cytoskeletal subdomain represented by the tendrils.

DISCUSSION

The fact that the NG2 chondroitin sulfate proteoglycan is a transmembrane molecule with both an extracellular and an intracellular domain suggests that the proteoglycan might be capable of interacting with both extracellular and intracellular components. Whereas several extracellular binding partners for NG2 have been identified, elucidation of cytoplasmic interactions involving NG2 has proved to be more difficult. Our current results support the idea that NG2 utilizes microfilamentous stress fibers as a means of anchorage to the cytoskeleton. On the surface of cells with a very flattened morphology, NG2 is expressed in highly ordered linear arrays that are well co-localized with cytoskeletal stress fibers. In contrast this organized expression pattern of NG2 bears no resemblance to the arrangement of other major cytoskeletal structures such as microtubules and intermedi-

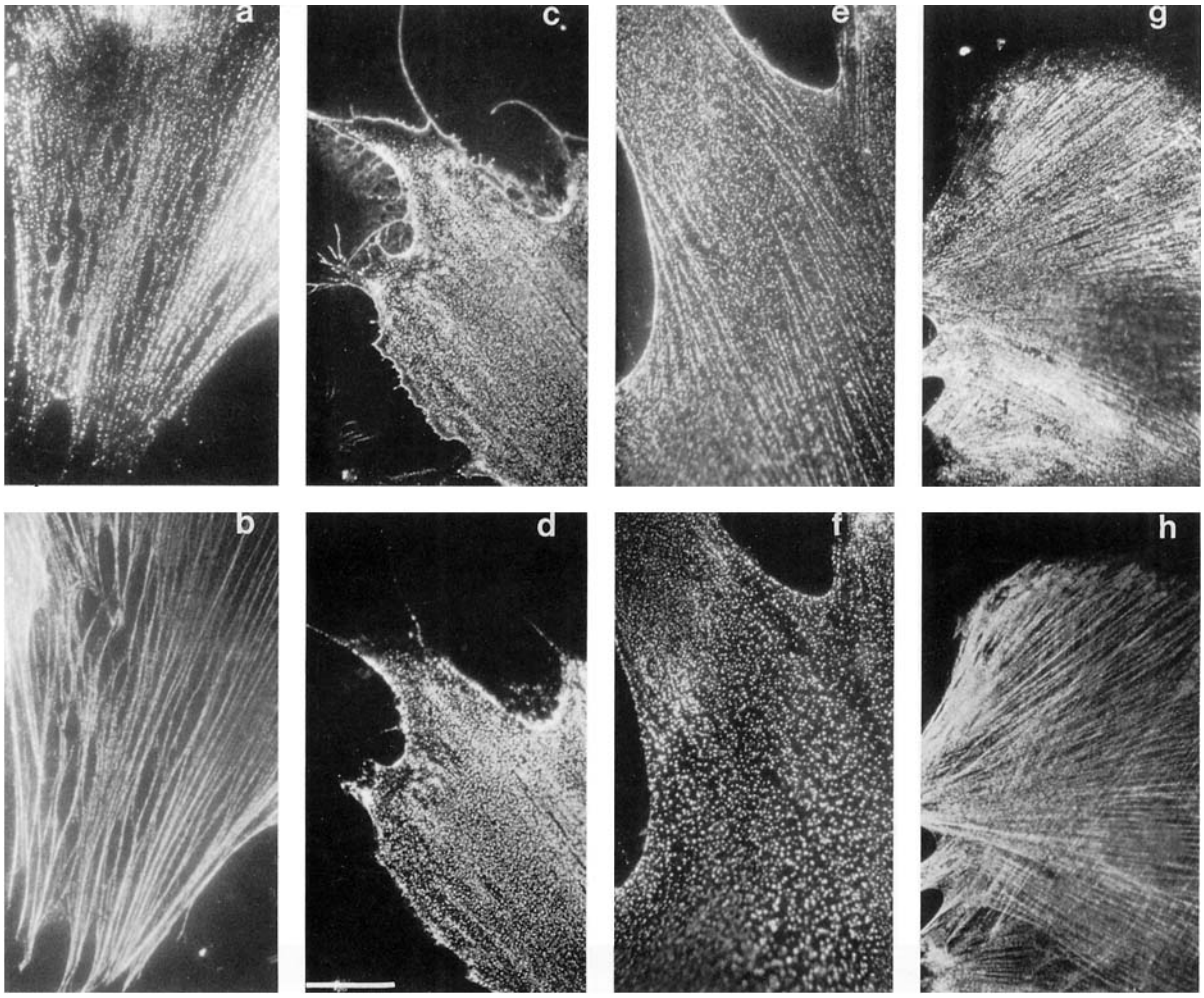

Fig. 6. Comparison of NG2 distribution with distribution of other cell surface molecules. B28 cells on poly L-lysine coated dishes were used to compare the cell surface distribution of NG2 with that of $\alpha_5\beta_1$ integrin, CD44, and L1. **a,b**: NG2 and myosin. NG2-transfected B28 cells. **c,d**: NG2 and $\alpha_5\beta_1$ integrin. NG2 transfected B28 cells. **e,f**: NG2 and CD44. NG2-transfected B28 cells. **g,h**: L1 and myosin. L1-transfected B28 cells. All four cell surface molecules have very similar patterns of distribution. Bar in d = 10 μm.

ate filaments. The possibility that NG2 might use the stress fibers as a means of cytoskeletal anchorage is further indicated by the ability of cytochalasin D, which interferes with actin polymerization, to disrupt the organization of both stress fibers and NG2. During cytochalasin treatment, NG2 not only aggregates into clusters on the cell surface but also is released from the cell surface at a much faster rate than from control cells. The soluble NG2 released into the tissue culture supernatant during cytochalasin treatment appears identical in nature to NG2 that is released under control conditions. This soluble form of the molecule is proteolytically truncated such that it lacks the transmembrane and cytoplasmic domains [Nishiyama et al., 1995]. One interpretation of the cytochalasin data is that the loss of cytoskeletal anchorage induces a conformational change in the NG2 polypeptide that is transmitted to the ectodomain, making it more susceptible to the cell surface proteolytic event that generates the soluble, released form of the molecule.

Additional evidence for conformational changes that stem from or influence the NG2 cytoskeletal interaction comes from observations on the effects of monoclonal antibodies on the detergent extractability of NG2. NG2 is normally quite amenable to extraction from the cell surface with 1% NP40. However, treatment with certain combinations of anti-NG2 monoclonal antibodies renders the proteoglycan relatively

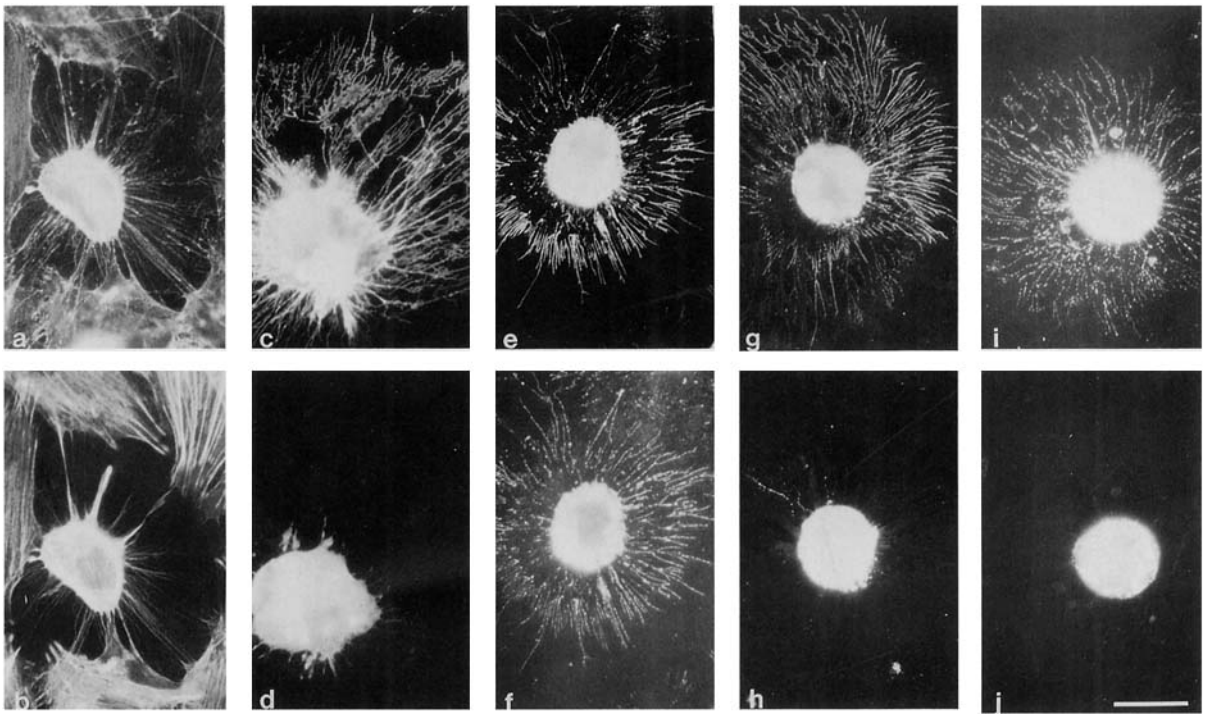

Fig. 7. Cytoskeletal remnants in colchicine-treated B28 cells. B28 cells on poly L-lysine coated dishes were treated with 2 x 10^{-5}M colchicine for 20 min at 37°C and then analyzed for localization of the indicated molecules. **a,b**: NG2 and actin (phalloidin staining). NG2-transfected B28 cells. **c,d**: NG2 and myosin. NG2-transfected B28 cells. **e,f**: NG2 and $\alpha_5\beta_1$ integrin. NG2-transfected B28 cells. **g,h**: NG2 and CD44. NG2-transfected B28 cells. **i,j**: $\alpha_5\beta_1$ integrin and L1. L1-transfected B28 cells. NG2 and $\alpha_5\beta_1$ integrin are present on actin-containing tendrils, while myosin, CD44, and L1 are absent from these cytoskeletal remnants. Bar in j = 10 μm.

resistant to NP40 extraction. In these cases SDS is required to achieve quantitative extraction of the proteoglycan. Since extraction of other cell surface (CD44) and extracellular matrix (laminin, type VI collagen) molecules appears to be unaffected by the monoclonal antibody treatment, we interpret the detergent resistance of NG2 to antibody-induced conformational changes in the proteoglycan which are transmitted to the cytoplasmic domain and enhance interaction with the cytoskeleton. Since a combination of at least three monoclonal antibodies seems to be required to produce this effect (even pairs of antibodies do not suffice), we conclude that multiple determinants must be involved in triggering the conformational change. All of the epitopes recognized by the N3/D4/D31 and N3/D4/N11 monoclonal cocktails are located in the amino terminal globular region of the NG2 core protein. It will be of considerable interest to determine if any of the proposed extracellular ligands for NG2 , either soluble [PDGF-AA: Grako and Stallcup, 1995; Nishiyama et al., 1996a, b], cell surface [PDGF α receptor: Grako and Stallcup, 1995; Nishiyama et al., 1996a, b], or matrix [type VI collagen, laminin, tenascin: Burg et al., 1996], are capable of inducing similar conformational changes in the proteoglycan. This would be strong evidence that NG2 is capable of mediating signalling between the extracellular environment and the cytoskeleton. Thus, changes in a cell's extracellular environment could lead to NG2-mediated changes in the morphological or motile properties of the cell. Conversely, changes in cytoskeletal architecture could lead to NG2-mediated changes in the cell's ability to interact with the local extracellular milieu.

The characteristic cell surface organization of NG2 is seen both in cells that endogenously express the molecule and in cells such as B28 which are normally NG2-negative, but express NG2 by virtue of cDNA transfection. This suggests that NG2 anchorage in the cytoskeleton depends on a molecule or molecules that are common to both NG2-positive and negative cells. We have attempted to gain further insight into the mechanism of NG2 anchorage by comparing

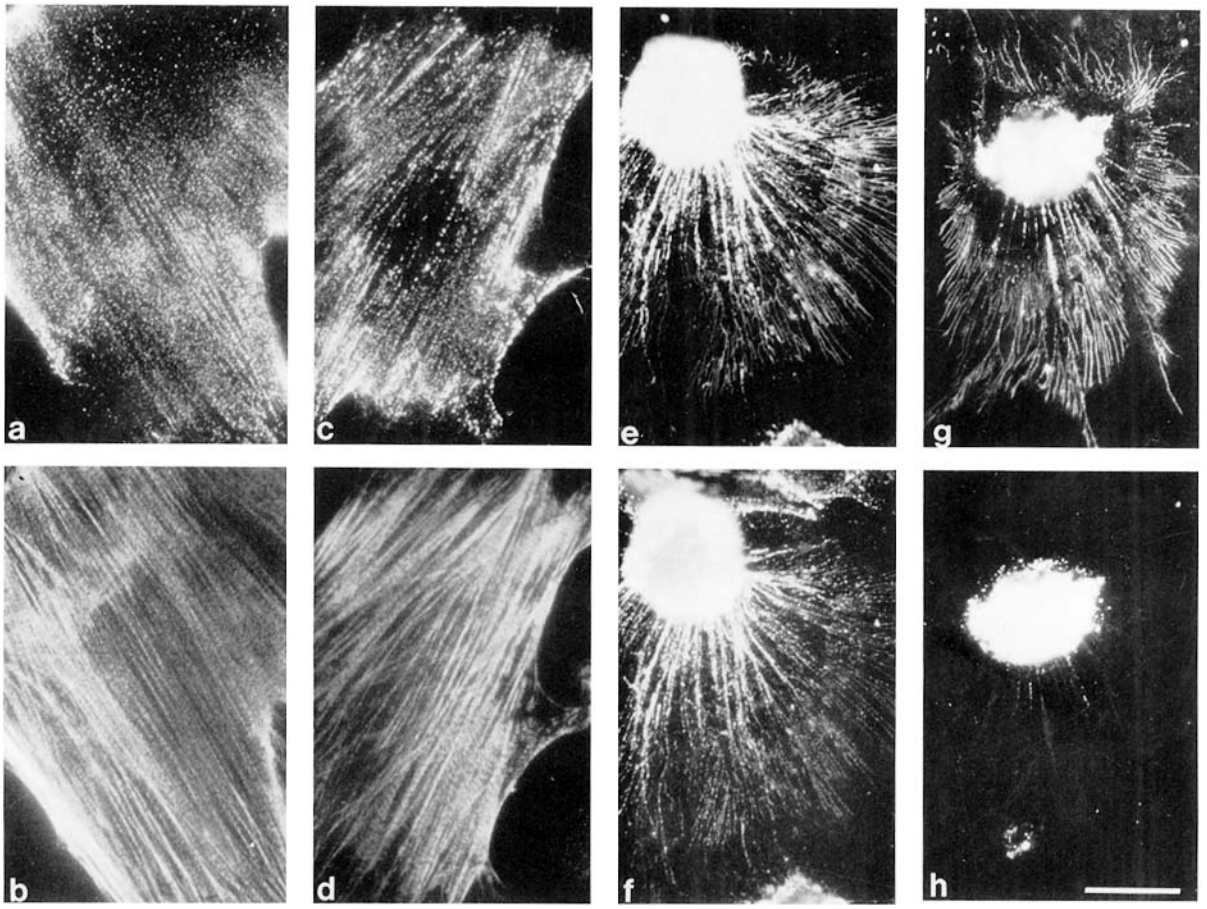

Fig. 8. Cytoskeletal association of chimeric molecules. The chimeric molecules $L1_E/NG2_C$ and $NG2_E/L1_C$ were compared for their ability to associate with the actin cytoskeleton in control and colchicine-treated B28 cells. **a,b**: L1.1 and myosin staining in $L1_E/NG2_C$ transfected cells. **c,d**: NG2 and myosin staining in $NG2_E/L1_C$ transfected cells. **e,f**: $\alpha_5\beta_1$ and L1.1 staining in $L1_E/NG2_C$ transfected cells treated with colchicine. **g,h**: $\alpha_5\beta_1$ and NG2 staining in $NG2_E/L1_C$ transfected cells treated with colchicine. Both chimeras associate normally with the cytoskeleton in untreated B28 cells. However, only $L1_E/NG2_C$ is present on colchicine-resistant tendrils. Bar in h = 10 μm.

NG2 to other molecules that are thought to utilize actin-containing microfilaments as a means of cytoskeletal anchorage. The $\alpha_5\beta_1$ integrin is the classical cell surface fibronectin receptor known to be responsible for cellular attachment to the RGD-containing cell binding site of fibronectin [Argraves et al., 1987]. CD44, which like NG2 is a membrane-spanning proteoglycan, has been identified as a cell surface receptor involved in lymphocyte homing and other adhesion phenomena [Brown et al., 1991; Faasen et al., 1992]. The neuronal cell adhesion molecule L1 is a member of the family of cell adhesion molecules that contain both immunoglobulin-like loops and fibronectin type III repeats [Moos et al., 1987; Hlavin and Lemmon, 1991]. $\alpha_5\beta_1$ integrin and CD44 are normally expressed by B28 cells, while L1 is introduced by transfection in the same manner as NG2. The cell surface organization of all four molecules is strikingly similar on untreated B28 cells. All seem to be present on the cell surface in linear arrays that coincide with the arrangement of stress fibers in the cytoskeleton.

Differences in the distribution of these molecules can be detected, however, when B28 cells are treated with colchicine to disrupt microtubule structure. The loss of microtubule integrity causes B28 cells to lose their flattened morphology and retract most of their cytoplasm into a rounded cell body or soma. These colchicine-treated cells nevertheless retain a portion of the organized actin cytoskeleton in the form of delicate tendrils projecting from the central soma. In contrast to the stress fibers seen in untreated cells, myosin is not associated with these actin-containing tendrils; myosin is present only within the cell soma. Thus the tendrils appear to

represent only a subdomain of the entire actin cytoskeleton. With regard to cell surface molecules, NG2 and the $\alpha_5\beta_1$ integrin both are found on the surface of the actin-positive tendrils, while CD44 and L1 are restricted to the central soma. This suggests that NG2 and $\alpha_5\beta_1$ integrin can interact with the actin cytoskeleton in a manner that is distinct from the mechanism utilized by CD44 and L1. The available evidence indicates that CD44 and L1 are anchored to the actin cytoskeleton by a different linker than that utilized by integrins. Both CD44 and L1 are reported to interact with ankyrin, which serves as their means of anchorage to actin microfilaments [Kalomiris and Bourguignon, 1988; Davis et al., 1993; Davis and Bennett, 1994]. Integrins, on the other hand, appear to associate with microfilaments via the linker molecules α-actinin and/or talin [Turner and Burridge, 1991; Schwartz, 1992; Clark and Brugge, 1995]. Our results indicate that the mechanism of NG2 anchorage to the actin cytoskeleton may more closely resemble that of integrins than that of molecules such as CD44 and L1 which utilize ankyrin as a linker. Further research will be required to identify the linker molecules involved in NG2 anchorage. It may also be of interest in future work to compare the cytoskeletal interactions of NG2 with those observed for syndecan, often considered to be the prototype for membrane-spanning proteoglycans. The syndecans comprise a family of heparan sulfate proteoglycans which are proposed to interact with the actin cytoskeleton and to influence cell morphology by relaying signals from the extracellular matrix to the cytoskeleton [Rapraeger et al., 1986; Bernfield et al., 1992; Carey et al., 1994]. Although diverse in terms of their extracellular domains, members of the syndecan family share a highly-conserved cytoplasmic domain [Elenius and Jalkanen, 1994], suggesting that various syndecan species may utilize similar mechanisms for cytoskeletal anchorage. It should be possible to compare the distribution of syndecan with that of NG2 as an initial means of evaluating similarities or differences in their modes of cytoskeletal attachment.

It is worthwhile to note that treatment with colchicine is not always required in order to observe differences in the distribution of NG2 and other cell surface molecules on B28 cells. Even in untreated cultures there are always a certain number of B28 cells which have assumed a rounded morphology, either in preparation for cell division or because of differences in the local microenvironment of the substratum. These cells usually retain NG2-positive tendrils that are indistinguishable from those seen in colchicine-treated cells. Processes of this type have previously been referred to in the literature as "retraction fibers" and have been observed in association with cells that are rounding up in preparation for mitosis [Mitchison, 1992; Cramer and Mitchison 1993]. Retraction fibers remain attached to the substratum during mitosis and are thought to provide a framework which allows postmitotic daughter cells to quickly re-acquire a flattened morphology. In addition to their presence on rounded cells, NG2-positive retraction fibers or filopodia are often observed in association with spreading lamellipodia on the edges of flattened B28 cells (see for example Fig. 6c). These NG2-positive projections have the same characteristics seen in colchicine-resistant tendrils: i.e., they are actin-positive, myosin-negative, and are devoid of CD44 and L1. Thus, the association of NG2 with filopodial and lamellipodial projections is a natural phenomenon that may be important in the dynamic processes of cell motility and cell spreading. Indeed, the human homolog of NG2, the melanoma proteoglycan, has been proposed to have a role as a co-receptor in $\alpha_4\beta_1$ integrin-mediated cell spreading on fibronectin [Iida et al., 1993, 1995]. One of our future goals will be to determine whether the cytoskeletal association of NG2 is important for its participation in this particular spreading phenomenon. It is of interest to note in this regard that human NG2 has been shown by electron microscopy to be especially enriched in cell surface microspikes [Garrigues et al., 1986]. Although the relationship between these microspikes and the tendrils/filopodia we have described is not yet established, it seems possible that localization in these types of structures could provide maximal opportunity for NG2 to interact with both cytoskeletal and extracellular components.

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