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NG2 Proteoglycan Mediates β_1 Integrin-Independent Cell Adhesion and Spreading on Collagen VI

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Abstract Collagens V and VI have been previously identified as specific extracellular matrix (ECM) ligands for the NG2 proteoglycan. In order to study the functional consequences of NG2/collagen interactions, we have utilized the GD25 cell line, which does not express the major collagen-binding β_1 integrin heterodimers. Use of these cells has allowed us to study β_1 integrin-independent phenomena that are mediated by binding of NG2 to collagens V and VI. Heterologous expression of NG2 in the GD25 line endows these cells with the capability of attaching to surfaces coated with collagens V and VI. The specificity of this effect is emphasized by the failure of NG2-positive GD25 cells to attach to other collagens or to laminin-1. More importantly, NG2-positive GD25 cells spread extensively on collagen VI. β_1 integrin-independent extension of ruffling lamellipodia demonstrates that engagement of NG2 by the collagen VI substratum triggers signaling events that lead to rearrangement of the actin cytoskeleton. In contrast, even though collagens V and VI each bind to the central segment of the NG2 ectodomain, collagen V engagement of NG2 does not trigger cell spreading. The distinct morphological consequences of NG2/collagen VI and NG2/collagen V interaction indicate that closely-related ECM ligands for NG2 differ in their ability to initiate transmembrane signaling via engagement of the proteoglycan. J. Cell. Biochem. 86: 726–736, 2002. © 2002 Wiley-Liss, Inc.

Key words: proteoglycan; NG2; cell adhesion; cell spreading; collagen V; collagen VI

The ability of cells to recognize and interact with the extracellular matrix (ECM) is fundamentally important for normal development and for continued maintenance of tissue architecture and function throughout adulthood. Interactions between cells and the ECM are mediated by several classes of cell surface molecules, the most prominent being the integrin family of heterodimeric proteins [Hynes, 1992].

The contribution of cell surface proteoglycans in cell/matrix interaction is also now well established. The literature describes a variety of structurally diverse cell surface proteoglycans that fulfill specialized developmental functions [Perrimon and Bernfield, 2000]. These include the syndecan and glypican families of heparan sulfate proteoglycans [Bernfield et al., 1999], as well as cell surface chondroitin sulfate proteoglycans, such as phosphacan and NG2 [Yamaguchi, 2000]. Betaglycan exemplifies a cell surface proteoglycan that contains both heparan sulfate and chondroitin sulfate chains [Lopez-Casillas et al., 1994].

Cell surface proteoglycans are often regarded as co-receptors that are able to modulate the ligand-dependant activation of primary signaling receptors, including receptor tyrosine kinases and cell adhesion molecules of both the integrin family and the Ig superfamily [Carey, 1997]. One of the best examples of a cooperative role for proteoglycans in cell adhesion is the dual involvement of integrins and syndecan-4 in the formation of focal adhesions following cell attachment to fibronectin [Woods and Couchman,

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1998; Woods et al., 1998; Echtermeyer et al., 1999; Longley et al., 1999; Saoncella et al., 1999]. A similar type of cooperation has been described for the NG2 proteoglycan acting in concert with $\alpha_4\beta_1$ integrin to mediate melanoma cell adhesion and spreading on fibronectin [Iida et al., 1995; Eisenmann et al., 1999]. In addition to its co-receptor role, there is evidence to suggest that NG2 may in some cases directly mediate signaling that leads to cell spreading, polarization, and migration in response to engagement by certain substrates [Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001]. The mechanisms underlying these various processes are the subject of active investigation.

As a membrane-spanning molecule, NG2 has the potential to mediate communication between the extracellular and intracellular compartments of the cell. Both extracellular and cytoplasmic binding partners for the proteoglycan have been identified. Inside the cell, there is evidence that NG2 affects remodeling of the actin cytoskeleton by activating rho family GTPases, resulting in cell polarization and increased cell motility [Lin et al., 1996; Eisenmann et al., 1999; Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001; Majumdar et al., 2002]. Binding of the NG2 cytoplasmic domain to PDZ-containing scaffolding proteins, such as MUPP1 [Barritt et al., 2000] may be important for the formation of signaling complexes that control these processes.

External to the cell, several ECM ligands for NG2 have been identified by binding studies using purified proteins [Burg et al., 1996; Tillet et al., 1997]. The best characterized ECM binding partner for NG2 is collagen VI, a microfibrillar collagen that is widespread in connective tissue and vasculature [Timpl and Chu, 1994]. Little is known, however, of the cellular responses induced by engagement of NG2 by ECM components, because the contribution of NG2 is often masked by activation of integrin receptors that interact with these same ECM ligands. Most of the initial studies attempting to evaluate the role of NG2 in cell spreading and migration have, therefore, focused on surfaces coated with specific NG2 antibodies, allowing the analysis of cellular responses that result from direct engagement of the proteoglycan [Iida et al., 1995; Eisenmann et al., 1999; Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001; Majumdar et al., 2002].

In order to gain new insight into the interaction of NG2 with physiological ligands, we have now studied cell adhesion and spreading on ECM substrata using GD25 cells that are deficient in β_1 integrin, a widely expressed β subunit involved in many cell/ECM interactions. Transfection of these cells with NG2 has allowed us to determine that the proteoglycan itself provides a means for cell adhesion to surfaces coated with NG2 mAbs, collagen V, and VI. Moreover, cell attachment to collagen VI-coated surfaces is followed by extensive cell spreading. This finding demonstrates that engagement of NG2 by a physiological ECM component triggers morphological changes that are independent of cell surface β_1 integrins.

MATERIALS AND METHODS

Cell Lines

The GD25 cell line was derived from the embryonic stem cell clone G201 after transformation with the SV40 large T antigen. These cells are deficient in the β_1 integrin subunit due to the introduction of a null mutation in both alleles of the β_1 gene [Fassler et al., 1995]. The stably transfected cell line GD25-β_1A was obtained by electroporating wild-type integrin β_1A cDNA into GD25 cells [Wennerberg et al., 1996]. All cell lines were cultured in DMEM supplemented with 10% fetal calf serum (FCS) (Seromed, Berlin, Germany).

Antibodies

The rabbit antibody NG2/EC was raised against a recombinant fragment of NG2 comprising the whole extracellular domain [Tillet et al., 1997]. The same fragment was used for immunization of mice to obtain monoclonal antibodies (mAbs) following a previously described protocol [Lethias et al., 1993]. These mAbs were purified from ascites fluids on a protein-G Sepharose column. Other NG2 mAbs have been previously described [Nishiyama et al., 1991, 1995].

ECM Proteins

Bovine plasma fibronectin, pepsinized human collagen IV, and collagen V were purchased from Sigma (St. Louis, MO). Acid-soluble collagen I from calf skin was kindly provided by Bioetica (Lyon, France). Mouse EHS laminin was obtained from GIBCO-BRL Life Science

(Grand Island, NY). Pepsin-extracted collagen VI was kindly provided by Dr. R. Timpl, Max-Planck Institute for Biochemistry, Martinsried, Germany [Odermatt et al., 1983].

cDNA Constructs and Transfection

The full-length NG2 cDNA was excised from the pcDNAamp-NG2 plasmid [Tillet et al., 1997] using BamH1 and Not I sites, and subcloned into the eukaryotic expression vector pcDNA3.1hygro (Invitrogen, La Jolla, CA). GD25 and GD25-β_1A cells were transfected by electroporation (960 μF, 250 V) with either the NG2-containing vector or the empty vector. After selection with 300 μg/ml of hygromycin for 3–4 weeks, individual colonies were screened for NG2 expression using immunofluorescence and immunoblotting. Homogeneous NG2-positive populations were then derived by subcloning these cells at limiting dilution in 96-well plates.

Immunofluorescence

NG2 expression was evaluated in living cells by immunofluorescence staining with the NG2/EC polyclonal antibody. Cells were cultured overnight on glass coverslips, washed once with DMEM containing 1% FCS, and incubated for 20 min with the primary antibody. After several washes with DMEM containing 1% FCS, cells were incubated for 10 min with a secondary antibody coupled to either cyanine 2 or 3 (Jackson ImmunoResearch Laboratories, West Grove, PA). After additional washes with PBS, specimens were fixed with 95% ethanol, air-dried, and coverslipped in PBS–glycerol. They were examined using a Zeiss Axioplan epifluorescence microscope.

Gel Electrophoresis and Immunoblotting

Cell monolayers were harvested by scraping, washed in PBS, pelleted by centrifugation, and then solubilized for 10 min on ice in a solution of 50-mM Tris-HCl, pH 7.4 containing 1% Nonidet P-40 (NP-40), 1-mM phenylmethylsulfonyl fluoride (PMSF), and 2-mM *N*-ethylmaleimide. After removal of insoluble material by centrifugation, half of the supernatant was treated for 1 h at 37°C with 0.01 U of chondroitinase ABC (Seikagaku, Tokyo, Japan). After addition of SDS–PAGE sample buffer, extracts were fractionated on 3–12% acrylamide gradient gels, and then transferred to Immobilon P membranes (Millipore Corporation, Bedford, MA). Membranes were blocked in TBS (50-mM Tris pH 7.4, 150-mM NaCl) containing 3% BSA, and probed with the appropriate antibody. Immunoreactive bands were visualized with the Renaissance chemiluminescence kit (NEN Life Science Products, Boston, MA).

Cell Adhesion Assay

Ninety-six-well plates (Maxisorp, Nunc, Germany) were coated overnight at 4°C with various concentrations of ECM proteins, and then blocked with PBS containing 1% BSA for 2 hr. Cells were harvested using enzyme-free cell dissociation buffer (GIBCO-BRL Life Science), washed with DMEM in the absence of serum, and plated in DMEM + 1% BSA at a density of 60,000 cells/well. Attachment was allowed to proceed for 45 min at 37°C. Non-adherent cells were removed by floatation on a 1.10 g/L Percoll solution according to the method of Goodwin and Pauli [1995]. The remaining adherent cells were fixed with glutaraldehyde and stained for 30 min with 0.1% crystal violet in water. Wells were extensively washed with water, and fixed cells were lyzed with 0.2% Triton X-100 for 2 h. The absorbance in each well was then measured at 570 nm with a microtiter plate reader (Dynex MRX). Each assay point was determined in triplicate.

Cell Spreading Assay

Glass cover slips were coated overnight at 4°C with either poly-L-lysine (50 μg/ml), affinity purified mouse immunoglobulins (10 μg/ml), NG2 mAbs (10 μg/ml), or ECM proteins (fibronectin, laminin, collagens I, IV, V, and VI, 20 μg/ml). Before use, the cover slips were blocked for 2 h with a solution of 1% BSA in PBS.

Cells were washed twice with PBS and harvested by treatment for 5 min at room temperature with enzyme-free cell dissociation buffer. They were then washed with DMEM and plated on the treated coverslips in DMEM + 1% BSA at a density of 1,00,000 cells/cm^2. Cells were incubated at 37°C for 30–45 min and non-adherent cells were removed by several washings with PBS. Adherent cells were fixed with 2.5% paraformaldehyde in PBS and either observed with a phase contrast microscope or stained for filamentous actin. In the latter case, cells were permeabilized with 0.1% Triton in PBS for 10 min, incubated for 45 min with

rhodamine–phalloidin, washed with PBS, and coverslipped in PBS-glycerol.

RESULTS

Expression of NG2 in GD25 Cells

Both the β_1 integrin-deficient GD25 cells and the GD25-β_1A cells were transfected with a plasmid coding for the full length rat NG2 (pcDNAhygro/NG2) or with the same plasmid without the NG2 insert (mock-transfection). The NG2-transfected cell lines were designated GD25/NG2 and GD25-β_1A/NG2, respectively. Immunofluorescence shows that NG2 is expressed in these cells at high levels and with the correct cell surface localization, as judged by the punctate staining obtained on the surface of unpermeabilized cells with anti-NG2 antibody (Fig. 1b,d). The mock-transfected cells (Fig. 1a,c) are negative for the proteoglycan, illustrating the absence of endogenous NG2 on GD25 cells. Western blot analysis of transfectants (Fig. 2) reveals the presence of NG2 in extracts from the transfected cells, but not from the mock-transfected cells. Most of this expressed NG2 is substituted with chondroitin sulfate, since chondroitinase ABC treatment produces a sharp 300-kDa band, which is not easily seen prior to chondroitinase digestion. The size of this band is identical to that seen in the fibroblast cell line rat-1, which is known to express high endogenous levels of NG2 [Fang et al., 1999]. These results indicate that NG2-transfected GD25 and GD25-β_1A cells express NG2 with correct post-translational modifications.

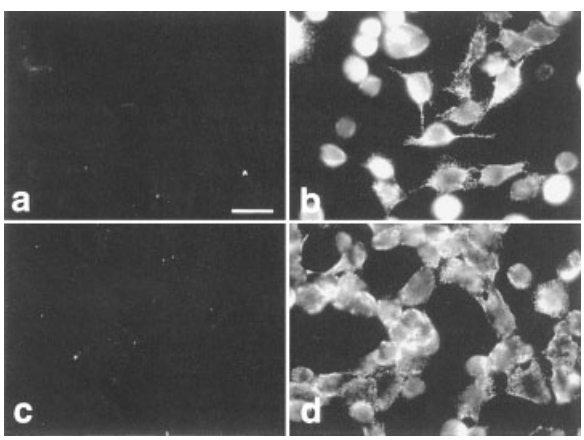

Fig. 1. Immunofluorescence labeling of NG2 in transfected cells. Immunofluorescence was performed on living cells with the polyclonal anti-NG2 antibody NG2/EC (**a**) mock-transfected GD25 cells; (**b**) GD25/NG2 cells; (**c**) mock-transfected GD25-β_1A cells; (**d**) GD25-β_1A/NG2 cells. Bar, 25 μm.

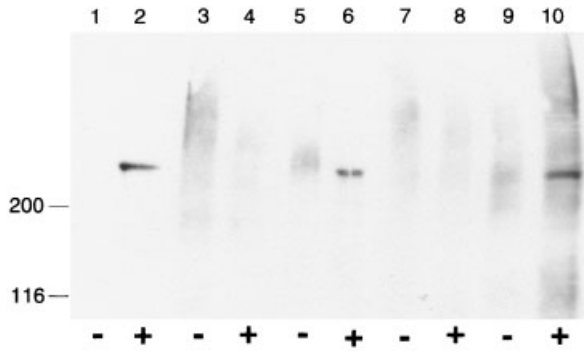

Fig. 2. Western blot analysis of NG2 expression by transfected cell lines. NP-40 cell extracts were digested (+) or not (–) with chondroitinase ABC. Samples were fractionated on a 3–12% gradient polyacrylamide gel, blotted and probed with the NG2EC antibody. **Lanes 1–2**: control cell line rat-1; **lanes 3–4**: mock-transfected GD25 cells; **lanes 5–6**: GD25/NG2 cells; **lanes 7–8**: mock-transfected GD25-β_1A cells; **lanes 9–10**: GD25-β_1A/NG2 cells. Molecular mass standards are indicated (left) in kDa.

Adhesion and Spreading of NG2-Transfected GD25 Cells on NG2 mAbs

It has been previously shown that engagement of NG2 by surfaces coated with NG2 mAbs can initiate cytoskeletal rearrangement and cell spreading [Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001]. We performed similar experiments with the GD25/NG2 and GD25-β_1A/NG2 cell lines, testing the ability of these cells to adhere and spread on several NG2 mAbs, including D4, D120 [Fang et al., 1999], and a newly produced mAb 2B3 that is able to promote spreading of NG2-transfected lymphoblastoma cells (unpublished communications). As expected, when cells are plated on poly-L-lysine, they attach and spread (Fig. 3a,b); when the same cells are plated on NG2 mAbs, they rapidly adhere, but do not spread. Even after 1 h on these mAb substrata, the cells retain a round morphology and phase-bright appearance as judged by phase contrast microscopy (Fig. 3c,d). We performed phalloidin staining in order to detect any fine, actin-positive cellular extensions that might be missed by phase microscopy. This phalloidin staining confirms the absence of actin fibers beyond the periphery of the cells (Fig. 3e,f). In control experiments, mock-transfected GD25 cells failed to attach to the mAb coated surfaces, confirming that the presence of NG2 is responsible for cell attachment

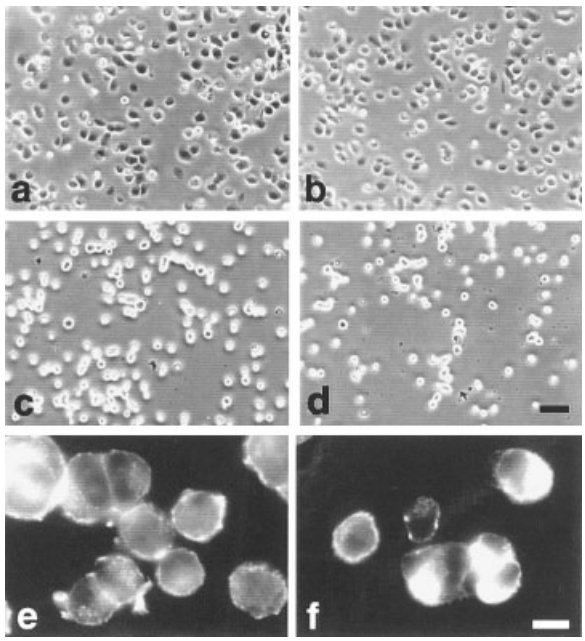

Fig. 3. Adhesion of NG2-transfected GD25 cells to mAb-coated surfaces. Glass cover slips were coated with either poly-L-lysine (50 μg/ml) (**a** and **b**) or the 2B3 monoclonal antibody against NG2 (10 μg/ml) (**c–f**). GD25/NG2 cells (a, c, and e) or GD25-β_1A/NG2 cells (b, d, and f) were plated on the treated coverslips and either observed with a phase contrast microscope (a, b, c, and d) or stained with rhodamine–phalloidin (e and f). Bar 50 μm (a–d), 10 μm (e and f).

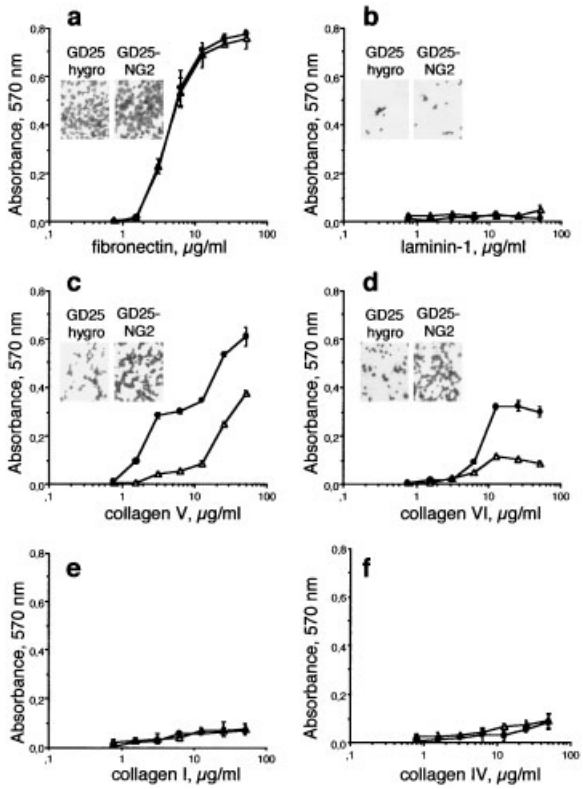

Fig. 4. Attachment of GD25 transfectants to ECM proteins. Mock-transfected GD25 cells (open triangles) or GD25/NG2 cells (●) were tested for their ability to adhere to wells coated with fibronectin (**a**), laminin-1 (**b**), collagen V (**c**), collagen VI (**d**), collagen I (**e**), and collagen IV (**f**). The insets show the stained cells prior to determination of the absorbance (for a substrate concentration of 20 μg/ml). GD25 and GD25/NG2 cells adhere equally well to fibronectin, but neither line attaches to laminin-1, collagen I, or IV. Expression of NG2 improves cell attachment to both collagen V and VI.

(not shown). These results demonstrate that although NG2 can promote attachment of GD25 cells, the presence of the proteoglycan on the cell surface is not sufficient to induce cell spreading on NG2 mAb-coated surfaces, even with cells expressing the β_1 integrin subunit.

Adhesion of NG2-Transfected GD25 Cells to ECM Ligands

Since, NG2 has previously been shown to bind to several ECM components, we next analyzed the ability of the proteoglycan to promote cell attachment to ECM ligands. Most cell types adhere to ECM-coated surfaces via integrin receptors. The behavior of β_1 integrin-negative, NG2-positive GD25 cells should, therefore, be very instructive as to the role of NG2 in cell attachment.

We find that NG2-negative, β_1 integrin-negative GD25 cells adhere well to fibronectin (Fig. 4a), possibly through engagement of the $\alpha_v\beta_3$ integrin [Wennerberg et al., 1996]. Not surprisingly, expression of NG2 does not improve this cell attachment to fibronectin. Both GD25 and GD25/NG2 cells fail to adhere to EHS laminin (Fig. 4b), which contains primarily the laminin-1 isoform. Although previous work indicated that NG2 might interact with laminin-1 in solid-phase binding assays [Burg et al., 1996], this new cell adhesion result suggests that the laminin-1 isoform does not serve as a functional NG2 ligand.

On collagens V and VI, the parental GD25 cells adhere poorly, and only at high concentrations of substrate. This level of adhesion is nevertheless higher than that seen for laminin, suggesting that it may represent a functional interaction. As with fibronectin, the $\alpha_v\beta_3$ integrin is a candidate for interaction with collagen VI [Pfaff et al., 1993], but the adhesion of GD25 cells to collagens V and VI may also involve receptors that do not belong to the integrin family. For example, we have found that GD25 cells are positive for CD44, which has been shown to be a

cell surface receptor for collagen VI [Gallatin et al., 1989]. It is also noteworthy that collagen V contains a heparin binding site in its triple helical domain that might be recognized by heparan sulfate proteoglycans of the syndecan family [Delacoux et al., 1998]. In contrast to this low level of parental cell adhesion, the expression of NG2 markedly enhances GD25 cell adhesion to both collagen substrates (Fig. 4c,d). This same result was obtained with two independent clones of GD25/NG2 cells. The inset micrographs of crystal violet-stained cells after adhesion for 45 min highlight the difference between NG2 positive and negative cells on the collagen V and VI substrates. In contrast, only background levels of adhesion are observed with either GD25 or GD25/NG2 cells on other collagens such as collagen I or IV (Fig. 4e,f), emphasizing the specificity of the NG2-mediated cell adhesion on collagens V and VI.

Expression of the β_1A integrin subunit by GD25 cells restores adhesion to laminin-1, collagen V, and VI (Fig. 5). In the presence of the β_1 integrin, we can no longer distinguish between the behavior of NG2-positive and -negative GD25 cells on surfaces coated with collagens V and VI (Fig. 5c,d). This masking effect of β_1A emphasizes the importance of our ability to study the contribution of NG2 to cell adhesion in the absence of β_1 integrins.

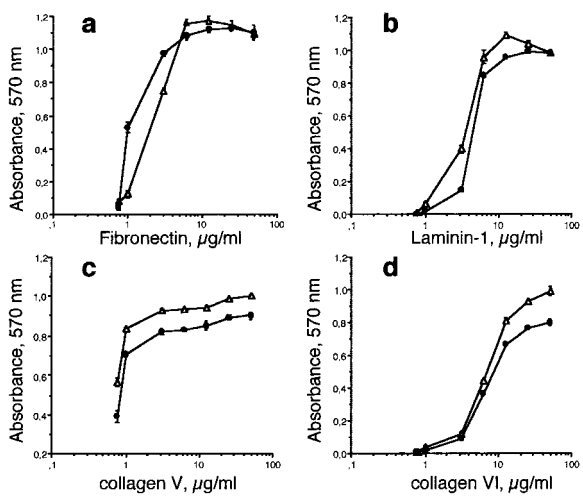

Fig. 5. Attachment of GD25-β_1A cells to ECM proteins. Mock-transfected GD25-β_1A cells (open triangles) or GD25-β_1A/NG2 cells (●) were tested for their ability to adhere to fibronectin (**a**), laminin-1 (**b**), collagen V (**c**), and collagen VI (**d**). Expression of β_1A promotes rapid attachment of GD25 cells to all four substrata. Against this background, the expression of NG2 has no additional effect.

Spreading of NG2-Transfected GD25 Cells on ECM Ligands

We next analyzed the ability of NG2 to mediate cell spreading on the same set of ECM proteins. For these experiments, we used phalloidin staining to compare the organization of the actin cytoskeleton in GD25 and GD25/NG2 cells (Fig. 6). Both cell lines spread on poly-L-lysine (Fig. 6a,b) and on fibronectin (Fig. 6c,d). The formation of stress fibers can be readily observed on the latter substratum. Although NG2-positive cells adhere much more efficiently than NG2-negative cells to the collagen V substratum, both cell types fail to spread on this surface, and are indistinguishable in terms of their diffuse cytoskeletal architecture (Fig. 6e,f). However, striking differences are observed between GD25 and GD25/NG2 cells plated on collagen VI. NG2-negative GD25 cells exhibit

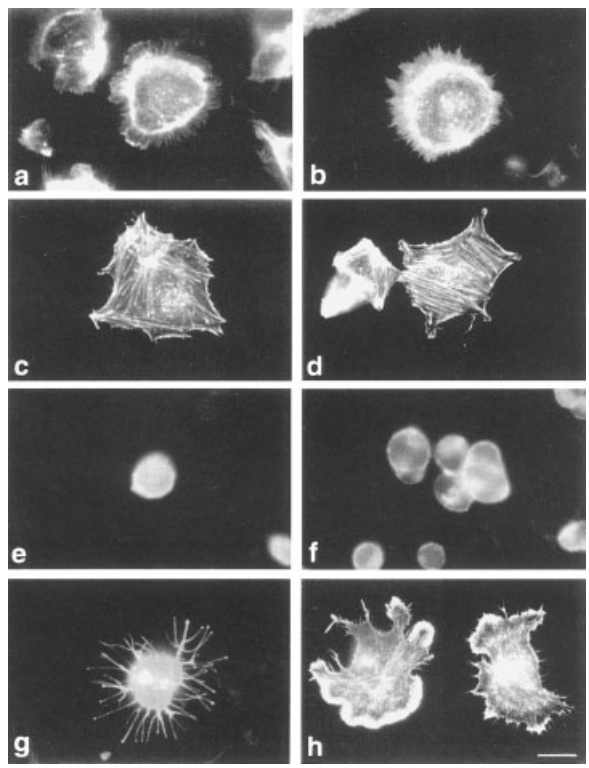

Fig. 6. Spreading of GD25/NG2 cells on ECM proteins. Glass coverslips were coated with polylysine (**a** and **b**) or 20 μg/ml of the following ECM proteins: fibronectin (**c** and **d**), collagen V (**e** and **f**) or collagen VI (**g** and **h**). Adhesion of mock-transfected GD25 cells (a, c, e, and g) or GD25/NG2 cells (b, d, f, and h) was monitored by staining with rhodamine–phalloidin. Bar, 20 μm. Both NG2-positive and -negative GD25 cells will spread on PLL and fibronectin, while only the GD25/NG2 cells spread effectively on collagen VI. Neither cell type spreads on collagen V.

minimal spreading on collagen VI, extending only fine actin-containing protrusions on this substratum (Fig. 6g). In contrast, GD25/NG2 cells on collagen VI develop a fully spread morphology with extensively ruffled actin-positive lamellipodia (Fig. 6h). In cells transfected with β_1A integrin, it was not possible to observe this NG2-mediated enhancement of cell spreading. Indeed, mock-transfected GD25-β_1A and GD25-β_1A/NG2 are indistinguishable in their abilities to spread and extend lamellipodia when plated on collagen VI (data not shown).

DISCUSSION

The NG2 proteoglycan is expressed by immature progenitor cells in several developmental lineages, including oligodendrocyte progenitors, chondroblasts, and pericytes/smooth muscle cells [Nishiyama et al., 1991, 1996; Grako and Stallcup, 1995; Grako et al., 1999; Ozerdem et al., 2001, 2002]. Although, NG2 expression is usually downregulated, when these cells undergo terminal differentiation, high levels of expression are once again observed in cell populations that are activated during wound healing and neovascularization [Schlingemann et al., 1990; Levine, 1994; Keirstead et al., 1998; Burg et al., 1999; Bu et al., 2001; McTigue et al., 2001; Ozerdem et al., 2001, 2002; Zhang et al., 2001]. NG2 expression is also upregulated in some types of malignant neoplasms, including melanomas, glioblastomas, chondrosarcomas, and lymphomas [Real et al., 1985; Schrappe et al., 1991; Leger et al., 1994; Smith et al., 1996].

Cell migration is a process that is often enhanced in both progenitor cells and tumor cells. We have been intrigued by the possibility that cell motility may be potentiated in these cell types via engagement of NG2 by ECM ligands, such as collagen VI [Burg et al., 1997]. These studies have been complicated by the fact that cell surface integrins also act as receptors for these ECM components, often obscuring the contribution of NG2 and other proteoglycan receptors. Two strategies have been employed to detect NG2-dependent changes in cell morphology and motility in the face of the overwhelming contribution of integrin receptors. The first approach has been to study the behavior of cells plated on surfaces coated with NG2 mAbs, in which case we can be assured that the primary stimulus occurs via engagement of the proteoglycan. We have shown that glioma cells will spread, polarize, and migrate on these mAb-coated surfaces, and have presented evidence that suggests that NG2-dependent spreading and polarization require the activation of rho family GTPases [Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001; Majumdar et al., 2002]. In the case of melanoma cells, direct evidence has been obtained for the NG2-dependent activation of cdc42, Ack-1, and p130cas following engagement by NG2 mAbs [Eisenmann et al., 1999].

In the present study, we have used a second approach, which allows examination of the role of NG2 in cell attachment and spreading on more physiological substrates, namely ECM components. This approach depends on use of the GD25 cell line, derived from mouse ES cells lacking the β_1 integrin subunit [Fassler et al., 1995]. These cells express the α_3, α_5, α_6, and α_v integrin subunits, but in the absence of the β_1 subunit are unable to assemble functional β_1 heterodimers on the cell surface [Wennerberg et al., 1996]. GD25 cells have therefore proved to be a valuable tool not only for studying the detailed function of β_1 integrins [Brakebusch et al., 1999], but also their functional relationship with other cell surface receptors, such as cadherins [Gimond et al., 1999]. Since β_1-containing integrins have been identified as primary cell surface receptors for collagen V [Ruggiero et al., 1994] and VI [Pfaff et al., 1993], we hoped that β_1-deficient GD25 cells would also be useful for defining the involvement of NG2 as a functional receptor for these collagen types. These two molecules were previously found to be specific ECM ligands for NG2 [Burg et al., 1996; Tillet et al., 1997]. By transfecting NG2 cDNA into GD25 cells and GD25-β_1A cells, we created pairs of cell lines that differed only in their expression of NG2.

As expected, the introduction of NG2 into the GD25 line endows these cells with the ability to attach to surfaces coated with NG2 mAbs. More interestingly, GD25/NG2 cells also exhibit an improved ability to attach to surfaces coated with collagens V and VI, but not to surfaces coated with collagens I and IV. This is consistent with the observed specificity for NG2 binding to different collagen species [Tillet et al., 1997]. Expression of the β_1A integrin subunit completely obscures the contribution of NG2 in cell attachment to collagens V and VI, underscoring the importance of seeking an NG2-dependent component of adhesion in the absence of β_1

integrins. The identity of the integrin species involved in GD25-β_1A adhesion to collagen V or VI has not been established. The primary receptors for both collagens are the integrins $\alpha1\beta_1$ and $\alpha2\beta_1$ [Pfaff et al., 1993; Ruggiero et al., 1994], neither of which have been detected in GD25 cells using biochemical methods [Wennerberg et al., 1996]. One possible receptor candidate would be the $\alpha_3\beta_1$ integrin, which has been previously shown to function as a collagen VI receptor in some transformed cell lines [Wayner and Carter, 1987] and during corneal stromal development [Doane et al., 1998]. Adhesion of some cell lines to native collagen V can also be inhibited by anti-α_3 integrin antibodies [Ruggiero et al., 1994], suggesting that the $\alpha_3\beta_1$ integrin may interact with both collagens V and VI.

In contrast to several other cell types we have examined, GD25/NG2 cells fail to spread on the mAb-coated surfaces. This behavior is observed not only with the newly produced 2B3 mAb, but also with several other NG2 mAbs previously found to induce spreading of glioma, fibroblast, and lymphoma cells. The reason for this failure to spread is unknown. However, this result is reminiscent of the behavior of human melanoma cells, which spread when both $\alpha_4\beta_1$ integrin and NG2 receptors are engaged, but fail to spread when either receptor is engaged individually [Iida et al., 1995]. This requirement for dual receptor activation is not apparent in the spreading of glioma cells on NG2 mAbs [Fang et al., 1999; Stallcup and Dahlin-Huppe, 2001]. Perhaps a specific pattern of clustering of NG2 receptors on the glioma cell surface renders them capable of signaling in the absence of a second receptor. This type of model would seem to imply the existence of a scaffolding or organizational entity (such as MUPP1 [Barritt et al., 2000]) which is present in the glioma cells, but absent or inoperative in melanoma cells and GD25 cells. Alternatively, engagement of NG2 in glioma cells may result in recruitment of a second type of receptor to form a functional signaling complex. Glioma cells express β_1 integrins, which might function in this context. However, it must be noted that expression of the β_1A subunit in NG2-positive GD25 cells still does not give them the capability of spreading on NG2 mAbs. Perhaps the absence of an α_4 subunit in these cells precludes formation of the $\alpha_4\beta_1$ heterodimer needed to serve as the co-receptor for NG2, as appears to be the case for melanoma cells. Further transfection of the GD25-β_1A/NG2 cells with α_4 integrin would be required to test this possibility.

Of more physiological interest is the spreading behavior of GD25/NG2 cells on the ECM ligands collagen V and VI. Although GD25/NG2 cells adhere to both collagens, cell spreading occurs only on collagen VI. The formation of ruffling lamellipodia on this substratum is highly reminiscent of the lamellipodia formed by glioma cells plated on some types of NG2 mAbs [Fang et al., 1999]. Since lamellipodia are thought to form in response to activation of the small GTPase rac [Ridley et al., 1992], it seems likely that the NG2/collagen VI interaction triggers activation of this signaling pathway. This idea is supported by our finding of rac activation in glioma cells in response to engagement of NG2 by monoclonal antibody-coated surfaces [Majumdar et al., 2002]. We have previously shown that collagen VI binds to the central, extended portion of the NG2 extracellular domain [Burg et al., 1997; Tillet et al., 1997]. This binding may trigger a conformational change in NG2 that results in signal transduction across the membrane. However, we can not exclude that collagen VI contains an additional binding site for a non-β_1 integrin ligand that is required for concomitant signaling along with NG2.

The case of collagen V appears analogous to that of the NG2 mAbs in terms of its ability to promote cell attachment, but not spreading. Although our previous evidence suggests that collagen V also binds to the central domain of NG2 [Tillet et al., 1997], this binding may not trigger the type of conformational change required for NG2 signaling. Alternatively, collagen V may lack a binding site for a second receptor needed to signal in concert with NG2.

In addition to their ability to bind to each other in solid phase assays, previous work has provided a few indications that NG2 and collagen VI may be involved in interactions that are functionally important. Expression of NG2 provides a means of anchoring collagen VI at the cell surface and presumably for the assembly of a more complex ECM [Nishiyama and Stallcup, 1993; Burg et al., 1997]. Exposure to collagen VI also promotes larger increases in cell motility in NG2-positive cells than in NG2-negative cells [Burg et al., 1997]. Reflecting a functional relationship, collagen VI is found in many of the same developmental sites as NG2, for example, in vasculature [Stallcup et al., 1990; Rand et al.,

1993; Sage and Vernon, 1994; Kuo et al., 1997] and various types of connective tissue [Stallcup et al., 1990; Nishiyama et al., 1991; Doane et al., 1998]. Like NG2, collagen VI expression is upregulated in healing wounds [Oono et al., 1993; Zhang et al., 1994] and in the stroma and vasculature of malignant glioblastomas and melanomas [McComb et al., 1987; Han et al., 1995; Daniels et al., 1996]. In addition to an anchoring role for the NG2/collagen VI complex, our current work provides the first clear evidence that the NG2/collagen VI interaction also offers a means of modulating cell adhesion and cell morphology. These processes may be operative not only during key periods of development when NG2 expression on progenitor cells is maximal, but also during pathological episodes of wound healing or tumor growth and metastasis when NG2 expression is again upregulated.

In summary, we have developed a unique and valuable model for studying NG2-mediated cell matrix interactions. Future work will be directed not only at understanding the developmental relationship between NG2 and collagen VI, but also at identifying in more depth the nature of the signaling complex formed by the two molecules, especially with regard to the activation of rac, cdc42, FAK, and p130cas. The use of NG2 mutants with altered cytoplasmic domains will also help to define structural motifs by which NG2 interacts with these signaling components.

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