NCAM regulates cell motility

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Accepted12 October 2001
Journal of Cell Science 115, 283-292 (2002) © The Company of Biologists Ltd

Summary

Cell migration is required during development of the nervous system. The regulatory mechanisms for this process, however, are poorly elucidated. We show here that expression of or exposure to the neural cell adhesion molecule (NCAM) strongly affected the motile behaviour of glioma cells independently of homophilic NCAM interactions.

Expression of the transmembrane 140 kDa isoform of NCAM (NCAM-140) caused a significant reduction in cellular motility, probably through interference with factors regulating cellular attachment, as NCAM-140-expressing cells exhibited a decreased attachment to a fibronectin substratum compared with NCAM-negative cells. Ectopic expression of the cytoplasmic part of NCAM-140 also inhibited cell motility, presumably via the non-receptor tyrosine kinase p59*fyn* with which NCAM-140 interacts.

Furthermore, we showed that the extracellular part of NCAM acted as a paracrine inhibitor of NCAM-negative cell locomotion through a heterophilic interaction with a cell-surface receptor. As we showed that the two N-terminal immunoglobulin modules of NCAM, which are known to bind to heparin, were responsible for this inhibition, we presume that this receptor is a heparan sulfate proteoglycan. A model for the inhibitory effect of NCAM is proposed, which involves competition between NCAM and extracellular components for the binding to membrane-associated heparan sulfate proteoglycan.

Key words: NCAM, Cell motility, Glioma, Heparan sulfate proteoglycan, Cell adhesion

Introduction

Migration of cells takes place during development, wound healing and tumor invasion. The correct formation and normal function of the nervous system require that the majority of neurons migrate throughout the developing nervous system from their sites of origin to their final positions. However, our understanding of the molecular mechanisms regulating neuronal migration is currently very limited. One possible regulatory molecule is NCAM, which plays a pivotal role during morphogenesis, mediating binding between neural cells and stimulating axonal outgrowth and fasciculation (Thiery et al., 1982; Fields and Itoh, 1996; Rønn et al., 1998). Early neural crest cells express NCAM, but the expression is downregulated gradually during their migratory phase and reinitiated at their final destination (Bronner-Fraser, 1993). Moreover, expression of NCAM has been shown to reduce migration and invasion of glioma cells in vitro as well as in vivo (Edvardsen et al., 1993a; Edvardsen et al., 1994; Gratsa et al., 1997; Owens et al., 1998).

NCAM belongs to the immunoglobulin (Ig) superfamily and is expressed as three major isoforms, depending on the cell type and stage of differentiation. There are two transmembrane isoforms, with either a short (NCAM-140 kDa) or a long (NCAM-180 kDa) cytoplasmic domain, and one isoform has a glycosyl-phosphatidylinositol (GPI) membrane anchor (NCAM-120 kD) (reviewed in Bock et al., 1997). The extracellular part of all NCAM isoforms consists of five Ig modules and two fibronectin type III (F3) modules.

NCAM mediates homophilic binding via its Ig modules. Homophilic binding may take place through dimerization of the third Ig modules (Rao et al., 1994), through a double reciprocal dimerization of the first and second Ig modules (Kiselyov et al., 1997; Atkins et al., 1999; Jensen et al., 1999; Kasper et al., 2000) or through all five Ig modules being arranged in an antiparallel manner (Ranheim et al., 1996). In addition to cell-cell adhesion, homophilic interaction of NCAM induces signal transduction, resulting in neuronal differentiation (Doherty and Walsh, 1996; Kolkova, 2000a) and inhibition of cell proliferation (Edvardsen et al., 1993a; Sporns et al., 1995; Crossin et al., 1997; Krushel et al., 1998).

The extracellular part of NCAM also interacts with several heterophilic ligands. In chicken brain NCAM colocalizes and copurifies with an abundant heparan sulfate proteoglycan (HSPG) (Burg et al., 1995), which is presumably bound to NCAM through heparin-binding sites localized to first and second Ig modules (Cole and Akeson, 1989; Reyes et al., 1990; Kallapur and Akeson, 1992; Burg et al., 1995; Kiselyov et al., 1997). More specifically, chicken NCAM interacts with agrin, a major HSPG of the brain expressed by neurons and glial cells (Storms et al., 1996; Cotman et al., 1999). Furthermore, NCAM binds to several chondroitin sulfate proteoglycans (CSPG), including neurocan expressed by neurons (Friedlander et al., 1994; Margolis et al., 1996) and phosphocan expressed by astroglial cells (Milev et al., 1994; Margolis et al., 1996).

The intracellular part of the 140 kDa isoform of NCAM

(NCAM-140), but not the intracellular part of the 180 kDa isoform of NCAM (NCAM-180), has been shown to associate constitutively with the non-receptor tyrosine kinase p59^{fyn} (Beggs et al., 1997). Stimulation of NCAM-140 by homophilic binding or by means of antibodies leads to recruitment and activation of the focal adhesion kinase p125^{fak}. Recently, p59^{fyn}, p125^{fak} and the Ras-MAP kinase pathway have been shown to be activated in connection with NCAM-mediated neurite outgrowth (Schmid et al., 1999; Kolkova et al., 2000a). In p59^{fyn}-null mice NCAM-stimulated axonal outgrowth is abrogated (Beggs et al., 1994), and in PC12 cells, inhibition of p125^{fak} by transfection with a dominant-negative construct results in a complete inhibition of NCAM-stimulated neurite outgrowth (Kolkova et al., 2000a), indicating that these two non-receptor tyrosine kinases play important parts in NCAM functions. A C-terminal five amino-acid sequence motif of the cytoplasmic part of NCAM-140 was identified as being essential for NCAM-stimulated neurite outgrowth (Kolkova et al., 2000b), but it is not yet known whether this motif constitutes the p59^{fyn}-binding site. The MAP kinases ERK1 and ERK2 activate the myosin light chain kinase and thereby regulate cell motility (Klemke et al., 1997), indicating that since NCAM signals via the MAP kinases, NCAM may regulate cellular migration through this pathway. However, the role of NCAM in cell motility has so far not been evaluated under conditions controlling the influence of cell-cell adhesion nor have the roles of the extracellular versus the intracellular parts of NCAM been assessed.

In the present study, we show that expression of NCAM in a neural cell line, the glioma line BT4Cn (Laerum et al., 1977), in the absence of NCAM-mediated cell-cell or cell-substratum interactions, leads to a strong downregulation of cell locomotion, probably owing to decreased cellular attachment. Expression of the intracellular parts of the transmembrane isoforms NCAM-140 (140-cyt) and NCAM-180 (180-cyt) by themselves had strong effects on cell motility. Furthermore, we show that the presence of NCAM, either in solution or on an adjacent cell, strongly affected the motile behavior of NCAM-negative cells, and this presumably is due to the interaction of the heparin and heparan-sulfate binding Ig modules of NCAM with a heterophilic membrane-associated receptor.

Materials and Methods

Expression plasmids

Expression plasmids encoding the human NCAM 120 kDa and 140 kDa isoforms (NCAM-120, NCAM-140) are described by Walsh et al. (Walsh et al., 1989) and the 180 kDa isoform (NCAM-180) by Doherty et al. (Doherty et al., 1992). The plasmids encoding the cytoplasmic parts of rat NCAM-140 (140-cyt) and NCAM-180 (180-cyt) are described by Kolkova et al. (Kolkova et al., 2000b). An expression plasmid encoding the enhanced green fluorescent protein (pEGFP-N1) was obtained from Clontech. A plasmid encoding a secreted form of NCAM was created by cloning the PCR amplification product encoding the first 692 amino acids of the cDNA of human NCAM-120 (GenBank accession number X16841) using primers generating a 5′ *Hin*dIII and a 3′ *Xba*I site and changing serine 693 to a stop codon (sense primer: 5′-TAGCATAAGCTTCAAGAACATCCCTCCCA-GCC-′3, antisense primer: 5′-TAGCATTCTAGATGGGCCTAGG-TCCTGAACAC′-3) into the expression vector pHβApr-1-neo (Gunning et al., 1987), which was modified to include an *Xba*I site in the multiple cloning site.

Transfection, cell lines and cell culture

The highly invasive rat glioma cell line BT4Cn is described in Laerum et al. (Laerum et al., 1977). This cell line does not express any isoforms of NCAM (Andersson et al., 1991). Transient transfections of rat glioma BT4Cn cells were performed using Lipofectamine Plus (GibcoBRL) with pEGFP-N1 in a 1:10 ratio together with plasmids encoding either human NCAM-140, the cytoplasmic part of rat NCAM-140 (140-cyt), the cytoplasmic part of rat NCAM-180 (180-cyt) or the vector as a control. Cells were replated 24 hours after transfection, and only transfected cells (identified by expression of EGFP) were analyzed. Cells were dislodged with 5 mM EDTA in PBS from semi-confluent cultures and replated at a density of 4×10^3 cells/cm^2. Cells were plated on cell culture dishes (NUNC) coated with 1 μg fibronectin/cm^2 (Sigma-Aldrich) and analyzed 16 hours after plating. For co-culture and interference reflection microscopy (IRM) experiments, no coating was employed, and the cells were recorded 4 hours after plating.

Stably transfected rat glioma BT4Cn cell lines expressing human NCAM-140, NCAM-120 and control lines have previously been described (Edvardsen et al., 1993a; Edvardsen et al., 1993b), and NCAM-180-expressing lines were generated in the same way. For motility of single cells, pools of three clones from three individual transfections were employed that express similar amounts of NCAM as determined by western blotting. The level of expression of one of the NCAM-140 and NCAM-120 cell lines has previously been determined by ELISA (Edvardsen et al., 1993b) and was found at the same level as for the parental, NCAM expressing glioma cell line (BT4C) (Andersson et al., 1991)

For co-culture experiments, the NCAM-140-positive or control cell lines, which were additionally transfected to stably express EGFP, were seeded on a confluent layer of a fibroblast cell line expressing human NCAM-140 (LBN110) or a fibroblast control line (LVN212) (Kasper et al., 1996). Medium containing soluble NCAM was collected from a BT4Cn cell line stably transfected with the plasmid encoding the secreted, extracellular form of NCAM (Sol-NCAM). Control media and soluble NCAM-containing conditioned media were diluted with two volumes of fresh medium before application, resulting in a final concentration of approximately 0.17 μg/ml of soluble NCAM (as determined by ELISA). The medium was added to cells one hour before recording cell motility. NCAM-depleted conditioned medium was obtained after two series of immunoadsorption to polyclonal anti-human NCAM antibodies (Protein Laboratory) immobilized on Protein A Sepharose (Pharmacia). For inhibition of sulfatation, sodium chlorate (Sigma-Aldrich) was added directly to the medium at a concentration of 50 mM 20 hours before recording. Low molecular weight heparin (Sigma-Aldrich) (50 μg/ml) or recombinant NCAM immunoglobulin domains I and II (NCAM IgI-IgII) (100 μg/ml) (Jensen et al., 1999) were added to the medium one hour prior to recording.

Measurements of cell motility

Time-lapse video recording of live cells was performed in sealed dishes on a thermostatically controlled and motorized stage (Lincam Scientific Instruments) mounted on a Diaphot 300 inverted microscope equipped with phase-contrast optics (Nikon) using a black and white CCD video camera (Burle). Images were recorded from 8-12 different fields/well for 48 minutes with four-minute intervals (for 140- and 180-cyt 60-70 fields, 120 minutes with six-minute intervals) using the PRIGRA software (Protein Laboratory). Cells transiently transfected with EGFP and the various NCAM or control plasmids were recorded live using standard fluorescence microscopy settings for FITC for the first image only. Glioma cells permanently transfected with EGFP in co-culture experiments were recorded using an automatic shutter with an opening time of 1/50 seconds to reduce the time of exposure to light. The track of an individual cell was defined as the sequence of positions of the center of its nucleus over

time. The dispersion of a cell is the Euclidean distance between two points on a plane measured in μm. The displacement of a single cell after a given time of observation, dt_{obs}, was calculated as

$$dt_{obs} = \sqrt{(x(t_{obs}) - x(t_0))^2 + (y(t_{obs}) - y(t_0))^2}$$

where $x(\cdot)$ and $y(\cdot)$ are the x- and y-coordinates of the cell, respectively, and t_{obs} is the time during which a given displacement dt_{obs} takes place with starting time t_0. R.m.s. (S) and persistence time, P, were estimated by plotting the mean-squared displacement, $<d^2>$ against time with subsequent curve fitting to the equation

$$<d^2> = 2S^2P(t_i - P(1 - e^{(-t_i/P)}))$$ (Dunn, 1983)

where t_i is the time interval of interest. The rate of diffusion, R, was calculated using the equation

$$R=S^2P$$

The average speed of the individual cells (mean cell speed), termed S_τ, was calculated as the mean displacement of each cell for several identical time intervals with different starting points. This was performed according to the equation

$$S_\tau = \frac{1}{N} \times \frac{\tau}{t_{tot}} \sum_{k=1}^{N} \sum_{t=\tau \text{ by } \tau}^{t_{tot}} \frac{\sqrt{(x_k(t) - x_k(t-\tau))^2 + (y_k(t) - y_k(t-\tau))^2}}{\tau}$$

where k denotes a given cell, N is the size of the investigated sample of a population of cells, $x(\cdot)$ and $y(\cdot)$ are the x- and y-coordinates of the cell, t_{tot} is the time elapsed from the first to the last image constituting a recording and τ is the time interval between discrete observations. The number of cells used for calculation in each individual experiment varied from 100 to 180 with an average of 120. A minimum of four independent experiments were performed for each test.

Attachment assays

For IRM analysis, cells were plated in one-well coverglass chambers (NUNC) and recorded live at 37°C using a Zeiss Axiovert 100 inverted microscope equipped with a Plan-NEOFLUAR 63×1.25 Oil Ph3 antiflex objective and a black and white CCD video camera (Burle). For trypsin attachment assays, 96-well plates (NUNC) coated with 1 μg fibronectin/cm^2 were additionally blocked with 0.2% (w/v) BSA. 16 hours after plating, cells were washed in PBS. Trypsin (Sigma-Aldrich) was added to final concentrations between 0-40 μg/ml for five minutes at 37°C, and dislodged cells were subsequently removed. The relative amount of attached cells was measured by absorbance at 550 nm after washing, fixing, staining with 0.5% (w/v) crystal violet and solubilising by 0.1 M sodium citrate in 50% (v/v) ethanol.

Immunostaining and staining of F-actin

After recording the motility in co-culture, cells were fixed in 3% (w/v) paraformaldehyde and immunostained with mouse monoclonal antibodies against human NCAM using a mixture of CD56, UJ13A (DAKO) and Leu19 (Becton Dickinson) each in a dilution of 1:50. For staining for 140-cyt, the cells were fixed in 3% (w/v) paraformaldehyde, permeabilized with 0.02% (w/v) saponin and immunostained with rabbit polyclonal anti recombinant rat NCAM exons 16, 17 and 19 (Protein Laboratory). Staining for vinculin was performed by fixation in 3% (w/v) paraformaldehyde, permeabilization with 0.2% (v/v) Triton X-100 and incubation with a mouse monoclonal antibody to vinculin (clone hVIN-1, Sigma-Aldrich). After incubation with FITC-coupled secondary antibodies (DAKO), the stainings were evaluated using confocal laser microscopy. For staining of F-actin, the cells were washed in actin stabilizing buffer (10 mM Tris-Cl pH 7.4, 0.15 M NaCl, 2 mM $MgCl_2$, 10% (v/v) glycerol), permeabilized with 0.2% (w/v) saponin and incubated with Texas-Red-X-conjugated phalloidin (Molecular Probes) and visualized as above.

Results

Effect of expression of NCAM-140 on single cell motility

BT4Cn cells were transiently transfected with an expression plasmid coding for the transmembrane NCAM-140 isoform together with a plasmid encoding EGFP (thus EGFP expression was a marker for transfected cells) (Fig. 1A). Control cells were transiently transfected with empty vector and the EGFP plasmid. Cells were plated as single cells on an NCAM inert substratum, in casu fibronectin (Probstmeier et al., 1989). Individual tracks of control cells and NCAM-140-expressing cells plated as single cells were determined using time-lapse video recording and computer-assisted image analysis. Fig. 1B shows so-called 'wind-rose' plots prepared by superimposing the starting points of tracks of individual cells. It can be seen that expression of NCAM resulted in a pronounced decrease in cellular displacement. To quantify the motile behaviour, we measured cell dispersion and calculated the rate of diffusion, R (Fig. 1C), the root mean square speed, S (Fig. 1D) and the mean cell speed, S_τ (Fig. 1E) (for definitions, see section of methods). Expression of NCAM-140 caused a 35-50% decrease in all three parameters. A cell typically moves along a relatively straight path, which can be described as a persistent random walk. At a fixed speed, a cell population with a high persistency will disperse more than a population with a low persistency. The persistence times, P, were therefore also calculated and found to be very low (five to 10 minutes) in both NCAM-negative and NCAM-positive cells with no statistically significant differences, indicating that the lower dispersion of NCAM positive cells was caused by a decrease in speed rather than a decrease in persistency. The rate of diffusion, R, was also measured for glioma cell lines stably transfected with NCAM-140 and control cell lines stably transfected with vector; values similar to those obtained using transiently transfected cells were obtained. Thus, NCAM-140-positive cell lines were found to have a mean R-value of 3.83±0.49 μm^2/min and control cell lines a mean R-value of 7.24±1.01 μm^2/min. In comparison, transiently transfected cells had mean R values of 4.63±2.13 and 7.01±2.58 μm^2/min, respectively. Normal astrocytes express NCAM-120, NCAM-140 and glial fibrillary acidic protein (GFAP), whereas the employed glioma cells do not express GFAP. Removal of GFAP expression has recently been shown to reduce the motility of normal astrocytes. In connection with these studies, primary astrocytes isolated from GFAP-null mice were found to have a mean R-value of 4.72 μm^2/min, which is in the same range as for the NCAM-140 expressing glioma cells (Lepekhin et al., 2001).

To determine how large the migratory fraction of the cells was, the distribution of the $S\tau$ for the populations of control and NCAM-140 expressing cells from both stable and transient transfections was analyzed. Fig. 1F,G show the distribution of the speed for individual cells from representative experiments. The x-axis shows the $S\tau$ for the individual cells; the y-axis shows the percentage of cells with a given $S\tau$. Although many of the cells moved relatively short distances during the recording time, most of the cells could be demonstrated to

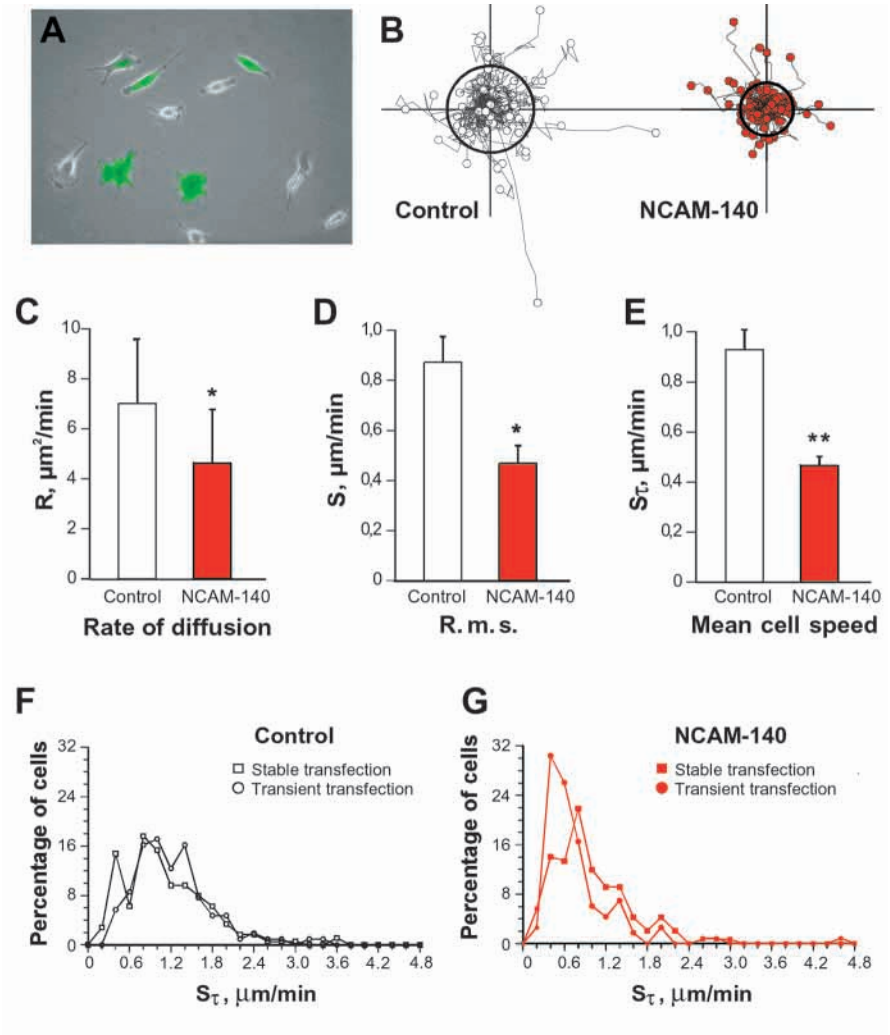

Fig. 1. Effect of expression of NCAM-140 on single-cell motility of glioma cells. (A) Phase-contrast image of glioma cells plated as single cells on fibronectin and transiently transfected with plasmids encoding NCAM-140 and EGFP overlaid with the fluorescence image of the EGFP-expressing cells. (B) 'Wind-rose' plots of tracks with superimposed starting points of glioma cells transiently transfected with a plasmid encoding EGFP and either co-transfected with a plasmid encoding NCAM-140 or a control plasmid. The circles mark the square root of the mean-squared displacement ($\sqrt{<d^2>}$) of the recorded cells. (C) Cell motility measured as rate of diffusion, R; (D) root mean square speed, S, and (E) mean cell speed, S_τ, of glioma cells transiently transfected with vector (control) or a plasmid encoding NCAM-140 (the mean from five experiments was $*P<0.05$, $**P<0.01$ using student's paired *t*-test). (F,G) The distribution of the Sτ for populations of cells from representative experiments of cells transiently and stably transfected with a control plasmid (F) or a plasmid encoding NCAM-140 (G).

be motile. Thus in all four populations presented, less than 6% of the cells had an Sτ below 0.2 μm/min. The distribution of Sτ was very similar for transient and stably transfected cells, and Fig. 1F,G shows that the average reduction of cellular speed in response to NCAM expression is not caused by a non-motile subpopulation but by a general reduction of the motility.

Effect of intercellular interactions on motility of glioma cells in co-culture with fibroblasts

To evaluate the role of NCAM-mediated cell-cell interactions in cell locomotion, EGFP-tagged glioma cells were co-cultured with fibroblasts. All four possible combinations of NCAM-140-positive or -negative glioma and fibroblast cell lines were analyzed, allowing examination of the effects of both homophilic and heterophilic NCAM-interactions on the rate of diffusion of the glioma cells. The expression of NCAM on both cell types was confirmed by immunostaining (Fig. 2B-D). The overall rate of diffusion of glioma cells in the co-culture system (Fig. 2E) was reduced to approximately 30% of that of sparsely seeded, single cells on fibronectin (Fig. 1C), probably reflecting cell-cell interactions not involving NCAM. However, when NCAM-140 was expressed either on the test (glioma) cells or on the supporting (fibroblast) cells or both, an additional strong reduction of the rate of diffusion of the test cells was observed. The fact that NCAM only had to be expressed by one of the two interacting cell types in order to affect the rate of diffusion of the glioma cells implies that the extracellular part of NCAM binds to a heterophilic receptor, which upon ligation influences cell locomotion.

Role of the cytoplasmic part of NCAM-140

One of the intracellular ligands of NCAM-140 is p59^{fyn}. The cytoplasmic part of NCAM-140 may compete with other p59^{fyn}-binding proteins, thereby affecting signalling pathways regulating motile behaviour. Ectopic expression of the cytoplasmic part of NCAM-140 (140-cyt) has previously been shown to inhibit NCAM-mediated neurite outgrowth, presumably by interfering with its signalling via the Ras-MAP kinase pathway (Kolkova et al., 2000b). Therefore, motility of NCAM-140-positive and -negative glioma cell lines transfected with a plasmid encoding 140-cyt was measured. The cytoplasmic NCAM-140 fragment was localized to the plasma membrane in control cells (Fig. 3C) in accordance with Little et al. (Little et al., 1998), who showed that the cytoplasmic part of NCAM can be palmityolated, thus providing membrane anchoring in the absence of the transmembrane region. In cells expressing intact NCAM-140, the antibody employed did not discriminate between 140-cyt and the cytoplasmic part of intact NCAM-140 (Fig. 3B,D). Expression of 140-cyt resulted in a significantly reduced rate of diffusion of the NCAM-negative control cells, showing that the cytoplasmic part in itself was capable of modifying cell motility (Fig. 3E). On the other hand, expression of 140-cyt did not influence the low motility of cells already expressing intact NCAM-140. Transfection with a plasmid encoding the intracellular part of NCAM-180 (180-cyt) (Fig. 3F) also resulted in a significant reduction in the rate of diffusion of the

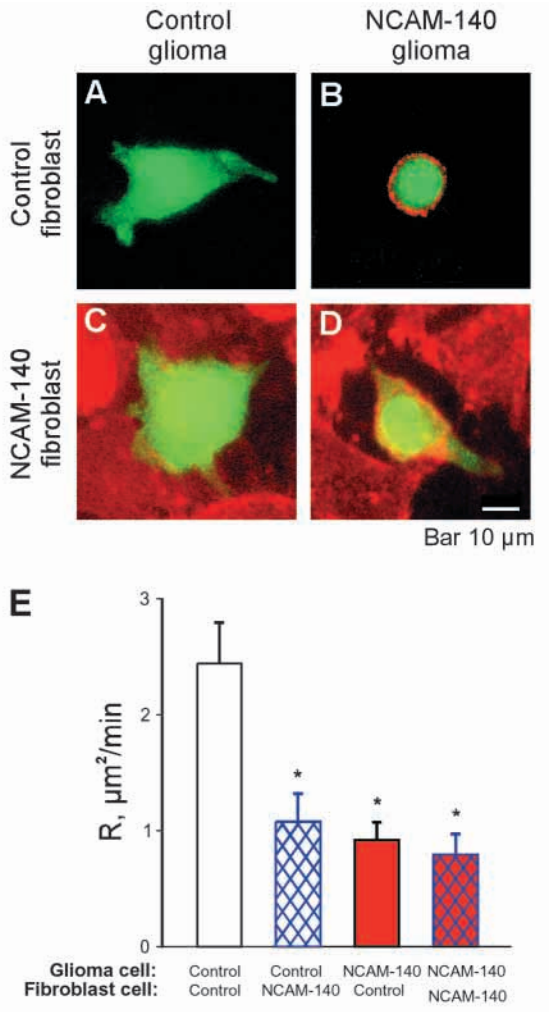

Fig. 2. Effect of NCAM-140 on motility of glioma cells in co-culture with fibroblasts. (A-D) Immunostaining of NCAM (red) of NCAM-140-positive or -negative glioma cell lines permanently transfected to express EGFP (green) in co-culture with NCAM-140-positive and -negative fibroblast cell lines. (E) Rate of diffusion, R, of the EGFP expressing NCAM-140-positive and -negative glioma cells in co-culture (the mean of four experiments was **P*<0.05 compared with vector-transfected controls, paired *t*-test).

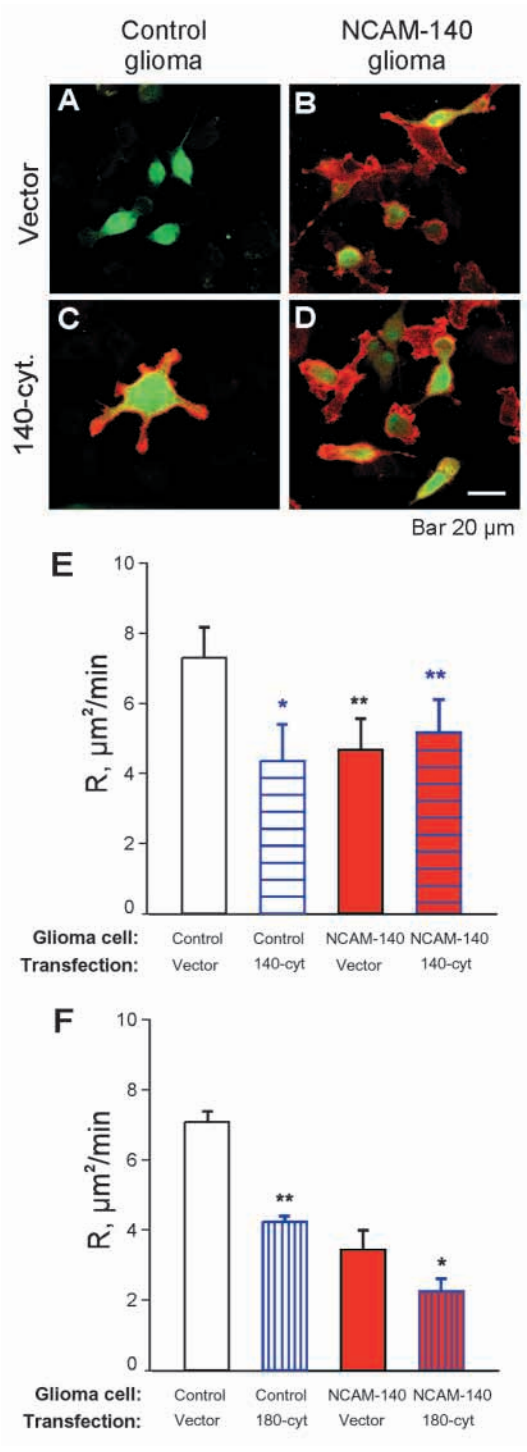

Fig. 3. The effect of the intracellular parts of NCAM on glioma-cell motility. (A-E) NCAM-140-positive or -negative glioma cell lines plated as single cells on fibronectin transiently transfected with a plasmid encoding EGFP together with either a plasmid encoding the cytoplasmic part of NCAM-140 (140-cyt) or a control plasmid (vector). (A-D) Immunostaining with antibodies against the cytoplasmic part of NCAM (red) of transiently transfected cells (green). (E) Rate of diffusion, R, of the transiently transfected cells (mean of six experiments, **P*<0.05, ***P*<0.01 compared with vector-transfected control cells, paired *t*-test). (F) NCAM-140-positive or -negative glioma cell lines plated as single cells on fibronectin transiently transfected with a plasmid encoding EGFP together with either a plasmid encoding the cytoplasmic part of NCAM-180 (180-cyt) or a control plasmid (vector). Rate of diffusion, R, of the transiently transfected cells (mean of 4 experiments, **P*<0.05 of 180-cyt-transfected NCAM-140 cells versus vector-transfected NCAM-140 cells and versus 180-cyt-transfected control cells, ***P*<0.01 for 180-cyt versus vector-transfected control glioma cells, paired *t*-test).

NCAM-negative glioma cells. However, expression of the 180-cyt in NCAM-140 expressing cells caused a statistically significant further reduction of the rate of diffusion, indicating different modes of action between the cytoplasmic domains.

Role of NCAM expression on cellular attachment

The speed of a cell is dependent on the strength by which it is attached to its surroundings (extracellular matrix or other cells), with maximal motility observed at an intermediate attachment strength (DiMilla et al., 1993). Using IRM, it is

possible to evaluate the degree of attachment of a cell to glass. Cell areas with close contact to the glass, for example, focal adhesions, are observed in IRM as dark structures, whereas areas with less contact appear brighter, reflecting the physical distance between the plasma membrane of the cell and the glass surface. IRM images in pseudo-colors are shown for the control (Fig. 4A) and for the NCAM-140-expressing cell (Fig. 4B) (white-yellow indicating a strong attachment and purple-blue a weak attachment). The degree of contact was measured for cells with or without NCAM-140 expression, and from the cumulative curves shown in Fig. 4C it can be seen that expression of NCAM resulted in a shift to the right, indicating a decreased attachment to the substrate. Attachment strength of the two cell populations was also estimated by an assay in which cells plated on fibronectin were incubated with different concentrations of trypsin (Fig. 4D). NCAM-140-expressing cells were found to detach at a significantly lower trypsin concentration than control cells with IC50 values of 11.2±1.2 and 22.0±4.8 μg/ml trypsin, respectively, reflecting a weaker attachment of the NCAM-140-positive cells. Thus, we conclude that the lower motility observed for the NCAM-expressing cells reflects a weaker attachment to the substratum.

Immunostaining of NCAM-140-expressing cell lines for the focal adhesion component vinculin revealed that these cells indeed displayed a marked decrease in focal adhesions compared with control lines (Fig. 4E,F), and phalloidin staining revealed that NCAM-140 expressing cells also contained fewer actin stress fibers than control cells (Fig. 4G,H). In contrast, cell lines expressing the lipid-anchored NCAM-120 kDa isoform and the transmembrane isoform NCAM-180 kDa displayed focal adhesions and stress fibers similar to control cells (not shown), indicating that only expression of the NCAM-140 isoform affected the number of focal adhesions and the arrangement of filamentous actin. This may probably reflect the fact that only the NCAM-140 isoform interacts with p125^{fak} (Beggs et al., 1997).

The role of the extracellular part of NCAM

Intracellular interactions of NCAM in glioma cells cannot account for the finding that NCAM-negative glioma cells plated on NCAM-140-positive fibroblasts had a significantly reduced rate of diffusion (Fig. 2E). Although plated on a monolayer of fibroblasts, the glioma cells did not remain on the surface of the monolayer but descended into the fibroblast layer. Therefore, the decreased motility of NCAM-negative glioma cells on NCAM-expressing fibroblasts might be caused by increased adhesion between the fibroblasts, thereby creating a mechanical barrier, rather than by a heterophilic binding between the glioma cells and the NCAM-expressing fibroblasts. To determine if this was the case, soluble NCAM was added to glioma cells plated as single cells on fibronectin using conditioned medium from a glioma cell line transfected to express a secreted form of the extracellular part of NCAM (sol.-NCAM). Conditioned medium from NCAM-negative control cells prepared in an identical manner was used as control. Addition of medium with soluble NCAM caused a strong inhibition of the rate of diffusion of control cells but had no effect on the reduced rate of diffusion already achieved by cells endogenously expressing NCAM-140 (Fig. 5A). Medium depleted of soluble NCAM by immunoprecipitation by specific antibodies against NCAM had no effect on either cell type (not shown). This indicates that the extracellular part of NCAM participates in interactions with a heterophilic membrane-associated receptor capable of modulating motility. The influence of the extracellular part of NCAM was also demonstrated by analysis of the motility of cell lines expressing the NCAM-120 kDa isoform, which is lipid anchored and therefore has no intracellular domain. The NCAM-120-expressing lines had a mean R value of 8.68±1.8 compared with control cells with a mean R value of 10.78±1.8 (mean of four experiments, $*P<0.01$, paired t-test), corresponding to a 20% decrease in the rate of diffusion, which shows that the expression of the extracellular part of NCAM resulted in reduced motility of the cells. As the cells are plated as single cells (therefore preventing cell-cell interactions), this interaction presumably is a cis interaction on the same cell.

Characterization of the heterophilic receptor

The binding of both the NCAM-positive and -negative cells to the fibronectin substratum is mainly achieved via integrins. Membrane-bound HSPGs also bind to fibronectin, thereby strengthening the adhesion, as shown for syndecans, which assist and strengthen substrate attachment of integrins by interaction with the heparin binding site of fibronectin and which are essential for formation of focal adhesions (for a review, see Carey, 1997). It is therefore possible that the extracellular part of NCAM sequesters a sulfated proteoglycan, which in NCAM-negative cells participates in the binding to fibronectin, thereby reducing the attachment strength. One approach to evaluate the involvement of CSPGs or HSPGs is to inhibit sulfatation of the glycosaminoglycan chains by treatment with chlorate. Pretreatment of NCAM-negative glioma cells with 50 mM sodium chlorate, a concentration shown to inhibit at least 70% of *N*-sulfation (Safaiyan et al., 1999), caused a marked, statistically significant reduction in the rate of diffusion (Fig. 5B), indicating an involvement of sulfated proteoglycans in the modulation of cell motility. By addition of heparin, a functional analogue of heparan sulfate, to the medium, a blocking of the heparin-binding site on fibronectin can be achieved, making it inaccessible for HSPGs. Treatment with heparin caused a statistically significant reduction in the rate of diffusion of NCAM-negative glioma cells compared with untreated cells (Fig. 5B), indicating an involvement of HSPGs, rather than CSPGs.

The first and second Ig modules of NCAM bind to heparin (Cole and Akeson, 1989; Kiselyov et al., 1997), and a heparin-binding site has been identified in the second Ig module (Cole and Akeson, 1989). A peptide corresponding to this site was shown by Kallapur and Akeson (Kallapur and Akeson, 1992) to bind to both NCAM-positive and -negative cells, and the binding could be reduced by addition of heparin or by chlorate treatment of the cells. The binding of NCAM to HSPGs is believed to strengthen NCAM homophilic binding (Cole et al., 1986). If the first and second Ig modules of NCAM are responsible for the inhibitory effect of soluble NCAM on motility, these two modules may likewise be expected to cause a reduction in cell motility. Indeed, addition of the combined first and second Ig modules of NCAM (Ig I-II) had a motility-reducing effect identical to that of soluble NCAM (Fig. 5B), confirming this assumption. Although the recombinant first and

second Ig modules form a dimer, owing to a homophilic interaction between these modules (Jensen et al., 1999; Atkins et al., 1999), it was shown by X-ray crystallography that the heparin-binding site on the second Ig module is localized to a loop positioned opposite the dimer interface and therefore is accessible to heparin and heparan sulfate (Kasper et al., 2000).

Discussion

In this study, we have evaluated the effects of NCAM expression on cell motility. Previous reports showing that the expression of NCAM reduces invasion and motility of glioma cells (Edvardsen et al., 1993a; Gratsa et al., 1997; Owens et al., 1998) have employed dense cell cultures, allowing cell-cell interactions including NCAM-NCAM homophilic binding, which probably affect motility profoundly. However, we show here that expression of NCAM-140 significantly reduces motility of glioma cells through interactions of NCAM-140 with other molecules both intra- and extracellularly rather than through homophilic binding.

By seeding the glioma cells sparsely as single cells on a fibronectin substratum and by subsequent analysis of cell displacement by time-lapse video recording and computer-assisted image analysis, we found that glioma cells expressing NCAM-140 by have a reduced rate of diffusion compared to control cells. Analysis of cell attachment by IRM showed that NCAM-140-positive cells had less contact with the substratum than NCAM-negative cells. A trypsin detachment assay also showed that NCAM-140-expressing cells had a weaker attachment to fibronectin than control cells. As attachment is mediated, in part, by focal contacts associated with the actin cytoskeleton, this finding was in accordance with the observation that NCAM-140-expressing cells exhibited considerably less focal adhesions and less structured F-actin filaments than NCAM-negative control cells. The ectopic expression of NCAM in pancreatic tumor cells has no effect on attachment of these cells to fibronectin (Cavallaro et al., 2001). However, the cells utilized in that study express N-cadherin, another neural cell adhesion molecule, which also can interact with the FGF-receptor and thereby modulate cell attachment.

In our study, we employed glioma BT4Cn cells, which do not express N-cadherin (unpublished). We also investigated the roles of both the intra- and extracellular parts of NCAM-140 separately. Transfection with an expression vector encoding the cytoplasmic domain of NCAM-140 decreased the motility of NCAM-negative glioma cells to the same level as transfection with a plasmid encoding the entire NCAM-140 molecule, whereas transfection with the cytoplasmic part of NCAM-140 had no effect on glioma cells already expressing NCAM-140. The effect of the cytoplasmic part of NCAM-140 could be due to the capacity of this domain to associate with p59^{fyn} and p125^{fak}, thereby sequestering these molecules. This is in accordance with the fact that the lipid-anchored NCAM-120 kDa isoform, which has no cytoplasmic domain and which therefore cannot interact with p59^{fyn}, had focal adhesions and actin stress fibers comparable to the control cells. Another transmembrane isoform, NCAM-180 kD, which does not associate with p59^{fyn} or p125^{fak} (Beggs et al., 1997), also displayed normal focal adhesions and stress fibers. Therefore, the effect of NCAM-140 expression on cellular attachment and motility may, at least in part, be due to the capacity of the intracellular part of NCAM-140 to associate with p59^{fyn} and p125^{fak}. This interaction may cause an intracellular disruption of focal adhesions and rearrangement of the actin cytoskeleton, resulting in a reduction of cell attachment and motility. Ectopic expression of the intracellular part of the NCAM-180 isoform (180-cyt) also had a profound, reducing the motility of the NCAM-negative glioma cells. Moreover, expression of 180-cyt in the NCAM-140 expressing cells caused a significant, additional reduction of the rate of diffusion. This indicates that the motility-reducing effects observed by expression of the two different cytoplasmic domains may be mediated through different pathways. Indeed, the intracellular part of the NCAM-180 isoform does not interact with p59^{fyn} or p125^{fak} as does NCAM-140, and NCAM-180-expressing cells have normal focal adhesions and stress fibers unlike NCAM-140-expressing cells. NCAM-180, but not NCAM-140, binds to and copurifies with the membrane-cytoskeleton linker protein, spectrin (Pollerberg et al., 1987). Thus, the intracellular parts of NCAM-140 and NCAM-180 have different modes of interaction with the cytoskeleton, and expression of both modulates motility. However, as no direct evidence was presented indicating that the respective interactions with p59^{fyn}, p125^{fak} or spectrin are involved in the reduction of cellular motility, we cannot exclude the possibility that interactions with other intracellular molecules may be determining factors.

The cytoplasmic part of NCAM is not the only part that modulates cell motility. The extracellular part of NCAM was also capable of modifying cellular migration. This was demonstrated using experiments measuring motility of glioma cells plated on NCAM-negative and -positive fibroblasts. Here we found that if NCAM-140 was expressed by either cell type, the motility of the glioma cells was markedly reduced. This indicates that the extracellular part of NCAM in itself could influence glioma cell motility – either expressed by the glioma cells or by the fibroblasts. NCAM expressed by the fibroblasts inhibited the motility of NCAM-negative glioma cells, indicating that the extracellular part of NCAM must interact with a heterophilic receptor on the glioma cells, thereby influencing cell motility. This was confirmed by the observations that addition of the extracellular part of NCAM in solution likewise inhibited motility. The demonstrated motility-regulating capacity of soluble NCAM may explain the puzzling observation that mice with complete inactivation of the *ncam* gene are viable and fertile, although they have some learning deficiencies (Cremer et al., 1994), whereas mice expressing a secreted isoform of NCAM on an NCAM-null background die at an early embryonic stage and exhibit serious morphological defects of the neural tube (Rabinowitz et al., 1996). The effects of secreted NCAM in these animals must obviously be accomplished through a heterophilic and not a homophilic binding mechanism, as no membrane-bound NCAM is present. Thus, soluble NCAM (shed or secreted) may act as a paracrine motility regulator that strongly affects the locomotion of NCAM-negative migrating cells. This observation is important as soluble NCAM under normal conditions is present extracellularly in relatively high amounts (Dalseg et al., 1989; Krog et al., 1992; Olsen et al., 1993). Elevated levels have been demonstrated in several neuropsychiatric disorders (Poltorak et al., 1996; van Kammen et al., 1998; Vawter et al., 1999).

We therefore propose that in NCAM-negative cells, the attachment mediated by integrins to fibronectin via the binding

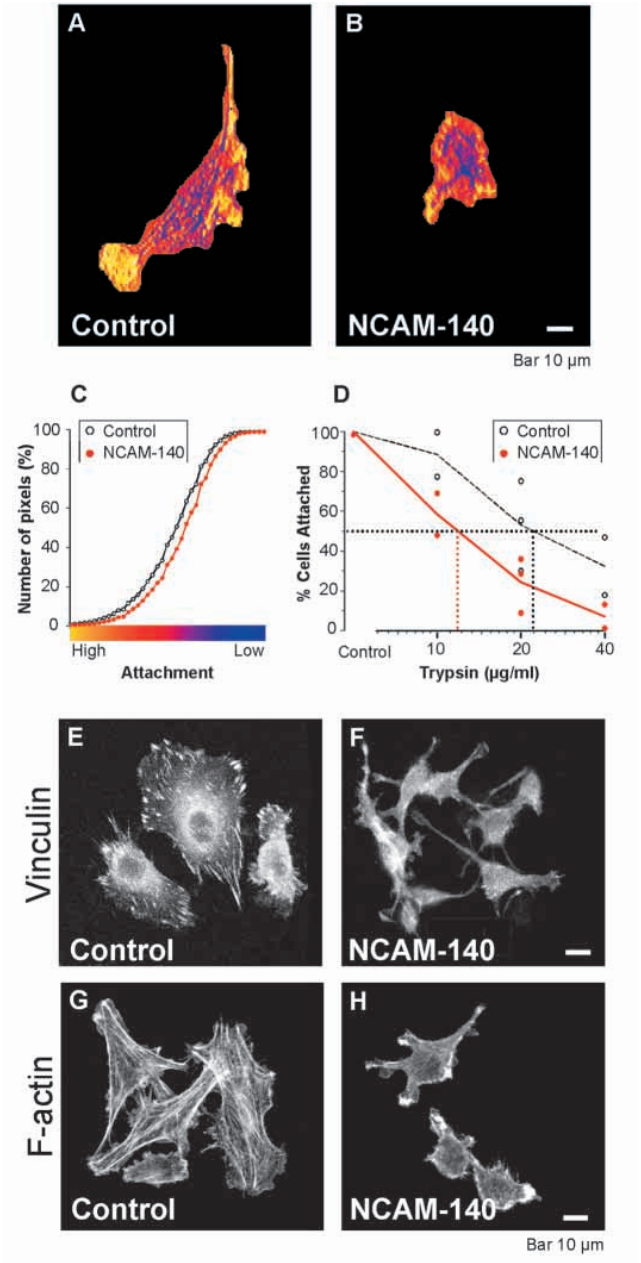

Fig. 4. Effect of expression of NCAM-140 on cell attachment. IRM images of a single cell from a control cell line (A) and a single cell from an NCAM-140-expressing glioma cell line plated on glass (B). Black and white images were transformed to pseudo-colors: white/yellow reflecting the strongest attachment and blue/purple the weakest. (C) Cumulative distribution of attachment strength determined by quantification of the grey levels after IRM (means of more than 150 cells in each group from a representative experiment). The difference in distribution was highly significant ($P<0.001$) in three independent experiments. (D) A detachment assay using trypsin on NCAM-140-positive or -negative cell lines plated as single cells on fibronectin (normalized to 100% attached cells for no trypsin added with values from three independent experiments). The difference in attachment was highly significant ($P<0.001$). (E,F) Immunostaining of focal adhesions of a control (E) and an NCAM-140-expressing cell line (F) plated as single cells on fibronectin using a monoclonal antibody against vinculin. (G,H) Staining of actin stress fibers (F-actin) of a control (G) and an NCAM-140 expressing cell line (H) with Texas-Red conjugated phalloidin.

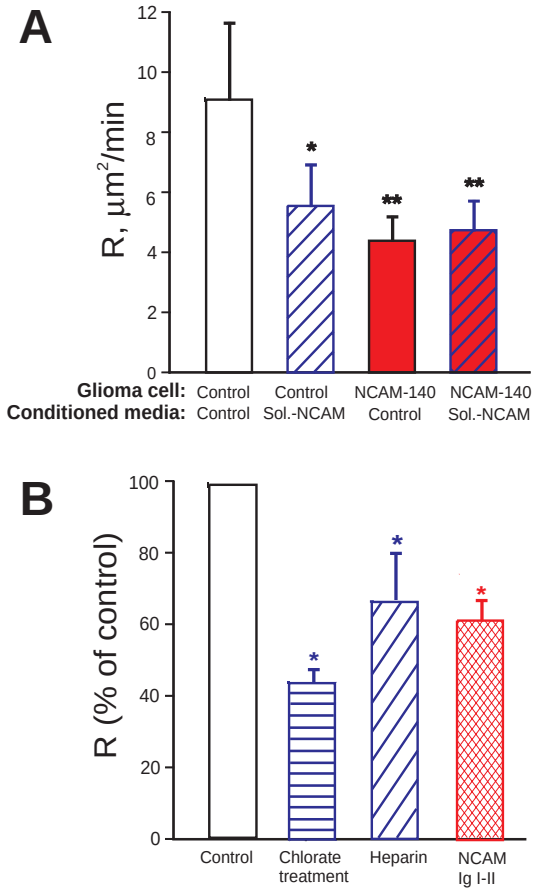

Fig. 5. Effect of extracellular interactions on cell motility. (A) Rate of diffusion, R, of NCAM-140-positive and -negative glioma cell lines after addition of conditioned medium containing a soluble form of the extracellular part of NCAM (Sol.-NCAM) or control conditioned medium (mean of 6 experiments, *$P<0.05$, **$P<0.001$, paired t-test). (B) Rate of diffusion, R, of NCAM-negative glioma cells treated for 20 hours with sodium chlorate (50 mM) or treated for one hour with heparin (50 μg/ml) or recombinant NCAM Ig modules I-II (IgI-II) (100 μg/ml) (mean of 4 experiments, *$P<0.05$, paired t-test).

to the RGD sequence in the latter protein is assisted by HSPGs adhering to fibronectin via an interaction with the heparin-binding domain (HBD), resulting in an adhesion strength compatible with high motility. This hypothesis is supported by the observation that impairment of sulfated proteoglycan function by inhibition of sulfatation by chlorate treatment markedly reduced cellular motility of glioma cells plated on fibronectin. Furthermore, addition of heparin, which can compete for binding to HBD on fibronectin, also induced a reduction of motility, indicating the proteoglycan to be an HSPG and not CSPG. However, our results do not exclude the involvement of CSPGs.

We also show that the first two Ig modules of NCAM can account for the motility-regulating effect of soluble NCAM. Because these two modules contain heparin-binding sites, it is conceivable that this effect is due to an interaction with a heterophilic HSPG receptor. Our data therefore indicate that the extracellular part of NCAM modulates cell motility by binding to a membrane-bound HSPG through the heparin-

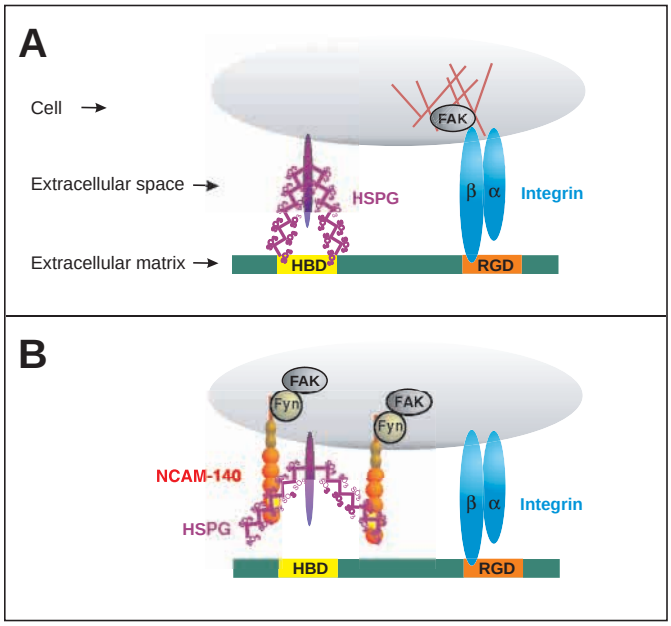

Fig. 6. A model of interactions of NCAM-140 that influence cell motility. (A) Optimal attachment for high motility of an NCAM-negative cell. (B) Decreased attachment and low motility owing to NCAM-140 expression.

binding sites in the first two Ig modules and that this binding affects attachment to the extracellular matrix, subsequently leading to a decreased motility. A potential HSPG candidate is agrin, a major brain HSPG, which binds with high affinity to NCAM (Storms et al., 1996) and which is expressed by both neurons and glial cells. However, agrin does not bind to fibronectin in a solid phase assay (Cotman et al., 1999). Other potential HSPG candidates are the syndecans. All adhesive cells express one or more syndecans in a cell- and development-specific manner. Several forms are predominantly localized at cell-cell contacts, and syndecan-4 colocalizes with focal adhesions. However, the major syndecan of neuronal cells, N-syndecan (syndecan-3) does not bind to fibronectin (reviewed in Carey, 1997). Thus the identity of the HSPG involved has yet to be determined.

In Fig. 6 a model is presented describing some intra- and extracellular interactions in the absence (Fig. 6A) and in the presence (Fig. 6B) of NCAM-140. In Fig. 6A, an NCAM-negative cell is shown with attachment to fibronectin via both HSPGs and integrins, with the integrins connecting to the cytoskeleton via focal adhesions involving $p125^{fak}$. These interactions lead to an attachment compatible with high cellular motility. Fig. 6B shows an NCAM-140-expressing cell, where $p59^{fyn}$ and $p125^{fak}$ have been sequestered by the cytoplasmic part of NCAM-140, leading to a disruption of intracellular structures involved in cell adhesion. In addition, the heparin-binding sites of the first two Ig modules of the extracellular part of NCAM can interact with HSPGs, thereby impairing binding of the latter to fibronectin. All these interactions presumably cause a decrease in attachment leading to a decreased cellular motility.

In conclusion, expression of or exposure to NCAM strongly affects glioma-cell locomotion by both intra- and extracellular mechanisms. Ectopic expression of intracellular domains of NCAM indicates that these domains play key roles in NCAM-mediated motility regulation. Furthermore, the extracellular heparin-binding domain(s) of NCAM were shown to interact with a heterophilic receptor, resulting in a pronounced reduction in cellular motility. Finally, our results identify this motility-regulating receptor to be a membrane-bound heparan sulfate proteoglycan.

We thank D. Bartels for generously providing a plasmid encoding rat NCAM-140 and F.S. Walsh for the plasmids encoding human NCAM-120, -140 and -180. This work was supported by the Danish Cancer Society, the Lundbeck Foundation, the Novo Nordisk Foundation, the Danish Medical Association Research Fund, Fonden til Lægevidenskabens Fremme, Direktør Jacob Madsen and Hustru Olga Madsens Fond, Fru Lily Benthine Lunds Fond af 1/6-78, Købmand M. Kristian Kjær og Hustru Margrethe Kjær, Født La Cour-Holmens Fond, Martha Margrethe og Christian Hermansens Legat and Fru Astrid Thaysens legat.

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