Functional Analysis of Posttranslational Cleavage Products of the Neuron–Glia Cell Adhesion Molecule, Ng-CAM

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Abstract. Neuron–glia cell adhesion molecule (Ng-CAM) mediates cell adhesion between neurons homophilically and between neurons and glia heterophilically; it also promotes neurite outgrowth. In the chick brain, Ng-CAM is detected as glycoproteins of 190 and 210 kD ($Ng\text{-}CAM_{200}$) with posttranslational cleavage products of 135 kD (F_{135}, which contains most of the extracellular region) and 80 kD (F_{80}, which includes the transmembrane and the cytoplasmic domains). To examine the functions of each of these components, we have expressed $Ng\text{-}CAM_{200}$, F_{135}, and F_{80} in murine L cells, and F_{135} and F_{80} as GST fusion proteins in the pGEX vector in bacteria. Appropriately transfected L cells expressed each of these proteins on their surfaces; F_{135} was also found in the media of cells transfected with $Ng\text{-}CAM_{200}$ and F_{135}. In addition to binding homophilically, cells transfected with $Ng\text{-}CAM_{200}$ and F_{135} bound heterophilically to untransfected L cells, suggesting that there is a ligand for Ng-CAM on fibroblasts that may be related to the glial ligand. Detailed studies using the transfected cells and the fusion proteins indicated that both the homophilic and the heterophilic binding activities of Ng-CAM are localized in the F_{135} fragment of the molecule. The results also indicated that proteolytic cleavage of $Ng\text{-}CAM_{200}$ is not required either for its expression on the cell surface or for cell adhesion and that there is an "anchor" for F_{135} on L cells (and presumably on neurons). In contrast to the cell binding results, the F_{80} but not the F_{135} fusion protein enhanced the outgrowth of neurites from dorsal root ganglion cells; this activity was associated with the Fn_{III} repeats of F_{80}. The observations that a protein corresponding to F_{135} contains the cell aggregation sites whereas one corresponding to the F_{80} has the ability to promote neurite outgrowth suggest that proteolytic cleavage may be an important event in regulating these Ng-CAM activities during embryonic development and neural regeneration.

THE neuron–glia cell adhesion molecule (Ng-CAM)[1] is a membrane glycoprotein of the chicken nervous system that is expressed by neurons and Schwann cells and is involved in neuron-neuron and neuron–glia adhesion. Antibody perturbation studies have indicated that it functions in the fasciculation of neurites and in the migration of neurons along Bergmann glial fibers during cerebellar development (9, 27). Ng-CAM on one neuron binds homophilically to Ng-CAM on another neuron. The molecule can also bind heterophilically to an as yet unidentified ligand on astrocytes. Both the homophilic and heterophilic interactions occur in a divalent cation–independent manner. Recently, Ng-CAM was found to bind to the extracellular matrix molecule laminin (22) and to the proteoglycans 3F8 and neurocan (21); it also interacts with the axon-associated proteins axonin-1 and F3/F11 (5, 30).

Expression of Ng-CAM is restricted to the nervous system where it first appears during periods of fiber tract extension and neuronal migration (11, 46). During development, it appears solely on postmitotic neurons in the central nervous system and on neurons and Schwann cells in the peripheral nervous system. The distribution of Ng-CAM on neuronal cell surfaces appears polarized as development progresses, becoming more prevalent on outgrowing axons than on cell bodies and dendrites (11, 46). The levels of expression are also modulated during regenerative events (12) and in mouse mutants with defects in myelination (42).

Ng-CAM has a multidomain structure similar to that of members of the N-CAM family of neural CAMs. It is a member of a closely related subfamily of CAMs that includes the chick neural molecules, Ng-CAM–related CAM (Nr-CAM)/Bravo, and neurofascin, the mammalian proteins L1 and NILE, and ankyrin binding glycoprotein (13, 25, 29, 36, 39, 47). The chick G4 (41) and 8D9 (32) antigens appear to be identical to Ng-CAM. All of these cell sur-

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1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM; FN, fibronectin; DRG, dorsal root ganglion; GST, glutathione-S-transferase.

face glycoproteins have extracellular regions containing six Ig-like domains and four or five fibronectin (FN) type III repeats, and are similar to each other in amino acid sequence, particularly in their cytoplasmic regions. All members of this subfamily are also posttranslationally cleaved in vivo; Ng-CAM, Nr-CAM, and L1 are cleaved at comparable sites in the middle of the third FN type III repeat, whereas proteolysis of neurofascin occurs between the Ig domains and the FN type III repeats (6, 16, 29, 47).

The predominant Ng-CAM component detected in chicken brain is a 135-kD glycoprotein but smaller amounts of a 80-kD glycoprotein and a doublet of 190 and 210 kD (which differ in glycosylation of a single polypeptide) are usually seen (23). All of these components are derived from a single gene and a single mRNA that encodes the larger 190/210-kD species, which we designate here Ng-CAM_{200} (6). The smaller components are generated by proteolysis yielding the amino-terminal 135-kD extracellular fragment (which we designate F_{135}), and the 80-kD transmembrane fragment (F_{80}). F_{135} and F_{80} each contain structural motifs that could contribute to the adhesive functions of Ng-CAM. The F_{135} contains all six Ig-like domains, which in N-CAM and other members of the N-CAM family have been demonstrated to mediate adhesion (4, 10, 40, 49). Furthermore, the amino-terminal segment of F_{80} includes within the third FN type III domain an Arg-Gly-Asp (RGD) sequence that in fibronectin has been demonstrated to mediate adhesion to integrin receptors (43). Both the Ig domains and the FN type III repeats of N-CAM (18) and of L1 (1) have been postulated to promote neurite outgrowth and spreading of neuronal cell bodies, and the FN type III repeats of the axonal protein F3 are thought to be essential for F3-mediated cell adhesion (15).

The diverse pattern of adhesive interactions of Ng-CAM and its complex spatiotemporal distribution during development suggest that it may play multiple roles during morphogenesis. The multidomain structure of this protein and its cleavage into several components further suggest that its individual components may carry out independent functions. To test this hypothesis, we have analyzed each of the components of Ng-CAM in isolation for cell–cell adhesion and the ability to promote neurite outgrowth. cDNAs encoding Ng-CAM_{200}, F_{135}, and F_{80} were each transfected into mouse LM (TK^-) cells, which neither aggregate nor display neural CAMs. Recombinant fusion proteins were also generated for F_{135} and F_{80}. The binding of the transfected cells and untransfected L cells to each other and the binding of these cells, neurons and glia to the fusion proteins and effects on neurite outgrowth were then tested. The combined data indicate that the structural domains within F_{135} mediate the homophilic binding and heterophilic glial binding activities of Ng-CAM, as well as a previously unknown binding to fibroblasts, whereas the F_{80} region contributes to the ability of Ng-CAM to promote neurite outgrowth.

Materials and Methods

cDNA Constructs

The three Ng-CAM constructs (Fig. 1) were prepared using cDNA clones for chicken Ng-CAM (6) (EMBL/GenBank/DDBJ under accession number X56969). A reanalysis of the Ng-CAM sequence revealed a double frame shift in the originally reported sequence (6). Correction of the frame shifts add one amino acid to the reported sequence and changes the reported amino acid sequence only over the segment of residues 54-72 from ISPSSPRSTGGSRWSPDRH to DQPFVPEEHGGVSVVPGSGT. In addition, the nucleotide at the reported position 464 is "G" instead of "A", changing the translated amino residue at that position from a lysine to a glutamic acid. (These corrections were entered in the Genbank/EMBL/DDBJ database on June 1, 1994.)

The λgt11 clones 906, 912, 903, 908, 922, and 913 were subcloned into the M13mp18 vector and linked together using the following strategy (Fig. 1): for the F_{80} construct clone 922 was digested with HphI and ligated to the HphI site of clone 913. The EcoR1 fragment of the 922-913 clone was treated with mung bean nuclease to generate blunt ends and ligated to a cDNA fragment encoding a signal peptide (714) from Nr-CAM (25). Clone 714 was digested with FokI and treated with Klenow to generate blunt ends. The resulting 630-bp fragment was digested with SalI (pBS polylinker site) and the 360-bp FokI/SalI fragment was ligated at the 5′ end of the blunt-ended 922-913 fragment, into the SalI/SmaI-digested Bluescript SK vector. The 714-922-913 construct was excised from Bluescript with HindIII/SalI, ligated to BglII linkers (New England Biolabs, Beverly, MA), and the BglII-digested insert was then ligated into the BamH1 site of PSVK3 (Pharmacia LKB Biotechnology, Alameda, CA), directly behind the SV-40 early promoter. This 80-kD construct was prepared from cDNA clones which lacked the first six amino acid residues of the 80-kD component, but in all other respects would be expressed at the cell surface as the intact 80-kD component.

For the Ng-CAM_{200} construct, the EcoR1 fragment of the 922-913 clone above was ligated into the pRSET B vector (Invitrogen Corp., San Diego, CA), digested with PvuII and ligated to the 856-bp PvuII/PvuII fragment of the λgt11 clone 908. The HindII/HindIII fragment from the 908-922-913 clone was blunt-ended with Klenow and ligated as the last step into the Ng-CAM_{200} construct. For the 5′ end of this construct, the HindIII (M13 polylinker site)/BalI fragment of clone 906 and the EcoR1 (M13 polylinker site)/BalI fragment of clone 912 were ligated together. The resulting 906-912 clone was digested with EcoR1 and partially digested with BstX1. The 630-bp EcoR1/BstXI fragment was ligated to the 2,273-bp EcoR1/BstX1 fragment of clone 903 and into the EcoR1 site of Bluescript. The 906-912-903 clone was digested with HindII and the vector-containing fragment was ligated to the blunt-ended HindII/HindIII fragment of clone 908-922-913. From this final clone the entire EcoR1 fragment was ligated into the EcoR1 site of the PSVK3 vector, behind the SV-40 early promoter.

For the F_{135} construct the 760-bp FokI/FokI fragment from the 200-kD clone in PSVK3 was treated with Klenow to generate blunt ends and ligated to XbaI linkers (New England Biolabs), containing an AMBER stop codon in frame at the 3′ end. This fragment was digested with HindII/XbaI and the 525-bp fragment was ligated into the HindII/XbaI (vector polylinker site) sites of the 200-kD clone which had been recloned into the EcoR1 site of pRSET B (Invitrogen Corp., San Diego, CA). The EcoR1/XbaI (PSVK3 polylinker sites) fragment of this clone was treated with Klenow to generate blunt ends and ligated in the + orientation into the SmaI site of the pcDNA1neo vector (Invitrogen Corp.), directly behind the CMV promoter. The Fok1 site in the Ng-CAM sequence used to add the stop codon is four amino acid residues amino terminal from the beginning of the F_{80} sequence. The F_{135} component terminated at this site may represent a slightly shorter form of the component, although attempts to characterize the carboxyl end of the native chicken F_{135} component have not clearly identified its carboxyl terminal residues (6). The correct orientation and order of the fragments in each of the final constructs were confirmed by restriction analysis and sequence analysis across the ligation junctions.

Cell Culture and Transfection

The Ng-CAM cDNA constructs were transfected into mouse L-M (TK^-) cells (CCL1.3; American Type Tissue Culture Collection, Rockville, MD) using calcium phosphate precipitation of the DNA (14). The Ng-CAM_{200} and F_{80} eukaryotic expression constructs in the PSVK3 vector were cotransfected into L cells with the PSV2neo vector. The F_{135} construct was expressed in the pCDNA1neo vector (Invitrogen Corp.). Clones were selected using G418 (GIBCO BRL, Gaithersburg, MD) at 500 μg/ml (244 μg/ml active). Clones resistant to G418 were cloned by limiting dilution up to three times and selected by immunofluorescent staining with anti-Ng-CAM antibodies.

Primary rat glial cells were prepared according to standard protocols (20).

GST Fusion Proteins

cDNA constructs for the pGEX fusion proteins were modifications of the transfection constructs. For the F_{80} construct, the 922–913 insert from Bluescript was excised with EcoR1, treated with Klenow to generate blunt ends, and ligated into the Sma1 site of pGEX2T (Pharmacia LKB Biotechnology). For the constructs of F_{135} and Ng-CAM_{200}, the signal peptide-encoding segment at the 5′ region was replaced with a short PCR product beginning at the amino terminus of the mature protein. A PCR product from nucleotide 119–685 of the Ng-CAM sequence and containing an EcoR1 site at the 5′ end was digested with EcoR1 and partially digested at nucleotide 630 with BstXl, yielding a 516-bp fragment. This EcoRl/BstXl fragment was ligated to the BstXl site of the 3,359 fragment from the EcoRl/BstXl digest of the Ng-CAM_{200} construct in PSVK3. The resulting ligation product was ligated into the EcoRl site of pGEX1λT (Pharmacia Fine Chemicals, Piscataway, NJ). For the F_{135} pGEX construct, the 516-bp EcoRl/BstX1 PCR fragment from above was ligated into the EcoRl/BstXl sites of the F_{135} construct in pSVK3. The PCR-F_{135} was digested at its 3′ end only with Xbal, blunt-ended with Klenow, and ligated to EcoRl linkers. Upon excision by EcoRl digestion, the entire PCR-F_{135} insert was ligated into the EcoRl site of pGEX1λT.

Fusion proteins spanning Fn_{III} repeats 3–5 and 4–5 of Ng-CAM were generated by PCR using a 5′ primer corresponding to the amino terminal boundary of either the third or fourth Fn_{III} repeat and a common 3′ primer corresponding to a region just before the transmembrane domain. In both cases the 5′ primer contained a BamH1 restriction site and the 3′ primer contained an EcoR1 restriction site. DNA fragments of 980 bp for FN3-5 and 635 bp for FN4-5 were amplified from Ng-CAM cDNA, excised and purified from a 1.5% agarose gel, digested with BamH1/EcoR1, and cloned into the BamH1/EcoR1 sites of pGEX4T2. The 5′ and 3′ PCR primers used were as follows:

FN3-5: GCGGGATCCAATGTGGGGGTGGAACTGCTG,
FN4-5: GCGGGATCCCCCGGCCCCCCCGAGGAGCTC,
FN3-5 and FN4-5: GCGGAATTCCCACCCCTTGGTGCAAACCCC.

The fusion proteins were produced by transforming *Escherichia coli* NM522 cells (Stratagene Corp.) with the pGEX constructs. Fusion proteins were produced using modifications (38) of standard protocols (34). SDS-PAGE of the F_{135} fusion protein yielded a major protein of 140 kD and several minor components at ~100 kD. The F_{80} fusion protein yielded a major component at ~70 kD, corresponding to the predicted size of the glutathione-S–transferase segment plus the Ng-CAM insert, and several small components. All of these components immunoblotted with anti-Ng-CAM antibodies. Attempts were made to prepare the Ng-CAM_{200} fusion proteins, but yields were too low for further analysis, possibly due to its large size. Expression of the Fn3-5 construct yielded a protein product of ~70 kD with some degradation products of 40 kD. The Fn4-5 construct yielded a product of ~60 kD with degradation products of 35 kD.

Antibodies

Polyclonal rabbit antibodies against all three components of chicken Ng-CAM were prepared as described (23). Antibodies raised against GST fusion proteins of the 200 kD (anti-Ng-CAM_{200}), 135 kD (anti-F_{135}), and cytoplasmic portions of Ng-CAM were obtained by immunizing rabbits at 4 wk intervals with 200 μg protein in PBS/Freund's Adjuvant as described (3). Rabbits were bled after the third injection and Fab′ prepared as described (3). Polyclonal rabbit antibodies to chick glia were previously described (20). Polyclonal antibodies to E4 chick embryos and embryonic fibroblasts were obtained by immunizing rabbits with embryonic lysates or dissociated fibroblasts by standard protocols. Polyclonal antibodies to all forms of mouse laminin were purchased (Sigma Chemical Co., St. Louis, MO) and human L1 monoclonal antibodies were a gift from Dr. John Hemperly (Becton Dickinson Research Center, Research Triangle Park, NC).

Immunofluorescent Staining

Cells were cultured on poly-L-lysine–coated 26-well Teflon slides (Celline Associates, Newfield, NJ) slides, fixed with 4% paraformaldehyde, and stained as described (35).

Cell–Cell Aggregation Assays

In aggregation assays, subconfluent cultures of cells transfected with Ng-CAM_{200} and F_{80} were treated for 12 h with medium containing 10 mM sodium butyrate to enhance expression of the proteins driven by the PSVK3 promoter. To prepare single cell suspensions, cells were incubated in PBS/2% FCS/5 mM EDTA for 10 min, collected in SMEM (GIBCO BRL) containing 20 mM Hepes (pH 7.4) and 50 μg/ml DNAse (SMEM-DNAse). The cells were centrifuged and resuspended in SMEM-DNAse. 4×10^5 cells in 600 μl SMEM-DNAse/3 mM EDTA were used for aggregation assays. Aggregations were done at 37°C at 100 rpm for 40 min in 24-well bacteriological plates that had been previously incubated for 2 h with PBS/2% BSA. The assays were stopped and the cells were fixed in PBS/1% glutaraldehyde. Calcium-containing experiments were done using SMEM-DNAse/2 mM $CaCl_2$. Cell suspensions were preincubated on ice for 30 min before being assayed for aggregation, with or without anti-Ng-CAM Fab′ fragments (0.2 μg/μl) as indicated in the tables and figure legends. Cell–cell binding was monitored by measuring the disappearance of single cells using a Coulter counter.

Coaggregation Experiments

Cells were removed from dishes with PBS/2% FCS/5 mM EDTA and labeled in SMEM-DNAse either with 3 μg/ml Fast diI or 20 μg/ml Fast diO (Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C. 3×10^5 cells of each type were mixed and aggregated as described above. Aggregates were viewed and photographed under a fluorescence microscope.

Cell–Substrate Adhesion: Gravity Assay

In the gravity binding assay, 3.5 cm bacteriological dishes (No. 1008; Falcon Plastics, Cocheysville, MD) were spotted in a circular dot pattern with 2 μl of Ng-CAM fusion proteins (5 μmol/ml). The positive binding control was 2 μl poly-L-lysine (100 μg/ml). Plates were incubated for 60 min at room temperature. The solutions were aspirated, and the dishes washed twice and blocked for 60 min at room temperature with 250 μl PBS/2% BSA. The blocking solution was aspirated and cells were added in SMEM-DNAse/2 mM EDTA, and incubated for 60 min at 37°C. The plates were washed with PBS/2 mM EDTA and fixed with 1% glutaraldehyde/PBS. The number of cells bound to the protein or poly-L-lysine substrates was determined by using an eyepiece grid to count the number of cells bound in each of 25 specific grid areas per spot (0.410 mm^2). Duplicate fusion protein or poly-L-lysine spots were used per dish to ensure consistency for the conditions for binding.

Cell Substrate Adhesion: Centrifugation Assay

In the centrifugation assay, 1.1×10^4 of indicated cells in 100 μl were centrifuged 60 s at 1,000 rpm in each U-shaped well of 96-well dishes (No. 3910; Falcon Plastics) that had been precoated with the protein to be tested, as previously described (19). The diameter of the cell-free area inside the ring of pelleted cells is used as a measure of the adhesivity of the substrate for the cells (19).

Quantitation of Protein Binding to Plastic

The fusion proteins were iodinated as previously described (38) and the protein concentrations were determined by the modified Lowry method to determine the specific activity. Volumes of 40 μl containing various concentrations of the iodinated proteins were incubated in the wells of 96-well dishes for 60 min at room temperature. After 60 min, the wells were washed three times with PBS, blocked 60 min with 2% BSA, and rinsed three times with PBS. The radioactivity bound to each well was released (>95%) with 100 μl 0.1 M KOH and counted in a Gamma counter. From the specific activity, the amount of protein bound was determined and found to be consistently 3–4% of the protein concentration in the incubation solution for F_{135}, and 8–10% for F_{80}.

Covasphere Aggregation

A modification of the cell aggregation assay was carried out with MX-Covaspheres (0.54 μm green or 0.71 μm red; Duke Scientific Corporation, Palo Alto, CA) covalently bound to the fusion protein. Each aggregation experiment used 5 μl green Covaspheres (850 cm^2/ml) or 3 μl red Covaspheres (850 cm^2/ml) bound with 2 μg 80GST protein or 2 μg 135GST protein, respectively. The amounts of each fusion protein bound to these two amounts of the Covaspheres was determined by the Lowry method (33) and were approximately equivalent in terms of moles of protein per surface area of the Covaspheres. Any untreated sites remaining on the

Covaspheres were neutralized with 2% BSA in PBS. The Covaspheres were preincubated with or without Fab' (0.5 mg/ml) on ice for 30 min, sonicated for 15–20 s, mixed, and aggregated without shaking in 110 μl vol of PBS/2% BSA/10 mM NaN_3 for 2 h at room temperature. The appearance of superthreshold aggregates was determined on a Coulter counter as previously described (20) or visualized by fluorescence microscopy.

Neurite Outgrowth Assay

For neurite outgrowth experiments, all steps were carried out in a 3.5 cm bacteriological culture dish as for the cell–substrate adhesion assay. Dorsal root ganglia were dissected from day six chicken embryos and placed in HBSS. The ganglia were placed in calcium, magnesium-free (CMF) HBSS and incubated at 37°C for 10 min. The ganglia transferred to 0.08% trypsin in CMF-HBSS and incubated at 37°C for 20 min. An equal volume of DME/F12, 10% FCS, 20 ng/ml NGF, 10 μg/ml gentamicin (10% medium), was added. The ganglia were pelleted and resuspended in 2 ml of 10% medium and triturated with a fire polished Pasteur pipette for 15 strokes. The single cell suspension was washed once with 10% medium, and preplated in a 10 cm tissue culture dish for 1 h at 37°C, 5% CO_2. After 1 h, the medium containing a cell population enriched for DRG neurons was removed and the cells were pelleted and washed two times with DME/F12, 1% FCS, 20 ng/ml NGF, 10 μg/ml gentamicin (1% medium). Cells were resuspended at a density of 2×10^4 cells/ml in 1% medium and 300 μl of cell suspension was added to the center of the plates. The plates were placed in a humidified chamber and incubated at 37°C, 5% CO_2 for 15 h. After the growth period, the cells were fixed with 1% glutaraldehyde and the number of cells that sprouted neurites were counted and neurite length was measured by phase contrast microscopy.

Immunoprecipitation and Immunoblotting

Cells were grown to confluence in 15 cm dishes in DME containing 10% bovine calf serum, penicillin/streptomycin (50 μ/ml), 1 mM L-glutamine at 37°C with 7% CO_2. Cells were treated with 10 mM sodium L-butyrate overnight prior to the experiment and solubilized in lysis buffer composed of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM EGTA, 1% Triton X-100, 10% glycerol, and proteases inhibitors (10 μg/ml, aprotinin; 10 μg/ml, leupeptin; 2 μg/ml, pepstatin; 1 mM PMSF). Cell lysates were immunoprecipitated with 20 μg/ml of rabbit polyclonal anti-Ng-CAM antibodies that recognize all three Ng-CAM components for two hours at 4°C followed by a 30 min addition of 5 mg protein A–Sepharose beads. Immunoprecipitates were washed in lysis buffer, boiled in Laemmli sample buffer, and the proteins were resolved on a 7.5% SDS–polyacrylamide gel and electrophoretically transferred to Immobilon membranes (Millipore Corp., Bedford, MA). Immunoblotting was performed with the same antibody at 5 μg/ml, unless otherwise specified, and blots were incubated with 50 μCi ^{125}I-labeled protein A, washed in PBS/0.1% Triton X-100 for 1 h and exposed for 15 h to XAR film at −70°C. To detect Ng-CAM in the culture supernatants of transfected cells, cells were grown for 5 d (1×10^7 cells/dish, final density), medium was harvested, cleared by centrifugation at 4°C for 15 min at 3,000 rpm, and immunoprecipitated as described above. For direct detection of Ng-CAM in cells, transfected cells were plated at 1×10^6 per well in a 24-well tissue culture dish, cultured overnight in the presence of 10 mM sodium butyrate, lysed, boiled in Laemmli buffer, and immunoblotted as described above.

F_{135} Binding to L Cells

Subconfluent cultures of L cells (1×10^6) were released into single cell suspensions by treatment with 5 mM EDTA. Cells were washed and incubated with 5 ml of 5-d-old culture supernatants derived from 1×10^7 Ng-CAM_{200} transfected cells or from control untransfected L cells for one hour at 4°C or for 30 min at 37°C in the presence of 3 mM EDTA. Cells were washed, and either solubilized in lysis buffer and immunoblotted with anti-Ng-CAM antibodies, or tested for cell–cell aggregation as described above. For immunofluorescent staining, L cells were plated overnight on poly-L-lysine–coated 26-well Teflon glass slides at 1,500 cells per well. Cells were then incubated with either culture media from 5-d-old Ng-CAM_{200} or from untransfected L cells, and stained with anti-Ng-CAM antibodies as described above.

Lactoperoxidase Labeling of Cell Surface Proteins

Ng-CAM_{200} transfected cells were freshly passaged overnight, washed in PBS and incubated in 4 ml PBS with 1 mCi ^{125}I, 20 U of lactoperoxidase (Calbiochem-Behring Corp., La Jolla, CA) 20 μl of 0.06% H_2O_2 for 10 min at room temperature, followed by addition of 20 μl 0.06% H_2O_2 twice for 5 min each. The enzymatic reaction was stopped by addition of 100 μl of 100 mM NaI, followed by five washes with PBS, including 1 mM NaN_3. Cells were subsequently solubilized and immunoprecipitated with anti-Ng-CAM antibodies. Proteins were resolved on 7.5% SDS-PAGE and the dried gels were exposed overnight on XAR film at −70°C with an intensifying screen.

Results

To examine the binding of the specific Ng-CAM components, cDNAs (Fig. 1) corresponding to the full-length Ng-CAM (Ng-CAM_{200}), the 135-kD fragment (F_{135}), and the 80-kD fragment (F_{80}) were constructed from previously described clones (reference 6; and for details see Materials and Methods), and transfected into murine LM (TK^-) cells. F_{135} and F_{80} were also prepared as fusion proteins with glutathione-S-transferase (GST) in bacteria in the pGEX expression vector.

Biochemical Characterization of Ng-CAM–transfected Cells

Permanently transfected L cell lines expressing Ng-CAM_{200}, F_{135}, and F_{80} were identified by immunofluorescent staining using polyclonal antibodies raised to all three components of Ng-CAM (Fig. 2). The antibodies showed no reactivity with untransfected L cells or with L cells

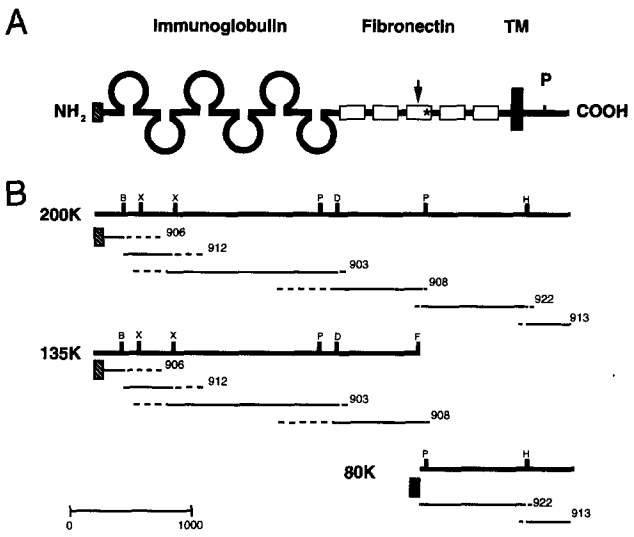

Figure 1. Structure of Ng-CAM and cDNA constructs. (*A*) Model of the domain structure of Ng-CAM. The six immunoglobulin-like domains (*circles*), five fibronectin-type III repeats (*open rectangles*), transmembrane region (*TM, vertical black rectangle*), and the phosphorylated cytoplasmic region (*P*) are shown. Cleavage of the protein occurs in the third fibronectin-type III repeat indicated by the arrow; the single RGD sequence is represented by an asterisk. (*B*) The regions of the protein encoded by the cDNA constructs are represented by heavy black lines below the model. Portions of the original cDNA clones used to create the constructs are indicated by unbroken lines below the construct, with the dashed lines indicating the remainder of each original clone. The signal peptides used in constructs are represented as shaded boxes. Restriction sites used for ligation of the constructs are noted: *B*, BalI; *X*, BstX1; *P*, Pvu II; *D*, Hind II; *F*, Fok1; *H*, Hph1.

transfected with the neomycin vector only. Seven lines expressing Ng-CAM$_{200}$, five expressing F_{135} and four expressing F_{80} were isolated. Those cell lines expressing the highest levels of each component were selected for further analysis.

Each cell line expressed proteins on the cell surface as detected by immunofluorescence (Fig. 2), and the proteins expressed were of the expected sizes as indicated by immunoblots (Fig. 3). Ng-CAM$_{200}$–transfected cells (Fig. 3 *A*, lane *2*) showed a doublet at 190/210 kD and a minor component at 135 kD, which comigrated with their respective counterparts in chick brain (lane *1*). ^{125}I labeling with lactoperoxidase confirmed that the 190 and 210 kD components were present on the surface (lane *5*). A 135-kD component was detected in F_{135}-transfected cells (lane *3*). In some experiments lower molecular weight components were observed that reacted with the anti-Ng-CAM antibodies indicating that they may be proteolytic products. The F_{135} transfectants showed a more diffuse staining pattern in the Golgi in addition to cell surface staining (Fig. 2 *d*) that may reflect processing of the F_{135} translation product that is different from the Ng-CAM$_{200}$ and the F_{80} products, which showed predominantly cell surface localization

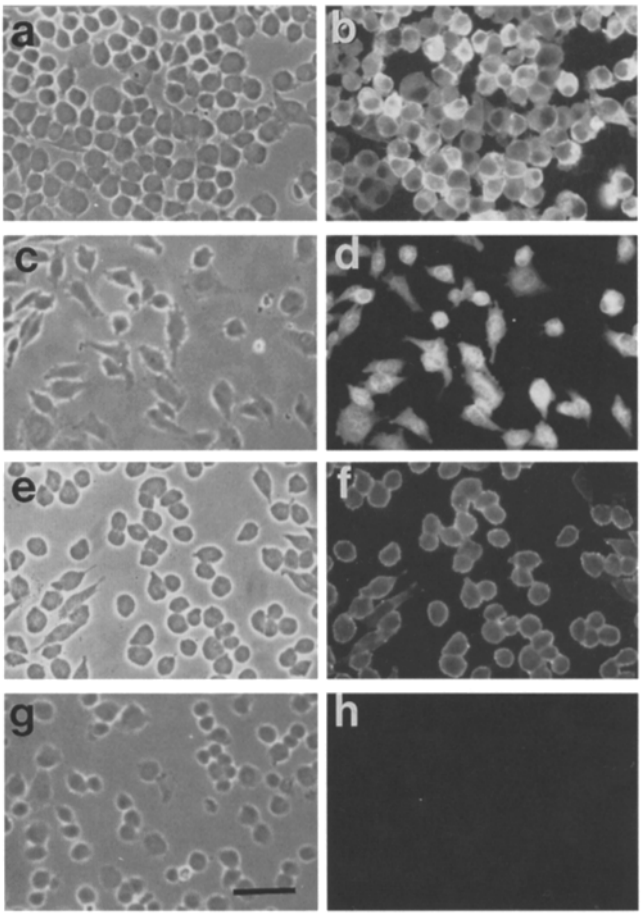

Figure 2. Cell surface expression of Ng-CAM in transfected cells. Matched phase-contrast (*a*, *c*, *e*, and *g*) and fluorescence photographs (*b*, *d*, *f*, *h*) of butyrate-treated L cells transfected with Ng-CAM$_{200}$ (*a* and *b*), F_{135} (*c* and *d*), F_{80} (*e* and *f*), or untransfected L cells (*g* and *h*), fixed with 4% paraformaldehyde, and stained with rabbit antibodies to all three components of Ng-CAM. Bar, 50 μm.

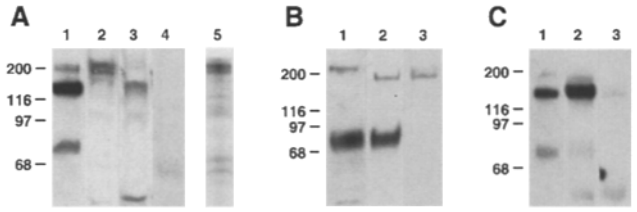

Figure 3. Biochemical analysis of Ng-CAM components in transfected cells. (*A*) Triton X-100–soluble extracts from Ng-CAM$_{200}$ (lane *2*), and F_{135} (lane *3*) transfected monolayers (1×10^6 cells), compared to extracts of untransfected L cells (lane *4*) and chicken brain extract (20 μg, lane *1*), were boiled in sample buffer. Lysate proteins were resolved on SDS-PAGE and immunoblotted with anti-Ng-CAM antibodies. Lane *5* contains Ng-CAM immunoprecipitated from 1×10^6 lactoperoxidase-labeled Ng-CAM$_{200}$ cells. (*B*) Extracts of Ng-CAM$_{80}$ transfected monolayers (1×10^6, lane *2*), L cells (lane *3*), and chick brain (lane *1*) were immunoblotted with antibodies to the cytoplasmic domain of Ng-CAM. (*C*) 5-d culture supernatants from 1×10^7 Ng-CAM$_{200}$ cells (lane *2*) and 3×10^7 F_{135} cells (lane *3*) were immunoprecipitated and blotted with anti-Ng-CAM antibodies, and compared to extracts of chick brain (20 μg, lane *1*).

(Fig. 2, *b* and *f*, respectively). Although F_{80} was recognized on transfected cells by immunofluorescence with the Ng-CAM antibodies, it was not recognized well in immunoblots with these antibodies. In contrast, this component was recognized very well by rabbit antibodies to the cytoplasmic portion of Ng-CAM (Fig. 3 *B*). These rabbit antibodies also reacted with a 180-kD component in untransfected L cells indicating that there is a molecule in L cells immunologically related to the cytoplasmic domain of Ng-CAM; this molecule was not recognized, however, by any antibodies that react with the extracellular portion of Ng-CAM. The cytoplasmic domain antibodies stained the Ng-CAM$_{200}$ and the F_{80} transfectants with equal intensity, suggesting equivalent amounts of protein on the two transfected cell lines.

In vivo, Ng-CAM is cleaved proteolytically to give F_{135} and F_{80}. This cleavage also occurred in the Ng-CAM$_{200}$-transfected L cells. Some F_{135} was apparent in immunoblots of lysates of Ng-CAM$_{200}$ transfected cell monolayers (1×10^7 cells/dish), and F_{135} could be immunoprecipitated from the culture media of these cells (Fig. 3 *C*, lanes *2* and *3*). The levels of F_{135} in the media increased with culture time and reached maximal levels at 3–4 d. The media from F_{135}-transfected cells (3×10^7 cells) also contained some F_{135}; the amount was less than that seen for the Ng-CAM$_{200}$ cells probably because the F_{135} cells generally expressed less Ng-CAM protein than the Ng-CAM$_{200}$ transfectants.

Although F_{135} was found in the media of the F_{135} transfected cells, a significant amount of the protein remained attached to the cells as revealed by immunoblots and immunofluorescence (Figs. 2 and 3). In addition, the F_{135} transfected cells mediated adhesion that was inhibited by antibodies to Ng-CAM (see below, Table I). The F_{135} has no transmembrane region and because the F_{135} transfected L cells contain no Ng-CAM$_{200}$ or F_{80}, the F_{135} must be anchored to the cell via another molecule. In accord with this notion, when untransfected L cells were incubated with media containing F_{135} from the Ng-CAM$_{200}$ transfected

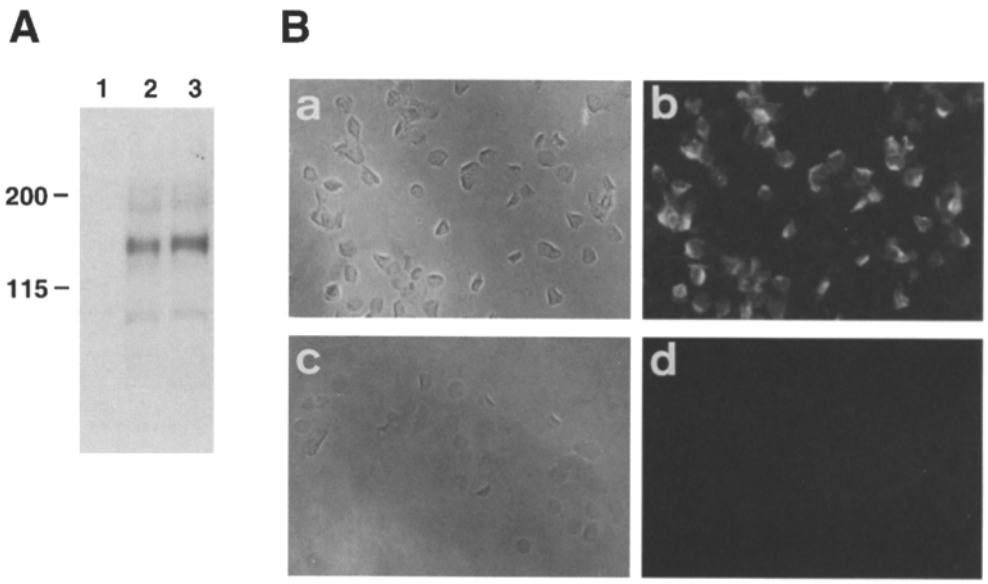

Figure 4. F_{135} binds to the surfaces of L cells. (*A*) EDTA-released L cell suspensions (1×10^6) were incubated with culture supernatants from untransfected L cells at 4°C for 2 h (lane *1*), or with culture supernatants from Ng-CAM_{200}-transfected cells at 4°C for 2 h (lane *2*), or at 37°C for 30 min (lane *3*). Cells were washed and lysed in lysis buffer. The proteins were then resolved on SDS-PAGE and immunoblotted with anti-Ng-CAM antibodies. (*B*) L cells (1.5×10^3) that were plated overnight on 26-well slides coated with poly-L-lysine were incubated with conditioned culture media from Ng-CAM_{200}-transfected cells for 1 h at 4°C (*a* and *b*) or with conditioned media from untransfected L cells (*c* and *d*). The monolayers were then washed, fixed with 4% paraformaldehyde and stained with anti-Ng-CAM antibodies as described in Materials and Methods. Fluorescent micrographs (*b* and *d*) are matched with phase contrast micrographs (*a* and *c*).

cells, the F_{135} bound to the untransfected L cells (Fig. 4 *A*). Equivalent amounts bound at 4° and at 37°C and immunofluorescent staining showed Ng-CAM reactivity on the cell surface (Fig. 4 *B*). Moreover, after binding the F_{135}, the cells aggregated under conditions in which native L cells did not aggregate, and this aggregation was inhibited by anti-Ng-CAM Fab′ (Table I, *bottom*).

Cell Aggregation

To test for cell–cell aggregation, the transfected cell lines were placed in rotating suspension cultures and cell aggregation was measured over time (Table I). Cells transfected with either Ng-CAM_{200} or F_{135} aggregated 18–28% in the absence of calcium, and the aggregation was inhibited 22–89% by Fab′ fragments of antibodies raised against native Ng-CAM or against the Ng-CAM-GST fusion proteins. Neither untransfected L cells nor F_{80} transfected cells aggregated under these conditions (Table I).

To test whether the aggregation had a heterophilic component, the ability of Ng-CAM_{200}- and F_{135}-transfected cells to aggregate with untransfected L cells was assessed. When Ng-CAM_{200}- or F_{135}-transfected cells were labeled with the fluorescent marker diO and incubated with untransfected L cells labeled with diI, the two cell types coaggregated (Fig. 5). No self-aggregation was seen with fluorescently labeled L cells alone (Fig. 5 *e*) or L cells transfected with F_{80} or with the neomycin resistance vector only (not shown). These results suggest that there is a heterophilic ligand for Ng-CAM on L cells, since these cells do not express Ng-CAM. Moreover, the co-aggregation of both Ng-CAM_{200}- and F_{135}- transfected cells with L cells was inhibited by heterologous antibodies that bind L cells but that do not detect Ng-CAM. When Ng-CAM_{200} transfected cells were preincubated with Fab′ fragments of antibodies to glial cells or an antibody to E4 chick embryos that do not recognize Ng-CAM, aggregation in the presence of EDTA (21%) was inhibited by 60–68% (Table II).

*Table I. Aggregation of Cells in the Absence of Divalent Cations**

Cell clone	Fab′ added	Percent aggregation	Percent inhibition
Ng-CAM_{200}/L cell transfectant	–	23 ± 4	–
	anti-Ng-CAM	11 ± 3	52
Ng-CAM_{200}/L cell transfectant	–	28 ± 3	–
	anti-Ng-CAM_{200}	15 ± 2	47
	anti-F_{135}	22 ± 1	22
F135/L cell transfectant	–	18 ± 3	–
	anti-F_{135}	2 ± 2	89
	anti-Ng-CAM_{200}	11	47
F_{80}/L cell transfectant	–	7 ± 3	–
L cells	–	4 ± 4	–
L cells + Ng- CAM_{200} cell media	–	16 ± 1	–
	anti-F_{135}	5 ± 1	69
L cells + L cell media	–	0	–

* Assays were performed in EDTA-containing SMEM as described in Materials and Methods and the accumulation of aggregates was determined using a Coulter counter. The results are averages ± SEM of a minimum of three separate experiments.

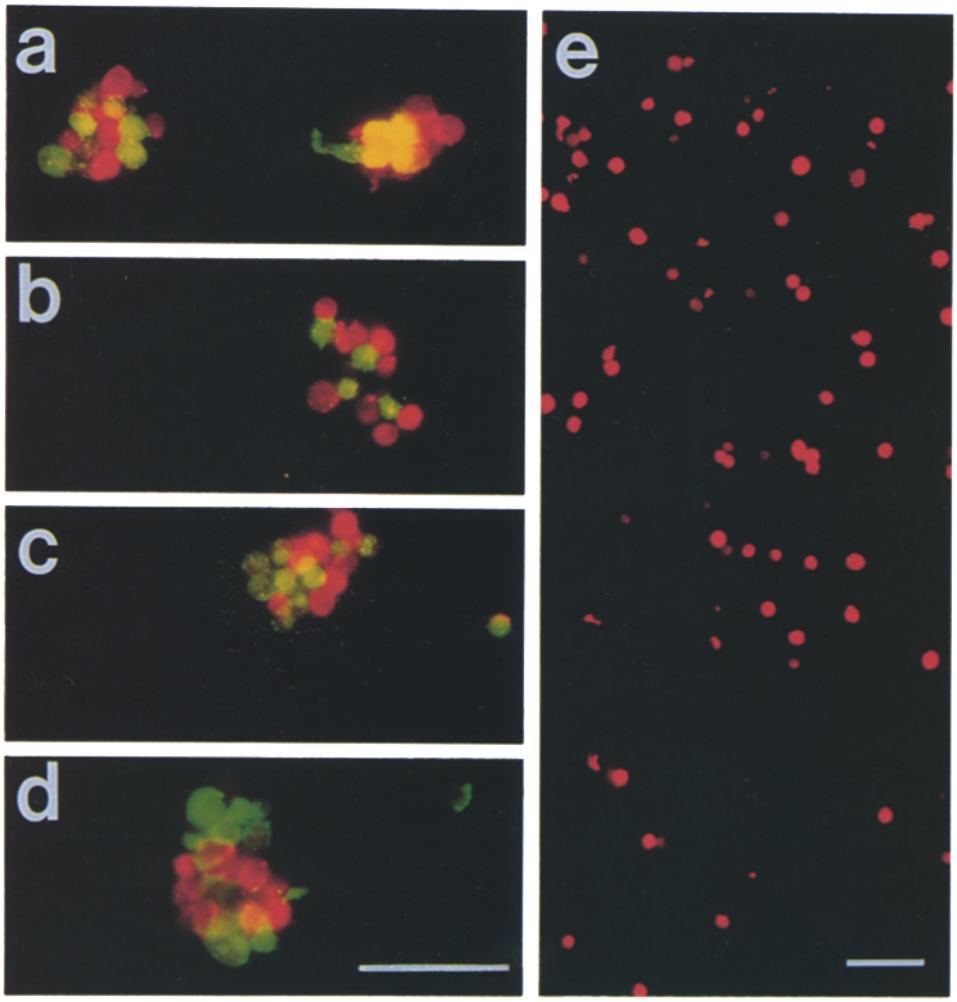

Figure 5. Coaggregation of Ng-CAM–transfected cells and untransfected L cells. Fluorescence photographs of coaggregates between untransfected L cells labeled with the fluorescent dye DiI (*red*) and fluorescent diO (*green*) labeled Ng-CAM_{200} (*a–c*) or F_{135}-transfected L cells (*d*), or untransfected L cells alone (*e*). Yellow appears where green and red cells are superimposed. Experiments were performed as described in Table I. Bar, 50 μm.

In contrast, an antibody to chick fibroblasts had no effect on aggregation.

Substrate Binding and Covasphere Aggregation

The cell aggregation assays suggested that Ng-CAM–expressing cells could bind heterophilically to an unidentified ligand on L cells in the absence of calcium. This heterophilic binding precluded our ability in these assays to analyze the ability of Ng-CAM to bind homophilically. We therefore produced fusion proteins of F_{135} and F_{80} with GST in bacteria and used these in cell substrate attachment assays (see Materials and Methods). Each fusion protein was produced in good yield and contained proteins of the size predicted for the unglycosylated Ng-CAM segment (6) plus 26 kD for the GST portion (not shown).

The fusion proteins were adsorbed onto plastic dishes and analyzed for their ability to bind single cells in a centrifugation assay; GST was also coated as a substrate to control for attachment to the GST portion of the bacterial fusion proteins. Binding of each protein to the plastic dishes was quantitated (see Materials and Methods) and was proportional to the concentration of the protein solution used to coat the dishes. Equimolar amounts of bound protein were used for each fusion protein.

Ng-CAM_{200}-transfected cells bound to the F_{135} fusion

*Table II. Effect of Heterologous Antibodies on Ca^{2+}-independent Cell Aggregation**

Cell clone	Fab′ added	Percent aggregation	Percent inhibition
Ng-CAM_{200}/L cell transfectant	–	25 ± 1	–
	anti-Ng-CAM_{200}	15 ± 1	40
	anti-chick embryo	10 ± 2	60
	anti-glia	8 ± 1	68
	anti-fibroblast	24 ± 3	4
	(anti-Ng-CAM_{200}) + (anti-chick embryo) + (anti-glia)	7 ± 2	72

* Ng-CAM_{200}-expressing L cells were preincubated for 30 min on ice with the antibodies indicated, washed 2× in EDTA-containing SMEM and aggregation assessed as described in Materials and Methods. Accumulation of aggregates was determined using a Coulter counter. The results are averages ± SEM of a minimum of three separate experiments.

protein substrate, but not to the F_{80} substrate or to control GST-coated substrates (Fig. 6 *a*). The extent of binding was proportional to the amount of F_{135} coated on the dishes and cells bound maximally to 1.0 pmol of F_{135} fusion protein. At least a portion of this binding was homophilic because the attachment of Ng-CAM_{200} cells to the F_{135} substrate was inhibited when the cells were preincubated with Fab′ fragments of antibodies to the Ng-CAM-fusion protein (Fig. 6 *c*). The attachment of the cells was also substantially inhibited when the same Fab′ fragments were incubated with the substrate.

To obtain additional evidence for homophilic binding mediated by the F_{135} region, the F_{135} and F_{80} fusion proteins were coupled to Covaspheres, and tested for their ability to aggregate (Table III). F_{135} Covaspheres aggregated and the aggregation was inhibited 89% by anti-Ng-CAM Fab′ fragments. In contrast, Covaspheres coupled with F_{80} GST-fusion protein, GST alone, or BSA did not aggregate with themselves. Moreover, F_{80}-GST Covaspheres did not coaggregate with the F_{135}-GST Covaspheres (Fig. 7).

Binding of untransfected L cells to the F_{135} fusion protein also provided additional support for heterophilic binding between Ng-CAM and L cells. L cells bound to the F_{135} substrate, but not to the F_{80} or GST substrates (Fig. 6 *b*). Binding of L cells to F_{135} had the same concentration dependence as that of Ng-CAM_{200}-transfected cells. In this case, however, binding of the untransfected cells was inhibited only when the F_{135} substrate, and not the cells, was preincubated with anti-Ng-CAM Fab′ (Fig. 6 *d*). In addition, adhesion was blocked (Fig. 6 *f*) when the

*Table III. Covasphere Aggregation**

Protein	Fab′ added	Superthreshold particles	Percent inhibition
F_{135}-GST	–	910 ± 7	–
	anti-Ng-CAM_{200}	100 ± 2	89
	anti-fibroblast	1,345 ± 50	0
F_{80}-GST	–	158 ± 19	–
	anti-Ng-CAM_{200}	154 ± 22	0
	anti-fibroblast	90 ± 9	43
GST	–	49 ± 3	
anti-Ng-CAM_{200}		44 ± 2	10
	anti-fibroblast	95 ± 36	0
BSA	–	51 ± 3	–
	anti-Ng-CAM_{200}	56 ± 6	0
	anti-fibroblast	83 ± 4	0

*Covaspheres were coupled to specific Ng-CAM-GST fusion proteins, GST alone, or BSA alone, preincubated on ice for 30 min alone or with Fab′ fragments of the antibodies indicated, sonicated, and after 2 h, the appearance of superthreshold aggregates was measured using a Coulter counter as described in Materials and Methods, using the following settings: lower threshold = 9.8, upper threshold = 100, 1/aperture = 1/4, 1/current = 1/4. Counts are averages ± SEM.

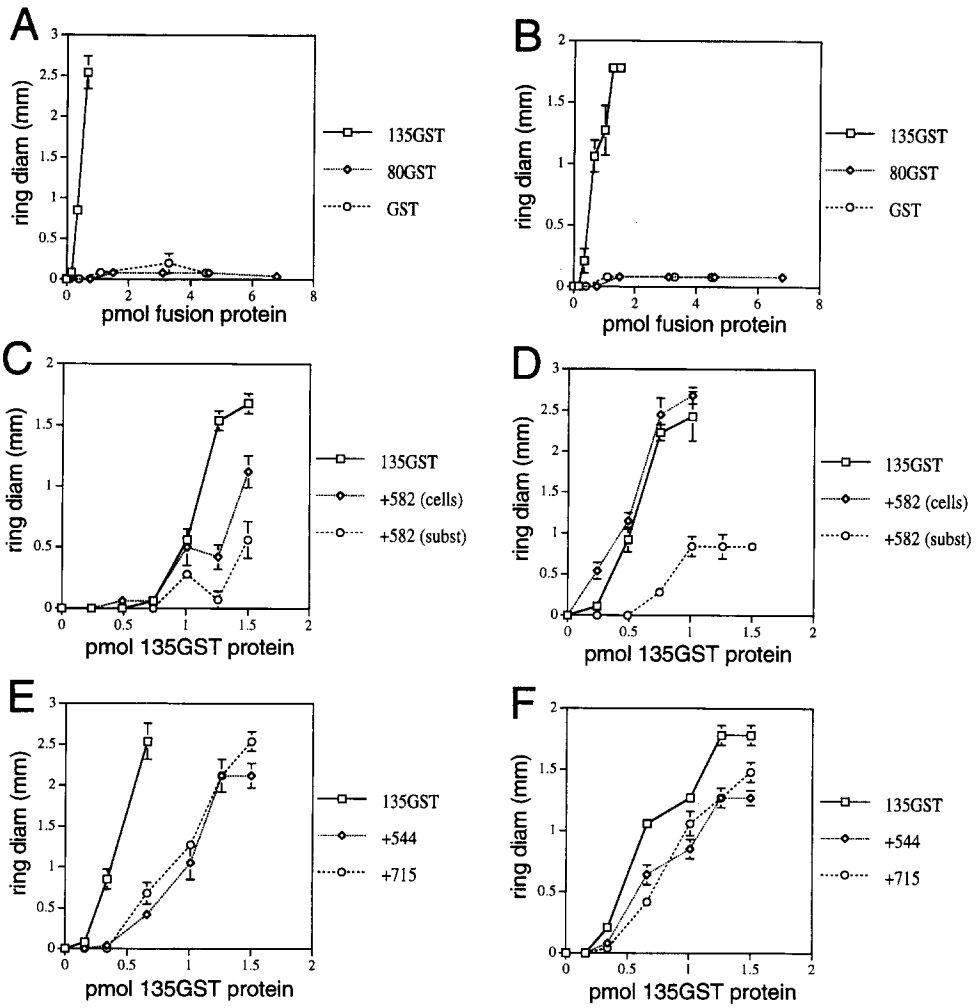

Figure 6. Quantitation of cell attachment to Ng-CAM fusion proteins. 96-well dishes were coated with increasing concentrations of fusion proteins and incubated in the centrifugation assay either with Ng-CAM_{200} transfected L cells (*a*, *c*, and *e*) or untransfected L cells (*b*, *d*, and *f*). The ring diameter of the pelleted cells is plotted against the amount of the GST protein that bound to the plate. (*a* and *b*) (□) F135 substrate; (◇) F80 substrate; (○) GST substrate. (*c* and *d*) (□) untreated cells to F135; (◇) cells preincubated with anti-Ng-CAM200 Fab′; (○) F135 substrate preincubated with anti-Ng-CAM200 Fab′. (*e* and *f*) (□) untreated cells to F135; (◇) cells preincubated with anti-chick embryo Fab′; (○) cells preincubated with anti-chick glia Fab′.

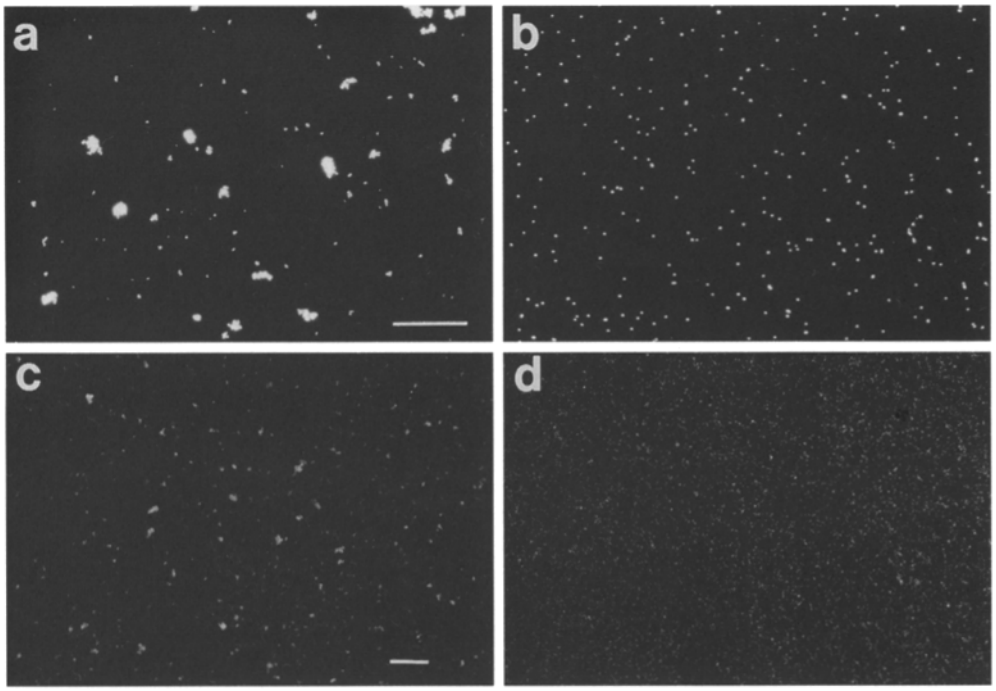

Figure 7. Coaggregation of Ng-CAM fusion protein-Covaspheres. Green-fluorescing F_{135}-Covaspheres (*a*) or red fluorescing F_{80}-Covaspheres (*b*) were allowed to aggregate as described in Materials and Methods and photographed under a fluorescence microscope. (*c* and *d*) The F_{135}-Covaspheres and F_{80}-Covaspheres were mixed, allowed to aggregate and identical fields visualized specifically for green-fluorescing F_{135}-Covaspheres (*c*) or the red-fluorescing F_{80}-Covaspheres (*d*). Bar, 50 μm.

cells were incubated with the anti-glial and anti-E5 embryo antibodies which react with L cells but not with F_{135}.

Glia Binding

The heterophilic binding of Ng-CAM to L cells closely resembles the binding of Ng-CAM to glia. Ng-CAM is not detected on CNS glia, but an unidentified ligand on the glial surfaces binds to Ng-CAM in a Ca^{2+}-independent manner (24). To determine which of the components of Ng-CAM binds to glia, the F_{135} and F_{80} fusion proteins of Ng-CAM were coated onto plastic dishes and tested as substrates in a gravity binding assay with astrocytes. Rat glial cells were used in these studies because they are easily identified by antibodies specific for the glial marker GFAP. The populations of glial cells used in these assays were >95% positive for GFAP immunostaining (not shown).

In the presence of EDTA, primary rat glia bound to the F_{135} fusion protein, but did not bind to the F_{80} fusion protein or to GST (Table IV). The binding was strongly inhibited by preincubation of the F_{135} substrate with Fab' fragments of antibodies raised to the fusion proteins. The Fab' fragments did not inhibit binding of glia to polylysine, used as a positive control for binding.

These results indicate that L cells and glia both bind to the F_{135} region of Ng-CAM in a calcium-independent manner. Moreover, the anti-glial antibody used to block Ng-CAM/L cell adhesion also blocked Ng-CAM–mediated neuron–glia adhesion (20), raising the possibility that the heterophilic ligand for Ng-CAM may be similar on the two cell types.

*Table IV. Glia Binding to Ng-CAM Fusion Proteins**

Substrate	Fab' added	Cells bound	Percent inhibition
F_{135}-GST	–	380 ± 26	–
	anti-Ng-CAM $_{200}$	94 ± 19	69
	anti-F_{135}	117 ± 26	75
F_{80}-GST	–	31 ± 6	–
	anti-Ng-CAM $_{200}$	39 ± 5	0
	anti-F_{135}	48 ± 5	0
GST	–	35 ± 4	–
	anti-Ng-CAM $_{200}$	74 ± 4	0
	anti-F_{135}	39 ± 6	0
poly-L-lysine	–	1,546 ± 108	–
	anti-Ng-CAM $_{200}$	1,811 ± 154	0

* Polystyrene dishes were coated with 2 μl spots containing equimolar concentrations of F_{135}-GST, F_{80}-GST, or GST, and 0.1 mg/ml poly-L-lysine. Dishes were blocked with 2% BSA and preincubated with Fab' fragments of antibodies for 30 min at room temperature where indicated. Primary rat glia were added and incubated as described in Materials and Methods. Adherent cells were visualized on an inverted microscope and counted on duplicate spots in a 0.410 mm^2 area. Counts are averages ± SEM.

Neuron Binding

In addition to neuron–glia binding, Ng-CAM also mediates binding between neurons. When brain cells from embryonic day 7 chicks (most of which are neurons at this stage) were incubated with the Ng-CAM components as substrates, a binding pattern different from that of glia was

*Table V. Brain Cell Binding to Ng-CAM**

Substrate	Fab' added	Cells bound	Percent inhibition
F_{135} GST	–	337 ± 10	–
anti-Ng-CAM_{200}		5 ± 4	98
F_{80}-GST	–	714 ± 53	–
	anti-Ng-CAM_{200}	35 ± 5	99
poly-L-lysine	–	849 ± 33	–
	anti-Ng-CAM_{200}	774 ± 28	9
GST	–	33 ± 10	–
	anti-Ng-CAM_{200}	30 ± 6	0

* Polystyrene dishes were coated with 2 μl spots of solutions of F_{135}-GST, F_{80}-GST, or GST, and 0.1 mg/ml poly-L-lysine. Dishes were blocked with 2% BSA and preincubated with Fab' fragments of the anti-Ng-CAM_{200} for 30 min at room temperature where indicated. Day seven chick embryo brain cells were prepared and incubated in the dishes as described in Materials and Methods. Adherent cells were visualized on an inverted microscope and counted on duplicate spots in a .410 mm^2 area. Counts are averages ± SEM.

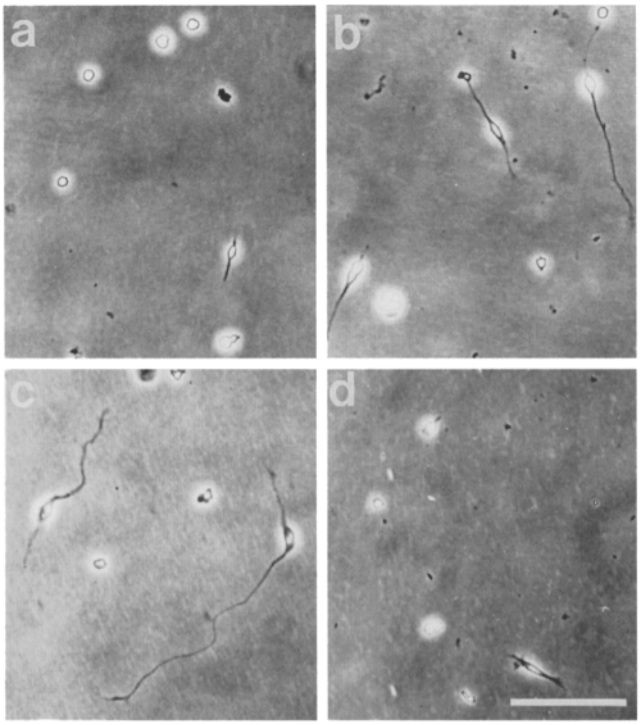

Figure 8. Outgrowth of dorsal root ganglia neurites on Ng-CAM fusion proteins. Phase-contrast photographs of dissociated dorsal root ganglia cells from E6 chick embryos cultured on surfaces coated with Ng-CAM F_{135} (*a*), F_{80} (*b*), $Fn_{III}3$–5 (*c*) fusion proteins, or GST (*d*). Cells were cultured for 15 h in plastic dishes precoated with equimolar amounts of F_{135} or F_{80} fusion protein, fixed with glutaraldehyde and photographed. Bar, 50 μm.

observed. Whereas glia showed no binding to F_{80} (Table IV), brain cells bound both to F_{80} and to F_{135} (Table V). Binding to both substrates was specifically inhibited (98–99%) in the presence of Fab' fragments of antibodies to Ng-CAM.

Neurite Outgrowth

Ng-CAM has been previously shown to promote neurite outgrowth (2, 7, 8, 30, 31). To assess the regions of Ng-CAM responsible for this activity, F_{135} and F_{80}-GST fusion proteins were used as substrates for culturing chick dorsal root ganglia cells (Fig. 8) over a 15-h time period. Although both substrates supported neuronal attachment in long-term culture, only the F_{80} fusion protein promoted extensive neurite outgrowth (Fig. 8 *b*). In contrast, the F_{135} had little effect on DRG neurite outgrowth (Fig. 8 *a*). When neurite outgrowth was tested on substrates coated with various concentrations of F_{135} and F_{80}, F_{80} supported neurite outgrowth at coating at concentrations as low as 0.2 mg/ml whereas F_{135} did not support neurite outgrowth even when coated at concentrations as high as 1.5 mg/ml.

To control for the possibility that the transmembrane domain present in F_{80} affected neurite outgrowth due to hydrophobic effects, we prepared fusion proteins corresponding to the three Fn repeats in F_{80}. One fusion protein included repeats 3–5 and a second spanned only repeats 4–5. Both of these fusion proteins supported neurite outgrowth comparable to F_{80}. The response to Fn 3–5 is shown in Fig. 8 *c* compared with the outgrowth observed on GST alone (Fig. 8 *d*). This result indicates that the neurite outgrowth promoting activity of Ng-CAM resides in the fibronectin type III domains. In accord with the finding that the Fn_{III} 4–5 supported neurite outgrowth, a peptide (FNGRGDGPPSEPIAC) corresponding to the RGD-containing region of $Fn_{III}3$ did not inhibit outgrowth. This suggests that while $Fn_{III}3$ domain may contribute to the neurite outgrowth-promoting activity, it is not essential.

Discussion

The results presented here localize the homophilic and heterophilic cell adhesion activity of Ng-CAM to the F_{135} region of the molecule, and the ability of Ng-CAM to promote neurite outgrowth to the F_{80} region. A previously unknown calcium-independent, heterophilic binding of Ng-CAM–expressing cells to L cells was also detected and was shown to mimic the adhesion of neurons to glia. In addition, the studies show that proteolytic cleavage to produce the two Ng-CAM fragments (F_{135} and F_{80}) occurs in L cells as well as in neurons, but is not required either for the expression of Ng-CAM on the cells or for its ability to support cell adhesion. On the other hand, cleavage does not destroy the ability of the molecule to promote adhesion because the F_{135} attaches and remains anchored on L-cells, as it apparently is on neurons, and mediates Ng-CAM–specific adhesion.

Earlier studies indicated that the Ng-CAM could bind homophilically on neurons and also heterophilically to an unknown ligand on glia (20, 23, 26). The Ng-CAM on neurons and Ng-CAM purified from brain that was used in those studies were predominantly F_{135}, suggesting that either the F_{135} carried the sites for binding or that the activity was inherent in a complex of F_{135} with the smaller amounts of Ng-CAM_{200} and F_{80}. The studies presented here resolve the issue and show clearly that both activities can be mediated by F_{135} in the absence of any other Ng-CAM component. Although the direct demonstration of homophilic binding using transfected cells was complicated by the ability of the cells and the F_{135} substrate to bind untransfected L cells, the ability of F_{135} Covaspheres to aggregate confirmed that the F_{135} can itself bind homophilically. Localization of homophilic binding to F_{135}, which contains all six Ig domains and 2 1/2 Fn_{III} repeats, is consistent with findings on other CAMs containing Ig-like domains. In all cases studied, the homophilic binding has been shown to be mediated by some combination of these domains (4, 10, 40, 49). There are far fewer comparative examples for heterophilic binding. An outstanding one, Nr-CAM, which is closely related to Ng-CAM, binds fibroblasts heterophilically in a calcium-dependent manner (35) in contrast to its homophilic binding which is not calcium-dependent. This activity has been localized in a segment that includes the six Ig domains and one Fn_{III} repeat (35).

The binding of Ng-CAM to L cells shown here is of special interest because it is heterophilic and is calcium-independent as is the binding of Ng-CAM to glia. Among the Ng-CAM subfamily, only Ng-CAM and possibly L1 bind glia and while Nr-CAM binds L cells, it does so only in the presence of calcium. The observation that antibodies that were made against glia and that block Ng-CAM/glia bind-

ing also block Ng-CAM/L cell binding suggests that the receptor for Ng-CAM on glia may be of the same family or even be identical to the L cell ligand. Moreover, a similar ligand may exist on a variety of cells; in preliminary studies, 3T3, LMH, and COS7 cells bound to F_{135} coated as a substrate. Because this binding is calcium independent, it probably does not involve integrins, which require divalent cations for binding (48). There is some suggestion that the N-CAM can bind sugars on L1 (28). This opens the possibility that the F_{135} might bind cells via its carbohydrates or via cell surface carbohydrates, but we have found no evidence for carbohydrate binding by Ng-CAM, and we found that the fusion proteins, which are not glycosylated, retain binding activity. The ability of the unglycosylated fusion proteins to reproduce the biological functions of the eukaryotic protein is in accord with similar binding properties reported for fusion proteins of other Ig-like CAMs, including Nr-CAM (35) and P_o (44). The operational significance of the binding of Ng-CAM to L cells is that these cells provide a much more accessible source than glia for isolating and characterizing the ligand. In a preliminary attempt to identify the L cell ligand, L cell lysates were immunoblotted with a variety of antibodies. Antibodies raised to human L1 and all forms of mouse laminin (22) did not recognize any proteins in L cells (data not shown) indicating that neither L1 nor laminin is the L cell ligand for Ng-CAM. We are currently using a variety of approaches to identify the L cell ligand.

Our studies show that the posttranslational proteolytic cleavage of Ng-CAM to F_{135} and F_{80} is not restricted to neurons, because it took place in our transfected L cells. This type of cleavage is shared among all members of the Ng-CAM subfamily; although the site of cleavage differs in neurofascin, it is essentially identical in Ng-CAM, Nr-CAM and L1. The cleavage is not a critical processing step for binding because Ng-CAM_{200} components were expressed on the cells and these cells mediated Ng-CAM specific adhesion. On the other hand, the limited proteolytic cleavage of Ng-CAM_{200} does not abrogate adhesion because the F_{135}, which has no transmembrane region, associates with the cell surface, both on neurons and on transfected L cells and can thereby mediate adhesion.

In earlier studies on neurons, it was not clear whether the F_{135} bound to another molecule or whether it in some way complexed with Ng-CAM_{200} and F_{80}. It has also been suggested that the comparable fragment of Nr-CAM/Bravo may remain associated with the neuronal membrane by interacting with the F_{80} equivalent. On F_{135} transfected L cells, however, the F_{135} must be anchored by another mechanism and it seems a reasonable hypothesis that this same mechanism operates on neurons. As indicated above, the possibility that carbohydrates on F_{135} serve as a ligand for an anchor molecule remains. Because F_{135} on transfected cells (presumably attached to the anchor) can itself promote heterophilic adhesion, this anchor is probably different from the heterophilic ligand for Ng-CAM on L cells; additional evidence will be required to establish this point.

The cleavage of Ng-CAM in Ng-CAM_{200} transfected L cells led to the appearance of F_{135} in the medium and F_{135} was also seen in the culture media of F_{135} transfected cells. No evidence for soluble F_{135} in brain tissue has been obtained, but it is possible that the F_{135} could bind to other cells and confer upon those cells the ability to mediate Ng-CAM–dependent adhesion.

Localization of the major portion of the neurite promoting activity of Ng-CAM to the F_{80} region in the present study suggests that posttranslational cleavage may to some degree segregate cell binding activity from the ability of Ng-CAM–expressing cells to promote outgrowth. The activity was localized to the extracellular Fn_{III} repeats of the F_{80} and apparently neither cleavage within the third Fn_{III} repeat nor the presence of the RGD sequence in $Fn_{III}3$ is essential for the activity. Because the F_{80} segment neither binds to itself nor to F_{135}, neuronal molecules other than Ng-CAM itself are likely to be involved in the ability of Ng-CAM to promote neurite outgrowth. Two candidates are contactin/F11 (5) and axonin-1 (30), both of which bind Ng-CAM heterophilically and affect neurite outgrowth (5, 15, 17, 30, 45). Other molecules such as integrins or laminin (22) may also be involved.

We have demonstrated that the Ig domains and Fn_{III} repeats of Ng-CAM exhibit different adhesive functions. Both F_{135} and F_{80} supported the short term attachment of DRG neurons; in contrast, only F_{135} and not F_{80} supported the attachment of glial cells. These results are consistent with the observations from cellular assays that F_{135} is responsible for the homophilic aggregation between neurons and the heterophilic aggregation between neurons and glia mediated by Ng-CAM and they suggest the presence of another cell binding activity for neurons within F_{80}.

In other molecules, both Ig domains (1, 5, 15, 18, 28, 44) and Fn_{III} repeats have been shown to support neurite outgrowth (1, 18). Ig domains, for example those in MAG (37), can also inhibit neurite outgrowth. These studies have employed a wide variety of techniques making direct comparisons between them difficult. Studies using fusion proteins of L1 (1) led to the conclusion that Ig domains 1–6 and Fn_{III} domains 1–2 supported neurite outgrowth from mouse small cerebellar neurons, whereas Ig domains 1–2 and 5–6 and Fn_{III} domains 3–5 supported neuronal attachment. All of these binding and neurite outgrowth promotion activities were mediated via interactions with L1 at the cell surface. Recent studies with the F11/contactin/F3 (5) revealed that Ng-CAM and restrictin bound to the Ig domains 1–3 of F11 and that the ability of F11 to promote neurite outgrowth occurred through a different but unidentified site (5). Another study demonstrated neurite outgrowth promoting activity in an Ig domain of F3 and a cell attachment activity in the Fn_{III} repeats. Inasmuch as neurite outgrowth is a very complex process of which cell binding is only one parameter, the resolution of how different molecular domains affect this process awaits further studies comparing a variety of molecules under identical assay conditions.

Overall, the results described here help to clarify our understanding of the binding of Ng-CAM and of the posttranslational proteolytic cleavage that gives rise to the F_{135} and F_{80} components. Many of the conclusions drawn here may extend to other members of the subfamily and aid in defining both their common and differential functions in neural development.

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