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-CAM200) with posttranslational cleavage products of 135 kD (F135, which contains most of the extracellular region) and 80 kD (F80, which includes the transmembrane and the cytoplasmic domains). To examine the functions of each of these components, we have expressed Ng-CAM200, F135, and F80 in murine L cells, and F135 and F80 as GST fusion proteins in the pGEX vector in bacteria. Appropriately transfected L cells expressed each of these proteins on their surfaces; F135 was also found in the media of cells transfected with Ng-CAM200 and F135. In addition to binding homophilically, cells transfected with Ng-CAM200 and F135 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 F135 fragment of the molecule. The results also indicated that proteolytic cleavage of Ng-CAM200 is not required either for its expression on the cell surface or for cell adhesion and that there is an "anchor" for F135 on L cells (and presumably on neurons). In contrast to the cell binding results, the F80 but not the F135 fusion protein enhanced the outgrowth of neurites from dorsal root ganglion cells; this activity was associated with the FnIII repeats of F80. The observations that a protein corresponding to F135 contains the cell aggregation sites whereas one corresponding to the F80 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) 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 anknyrin binding glycoprotein (13, 25, 29, 30, 39, 47). The chick G4 (41) and 8D9 (32) antigens appear to be identical to Ng-CAM. All of these cell sur-
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 (25). 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-CAM200 (6). The smaller components are generated by proteolysis yielding the amino-terminal 135-kD extracellular fragment (which we designate F135), and the 80-kD transmembrane fragment (F80). F135 and F80 each contain structural motifs that could contribute to the adhesive functions of Ng-CAM. The F135 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 F80 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-CAM200, F135, and F80 were each transfected into mouse LM (TK-) cells (CCLI.3; American Type Tissue Culture Collection, Rockville, MD) digested with PvuII and ligated to the 856-bp PvuII/PvuII fragment of the agt1 clone 908. The HindIII/HindIII fragment from the 908-922-135 clone was blunt-ended with KpnI and ligated to the BamHI site of clone 908, digested with PvuII and ligated to the 856-bp PvuII/PvuII fragment of the agt1 clone 908. The HindIII/HindIII fragment from the 908-922-135 clone was blunt-ended with KpnI and ligated as the last step into the Ng-CAM500 construct. For the 5’ end of this construct, the HindIII (M13 polynucleotide site) BalI fragment of clone 906 and the EcoRI (M13 polynucleotide site) BalI fragment of clone 912 were ligated together. The resulting 906-912 clone was digested with EcoRI and partially digested with BstXI. The 630-bp EcoRI/BstXI fragment was ligated to the 2,273-bp EcoRI/BstXI fragment of clone 903 and into the EcoRI site of Bluescript. The 906-912-903 clone was digested with HindIII and the vector-containing fragment was ligated to the blunt-ended HindIII/HindIII fragment of clone 908-922-913. From this final clone the entire EcoRI fragment was ligated into the EcoRI site of the PSVK3 vector, behind the SV-40 early promoter.

For the F135 construct the 760-bp FokI/FokI fragment from the 200-kD clone in PSVK3 was treated with KpnI 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 HindIII/ XbaI (the 725-bp fragment was ligated into the HindIII/XbaI (vector polynucleotide site) sites of the 200-kD clone which had been recloned into the EcoRI site of pRSV B (Intrivogen Corp., San Diego, CA). The EcoRI/ XbaI (PSVK3 polynucleotide sites) fragment of this clone was treated with KpnI to generate blunt ends and ligated in the 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 Culture Collection, Rockville, MD) using calcium phosphate precipitation of the DNA (14). The Ng-CAM500 and F8e, eukaryotic expression constructs in the PSVK3 vector were cotransfected into L cells with the PSV2neo vector. The F135 construct was expressed in the pCDNA1neo vector (Intrivogen Corp.). Clones were selected using G418 (GenBank 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 Fs0 construct, the 922-913 insert from cDNA constructs for the pGEX fusion proteins were modifications of the GST Fusion Proteins (Pharmacia LKB Biotechnology). For the constructs of F135 and Ng-CAM200, the signal peptide-encoding segment at the 5' end 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 EcoRI site at the 5' end was digested with EcoRI and partially digested at its 3' ends, and ligated into the Smal site of pGEX2T (Pharmacia LKB Biotechnology). For the constructs of F135 and Ng-CAM200, the signal peptide-encoding segment at the 5' region was replaced with a short PCR product encoding segment at the 5' region was replaced with a short PCR product. The F135 construct was digested at its 3' end only with XbaI, blunt-ended with Klenow, and ligated to EcoRI linkers. Upon excision by EcoRI digestion, the entire PCR-F135 insert was ligated into the EcoRI site of pGEX1XT (Pharmacia Fine Chemicals, Piscataway, NJ). For the F135 pGEX construct, the 516-bp EcoRI/BstXI PCR fragment from above was ligated into the EcoRI/BstXI site of the pGEX1XT vector. The Fs0 PCR product was digested at its 3' end only with XbaI, blunt-ended with Klenow, and ligated to EcoRI linkers. Upon excision by EcoRI digestion, the entire PCR-Fs0 insert was ligated into the EcoRI site of pGEX1XT.

Promoters spanning Fn3-5 repeats 3-5 and 4-5 of Ng-CAM were generated by PCR using a 5′ primer corresponding to the amino terminus of either the third or fourth Fn3-5 repeat and a common 3′ primer corresponding to a region just before the transmembrane domain. In both cases the 5′ primer contained a BamHI restriction site and the 3′ primer contained an EcoRI 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 BamHI/EcoRI, and cloned into the BamHI/EcoRI sites of pGEX4T2. The 5′ and 3′ PCR primers used were as follows: Fn3-5: GCGGGATCCAATGTGGGGGTGGAACTGCTG, Fn4-5: GCGGGATCCACCTGATTGCCTCCATCGTGGTGGGAACTGCTG. The fusion proteins were produced using modifications of Escherichia coli NM522 cells (Stratagene Corp.) with the pGEX constructs. Fusion proteins were produced using modifications of pGEX constructs (34). SDS-PAGE of the Fs0 fusion protein yielded a major protein of 140 kD and several minor components at ~100 kD. The Fs0 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 were immunoblotted with anti-Ng-CAM antibodies. Attempts were made to prepare the Ng-CAM 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 protein 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-CAM390), 135 kD (anti-F3), and cytoplasmic portions of Ng-CAM were generated by immunizing rabbits against 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 chicken glioma 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 (Cell-line 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-CAM390 and Fs0 were treated for 12 h with medium containing 10 mM sodium butyrate to enhance expression of the proteins driven by the P53 promoter. To prepare single cell suspensions, cells were incubated in PBS/2% FCS/1 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 × 104 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 CaCl2. 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/ml) 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 × 102 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, Cockeysville, 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 mm²). 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 × 104 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 K2HPO4 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 Fs0, and 8–10% for F390.

**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 cm²/ml) or 3 μl red Covaspheres (850 cm²/ml) bound with 2 μg BSA/g 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 NaNO₂ 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 NFG, 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% CO₂. After 1 h, the medium containing a cell population enriched for DRG neurons was removed and the cells were pelleted and washed twice with DME/F12, 1% FCS, 20 ng/ml NFG, 10 μg/ml gentamicin (1% medium). Cells were resuspended at a density of 2 × 10⁴ 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% CO₂ 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 μg/ml), 1 mM L-glutamine at 37°C with 7% CO₂. Cells were treated with 10 mM sodium L-butyrate in PBS/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% CO₂. After 1 h, the medium containing a cell population enriched for DRG neurons was removed and the cells were pelleted and washed twice with DME/F12, 1% FCS, 20 ng/ml NFG, 10 μg/ml gentamicin (1% medium). Cells were resuspended at a density of 2 × 10⁴ 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% CO₂ 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.

F₁₃₅ Binding to L Cells

Subconfluent cultures of L cells (1 × 10⁶) 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 × 10⁷ Ng-CAM₂₀₀ 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 ml DME medium. 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 over-night 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-CAM₂₀₀ or from untransfected L cells, and stained with anti-Ng-CAM antibodies as described above.

Lactoperoxidase Labeling of Cell Surface Proteins

Ng-CAM₂₀₀ transfected cells were freshly passaged overnight, washed in PBS and incubated in 4 ml PBS with 1 μCi [³¹⁵I] 20 U of lactoperoxidase (Calbiochem-Behring Corp., La Jolla, CA) 20 μl of 0.06% H₂O₂ for 10 min at room temperature, followed by addition of 20 μl 0.06% H₂O₂ twice for 5 min each. The enzymatic reaction was stopped by addition of 100 μl of 100 mM NaF, followed by five washes with PBS, including 1 mM NaNO₂. 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-CAM₂₀₀), the 135-kD fragment (F₁₃₅), and the 80-kD fragment (F₈₀) were constructed from previously described clones (reference 6; and for details see Materials and Methods), and transfected into murine LM (TK⁻) cells. F₁₃₅ and F₈₀ 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-CAM₂₀₀, F₁₃₅, and F₈₀ 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
transfected with the neomycin vector only. Seven lines expressing Ng-CAM200, five expressing F135, and four expressing F80 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-CAM200-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). 125I 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 F135-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 F135 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 F135 translation product that is different from the Ng-CAM200 and the F80 products, which showed predominantly cell surface localization (Fig. 2, b and f, respectively). Although F80 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 domain 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-CAM200 and the F80 transfectants with equal intensity, suggesting equivalent amounts of protein on the two transfected cell lines.

In vivo, Ng-CAM is cleaved proteolytically to give F135 and F80. This cleavage also occurred in the Ng-CAM200-transfected L cells. Some F135 was apparent in immunoblots of lysates of Ng-CAM200 transfected cell monolayers (1 × 10⁶ cells/dish), and F135 could be immunoprecipitated from the culture media of these cells (Fig. 3 C, lanes 2 and 3). The levels of F135 in the media increased with culture time and reached maximal levels at 3–4 d. The media from F135-transfected cells (3 × 10⁷ cells) also contained some F135; the amount was less than that seen for the Ng-CAM200 cells probably because the F135 cells generally expressed less Ng-CAM protein than the Ng-CAM200 transfectants.

Although F135 was found in the media of the F135 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 F135 transfected cells mediated adhesion that was inhibited by antibodies to Ng-CAM (see below, Table I). The F135 has no transmembrane region and because the F135 transfected L cells contain no Ng-CAM200 or F80, the F135 must be anchored to the cell via another molecule. In accord with this notion, when untransfected L cells were incubated with media containing F135 from the Ng-CAM200 transfected

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**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-CAM200 (a and b), F135 (c and d), F80 (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-CAM200 (lane 2), and F135 (lane 3) transfected monolayers (1 × 10⁶ 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 × 10⁷ lactoperoxidase-labeled Ng-CAM200 cells. (B) Extracts of Ng-CAM80 transfected monolayers (1 × 10⁵, 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 × 10⁷ Ng-CAM200 cells (lane 2) and 3 × 10⁷ F135 cells (lane 3) were immunoprecipitated and blotted with anti-Ng-CAM antibodies, and compared to extracts of chick brain (20 μg, lane 1).
Figure 4. $F_{135}$ binds to the surfaces of L cells. (A) EDTA-released L cell suspensions ($1 \times 10^6$) 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 $\times$ 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).

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

| Cell clone                  | Fab' added | Percent aggregation | Percent inhibition |
|-----------------------------|------------|---------------------|--------------------|
| Ng-CAM$_{200}$/L cell transfec tant | −          | 23 ± 4              | −                  |
| Ng-CAM$_{200}$/L cell transfec tant | anti-Ng-CAM | 11 ± 3              | 52                 |
|                            | −          | 28 ± 3              | −                  |
|                            | anti-Ng-CAM$_{200}$ | 15 ± 2              | 47                 |
|                            | anti-$F_{135}$ | 22 ± 1              | 22                 |
| F$_{135}$/L cell transfec tant | −          | 18 ± 3              | −                  |
|                            | anti-$F_{135}$ | 2 ± 2               | 89                 |
|                            | anti-Ng-CAM$_{200}$ | 11                   | 47                 |
| F$_{10}$/L cell transfec tant | −          | 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 Dil (red) and fluorescent diO (green) labeled Ng-CAM_200 (a–e) 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 to analyze the ability of Ng-CAM to bind homophilically. We therefore produced fusion proteins of F_135 and F_90 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 protein.

| Table II. Effect of Heterologous Antibodies on Ca^{2+}-Independent Cell Aggregation* |
|---------------------------------|-----------------|-----------------|
| Cell clone | Fab' added | Percent | Percent |
| Ng-CAM_200/L cell transfectant | | aggregation | inhibition |
| – | | 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 F80 substrate or to control GST-coated substrates (Fig. 6a). The extent of binding was proportional to the amount of F135 coated on the dishes and cells bound maximally to 1.0 pmol of F135 fusion protein. At least a portion of this binding was homophilic because the attachment of Ng-CAM200 cells to the F135 substrate was inhibited when the cells were preincubated with Fab' fragments of antibodies to the Ng-CAM-fusion protein (Fig. 6c). 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 F135 region, the F135 and F80 fusion proteins were coupled to Covaspheres, and tested for their ability to aggregate (Table III). F135 Covaspheres aggregated and the aggregation was inhibited 89% by anti-Ng-CAM Fab' fragments. In contrast, Covaspheres coupled with F80 GST-fusion protein, GST alone, or BSA did not aggregate with themselves. Moreover, F80-GST Covaspheres did not coaggregate with the F135-GST Covaspheres (Fig. 7).

Binding of untransfected L cells to the F135 fusion protein also provided additional support for heterophilic binding between Ng-CAM and L cells. L cells bound to the F135 substrate, but not to the F80 or GST substrates (Fig. 6b). Binding of L cells to F135 had the same concentration dependence as that of Ng-CAM200-transfected cells. In this case, however, binding of the untransfected cells was inhibited only when the F135 substrate, and not the cells, was preincubated with anti-Ng-CAM Fab' (Fig. 6d). In addition, adhesion was blocked (Fig. 6f) when the

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**Table III. Covasphere Aggregation**

| Protein       | Fab' added | Superthreshold particles | Percent inhibition |
|---------------|------------|--------------------------|--------------------|
| F135-GST     | –          | 910 ± 7                  | –                  |
| anti-Ng-CAM  | 100 ± 2    | 89                       |                    |
| anti-fibroblast | 1,345 ± 50 | 0                        |                    |
| F80-GST      | –          | 154 ± 22                 | 0                  |
| anti-Ng-CAM  | 154 ± 22   | 43                       |                    |
| anti-fibroblast | 90 ± 9     | 0                        |                    |
| GST          | –          | 49 ± 3                   | 0                  |
| anti-Ng-CAM  | 44 ± 2     | 10                       |                    |
| anti-fibroblast | 95 ± 36    | 0                        |                    |
| BSA          | –          | 51 ± 3                   | 0                  |
| anti-Ng-CAM  | 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.

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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-CAM200 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'.
cells were incubated with the anti-glial and anti-E5 embryo antibodies which react with L cells but not with F135.

**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 Ca²⁺-independent manner (24). To determine which of the components of Ng-CAM binds to glia, the F135 and F80 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

| Substrate | Fab' added | Cells bound | Percent inhibition |
|-----------|------------|-------------|-------------------|
| F135-GST  | –          | 380 ± 26    | –                 |
| anti-Ng-CAM 200 | 94 ± 19   | 69          |                    |
| anti-F 135 | 117 ± 26   | 75          |                    |
| F80-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         |                    |

* Polyethylene dishes were coated with 2 µl spots containing equimolar concentrations of F135-GST, F80-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 mm² area. Counts are averages ± SEM.

F135 fusion protein, but did not bind to the F80 fusion protein or to GST (Table IV). The binding was strongly inhibited by preincubation of the F135 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 F135 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.

**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

| Substrate | Fab' added | Cells bound | Percent inhibition |
|-----------|------------|-------------|-------------------|
| F135-GST  | –          | 337 ± 10    | –                 |
| anti-Ng-CAM 200 | 5 ± 4      | 98          |                    |
| F80-GST  | –          | 714 ± 53    | –                 |
| anti-Ng-CAM 200 | 35 ± 5    | 99          |                    |
| GST       | –          | 849 ± 33    | –                 |
| anti-Ng-CAM 200 | 774 ± 28  | 9           |                    |
| poly-L-lysine | –       | 33 ± 10     | –                 |
| anti-Ng-CAM 200 | 30 ± 6    | 0           |                    |

* Polyethylene dishes were coated with 2 µl spots of solutions of F135-GST, F80-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 antibody 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 0.410 mm² area. Counts are averages ± SEM.
precoated with equimolar amounts of Ng-CAM F135 (a), Fs0 (b), Fnm3-5 (c) fusion proteins, or GST (d). Cells were cultured for 15 h in plastic dishes precoated with equimolar amounts of F135 or Fs0 fusion protein, fixed with glutaraldehyde and photographed. Bar, 50 µm.

**Discussion**

The results presented here localize the homophilic and heterophilic cell adhesion activity of Ng-CAM to the F135 region of the molecule, and the ability of Ng-CAM to promote neurite outgrowth to the Fs0 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 (F135 and Fs0) 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 F135 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 F135, suggesting that either the F135 carried the sites for binding or that the activity was inherent in a complex of F135 with the smaller amounts of Ng-CAM320 and Fs0. The studies presented here resolve the issue and show clearly that both activities can be mediated by F135 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 F135 substrate to bind untransfected L cells, the ability of F135 Covaspheres to aggregate confirmed that the F135 can itself bind homophilically. Localization of homophilic binding to F135, which contains all six Ig domains and 2 1/2 Fnni 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 Fnni 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-
No evidence for soluble F135 in brain tissue has been observed. However, F135 was also seen in the culture media of F135 transfected cells. This suggests that the posttranslational proteolytic cleavage that gives rise to the F135 and F80 components is a natural process in the cells. F135 is probably different from the heterophilic ligand for Ng-CAM on transfected cells (presumably attached to the anchoring molecule). On transfected L cells, however, the F135 must be anchored by an unknown mechanism. 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 P0 (44). The operational significance of the binding of Ng-CAM to L cells is that these cells provide a 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 F135 and F80 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-CAM500 components were expressed on the cells and these cells mediated Ng-CAM specific adhesion. On the other hand, the limited proteolytic cleavage of Ng-CAM500 does not abrogate adhesion because the F135, 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 F135 bound to another molecule or whether it in some way complexed with Ng-CAM and F80. It has also been suggested that the comparable fragment of Nr-CAM/Bravo may remain associated with the neuronal membrane by interacting with the F80 equivalent. On F135 transfected L cells, however, the F135 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 F135 serve as a ligand for an anchor molecule remains. Because F135 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-CAM500 transfected L cells led to the appearance of F135 in the medium and F135 was also seen in the culture media of F135 transfected cells. No evidence for soluble F135 in brain tissue has been obtained, but it is possible that the F135 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 F80 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 FnIII repeats of the F80 and apparently neither cleavage within the third FnIII repeat nor the presence of the RGD sequence in FnIII is essential for the activity. Because the F80 segment neither binds itself nor to F135, 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 FnIII repeats of Ng-CAM exhibit distinct adhesive functions. Both F135 and F80 supported the short term attachment of DRG neurons; in contrast, only F135 and not F80 supported the attachment of glial cells. These results are consistent with the observations from cellular assays that F135 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 F80.

In other molecules, both Ig domains (1, 5, 15, 18, 28, 44) and FnIII 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 FnIII domains 1–2 supported neurite outgrowth from mouse small cerebellar neurons, whereas Ig domains 1–2 and 5–6 and FnIII 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 bind 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 FnIII 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 F135 and F80 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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