Neural Cell Adhesion Molecule (N-CAM) Homophilic Binding Mediated by the Two N-terminal Ig Domains Is Influenced by Intramolecular Domain-Domain Interactions*

Received for publication, August 10, 2004, and in revised form, September 13, 2004
Published, JBC Papers in Press, September 20, 2004, DOI 10.1074/jbc.M409159200

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The mechanism by which the neural cell adhesion molecule, N-CAM, mediates homophilic interactions between cells has been variously attributed to an isologous inter- action of the third immunoglobulin (Ig) domain, to reciprocal binding of the two N-terminal Ig domains, or to reciprocal interactions of all five Ig domains. Here, we have used a panel of recombinant proteins in a bead binding assay, as well as transfected and primary cells, to clarify the molecular mechanism of N-CAM homophilic binding. The entire extracellular region of N-CAM mediated bead aggregation in a concentration- and temperature-dependent manner. Interactions of the N-terminal Ig domains, Ig1 and Ig2, were essential for bead binding, based on deletion and mutation experiments and on antibody inhibition studies. These findings were largely in accord with aggregation experiments using transfected L cells or primary chick brain cells. Additionally, maximal binding was dependent on the integrity of the intramolecular domain-domain interactions throughout the extra-cellular region. We propose that these interactions maintain the relative orientation of each domain in an optimal configuration for binding. Our results suggest that the role of Ig3 in homophilic binding is largely structural. Several Ig3-specific reagents failed to affect N-CAM binding on beads or on cells, while an inhibitory effect of an Ig3-specific monoclonal antibody is probably due to perturbations at the Ig2-Ig3 boundary. Thus, it appears that reciprocal interactions between Ig1 and Ig2 are necessary and sufficient for N-CAM homophilic binding, but that maximal binding requires the quaternary structure of the extracellular region defined by intramolecular domain-domain interactions.

The factors affecting interactions of molecules on the cell surface including adhesion molecules are more complex than for comparable solution interactions. Molecules bound to the cell membrane experience restricted motion, and thus factors including molecular size and flexibility may be important determinants of binding strength or adhesiveness (1). In addition, the orientation of molecules on the cell surface may influence the presentation of binding sites and thereby affect recognition by an apposing cell (2). Furthermore, robust cell-cell binding requires multiple adhesive interactions, as the binding affinities determined for adhesion molecules in solution are weak (3, 4). Solution and crystallographic studies of cell adhesion molecules have revealed details of their domain structures and identified potential adhesive intermolecular interactions. However, such studies cannot readily assess the influences of the specific factors associated with cell-cell interactions on binding affinities. Alternative approaches developed for studying the interactions of molecules bound to surfaces including studies in hydrodynamic flow chambers, and surface force and atomic force microscopy are providing quantitative information of the interactions of molecules on a cell membrane (5, 6, 7).

The neural cell adhesion molecule, N-CAM,1 is a member of the immunoglobulin superfamily of cell adhesion molecules that mediate Ca2+-independent cell-cell adhesion, primarily through homophilic binding (8). In addition, N-CAM has also been shown to interact in a heterophilic way with several cell surface and extracellular binding partners (9), and to transduce cellular signals (10). N-CAM is first expressed at the stage of the neural tube, and in adults is found predominantly in neuronal cells and at neuromuscular junctions. This protein is expressed as various isoforms that arise from alternative splicing of the N-CAM mRNA. The three most abundant isoforms contain 5 tandem immunoglobulin (Ig) domains and 2 fibronectin type III (FnIII) homology domains, but differ in their association with the cell membrane; N-CAM 180 and N-CAM 140 are transmembrane proteins whereas N-CAM 120 is GPI-linked. The expression of the various N-CAM isoforms is developmentally regulated, suggesting specialized roles for each of the splice variants (11). In addition, the activity of N-CAM is further modulated through the post-translational addition of long polysialic acid chains to specific sites on the fifth Ig domain (12).

The molecular mechanism of N-CAM homophilic binding is unclear. Early electron microscopy studies on native N-CAM revealed a rod-shaped molecule with a flexible hinge (13, 14), consistent with the predicted requirement for flexibility in cell adhesion molecules (1). A later series of experiments suggested that self-association of the third Ig domain, Ig3 was required for homophilic binding, and showed that a peptide, P5, whose sequence is contained within the Ig3 domain could inhibit cell

* This work was supported by National Institutes of Health Grants HD16550 and HD09635 and a grant from the G. Harold and Leila Mathers Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: N-CAM, neural cell adhesion molecule; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody; Fn III, fibronectin type III domain.
attachment (15, 16, 17). Independent bead and cell-binding experiments also demonstrated self-association of Ig3. In addition, this study observed reciprocal interactions between the remaining Ig domains consistent with antiparallel binding of all 5 Ig domains (18). In contrast, solution and crystallographic studies have characterized an interaction between the N-terminal Ig domains Ig1 and Ig2 (3, 19–21), and failed to detect any self-association of Ig3. A cooperative binding mechanism was proposed for the Ig1:Ig2 interaction, based on the significantly stronger binding measured for concatenated Ig12 domains compared with isolated domains (4). In contrast, solution and crystallographic studies have characterized an interaction between the N-terminal Ig domains Ig3, Ig4, and Ig5 (20). In contrast, solution and crystallographic studies have characterized an interaction between the N-terminal Ig domains Ig3, Ig4, and Ig5 (20). Therefore, the reciprocal interaction between Ig1 and Ig2 is necessary for robust surface. The results of the bead studies indicate that the reciprocal interaction between Ig1 and Ig2 is necessary for robust N-CAM-mediated bead aggregation, and similar results were obtained with primary and transfected cells. Furthermore, the data suggest that intramolecular domain-domain interactions are required for optimal presentation of the molecule on the cell surface for adhesive interactions.

EXPERIMENTAL PROCEDURES

N-CAM Fc Expression Vectors—DNA encoding the Fc of human IgG, a generous gift from James Nelson (Stanford), was cloned into the 5‘-XbaI and 3‘-BamHI sites of the pCEP4 vector (Invitrogen). cDNAs corresponding to the signal sequence and the required domains from chick N-CAM were generated by PCR and cloned 5’ to the Fc to produce N-CAM Fc fusions. Constructs encoding the 5 Ig and the 2 fibronectin domains, (N-CAM, residues Leu1–Leu153), a deletion of Fn2 (ΔN2, residues Leu1–Glu95), the 5 Ig domains (ΔIg5, residues Leu1–Lys98) were cloned into the vector at the 5’-BamHI and 3’-HindIII and 3’-KpnI restriction endonuclease sites, respectively. The individual fragments were subcloned into the vector as described (4). Typically, 10 μl of a 1% solution of 1-μm blue-dyed polystyrene or carboxylate protein A microspheres (Poly-science) were incubated for 45 s on ice with 1–3 μg of recombinant Fc-fusion proteins, blocked with 10 mg/ml BSA, and pelleted by centrifugation (2 min at 15,000 rpm). The coated beads were dispersed in 500 μl of 10 mg/ml BSA by 10 s of sonication at 10 microns and transferred to borosilicate tubes. For inhibition experiments, Fab fragments, proteins, or peptides were added at this time. The beads were rotated at 90 rpm and 25°C, unless otherwise stated. The extent of aggregation was determined by the appearance of bead aggregates in the 2–4 μm range measured using a Beckman Coulter Counter (Fullerton, CA) on 20 μl aliquots diluted to 1 ml of Ison. Results are reported as the average of typically triplicate experiments repeated with proteins from different transfections. For mixed bead aggregation experiments, beads were incubated with the desired protein (or BSA for mock-treated beads) in batch, then washed a total of 4 times with 1 ml of 10 mg/ml BSA. The coated beads were dispersed by sonication, and aliquots combined prior to the initiation of aggregation.

Primary Cell Aggregation Experiments—Aggregation of E8 chick brain cells was determined as described (25). Briefly, Ca2+–independent aggregation of single brain cells generated by limited trypsin treatment (26), or digestion with CTB (27) and 3000 units of hyaluronidase (Sigma), was measured as the disappearance of single cells at 37°C using a Coulter Counter. For inhibition experiments, reagents were incubated with dispersed cells on ice for 15 min prior to initiation of aggregation.

Production and Aggregation of Transfected Cells—Construction of N-CAM 140 expression vector has been described previously (28). The F19S mutation was introduced into this vector using the standard protocol of the QuickChange in vitro mutagenesis kit (Stratagene, La Jolla, CA). Stably transfected L cells expressing human N-CAM 140 were prepared as described (28). The N-CAM Ag3 mutation point mutation was initially selected with G418 and subsequently, immunopanned using the SELLecTion pan mouse IgG kit (Dynal, Brown Deer, WI) and the human monoclonal antibody, 16.2 (BD Biosciences Pharmingen, San Diego, CA) for high expressing clones. FACs analysis was used to establish that comparable levels of cell surface expression of N-CAM were achieved in the two immunopanned cell lines. Ca2+-independent cell-cell aggregation experiments were performed as described (25).

RESULTS

Recombinant fusion proteins of chicken N-CAM and human Fe (Fig. 1A) were purified from the media of transiently transfected 293 EBNA cells as described (23). The Fe fusion facilitated high affinity binding of the recombinant proteins to protein A-coated beads through their C termini, thereby orienting the N-CAM domains in a manner similar to that observed on cells. In addition, Fe-mediated dimerization served to increase

agene, La Jolla, CA). A schematic representation of the N-CAM Fe fusion proteins is shown in Fig. 1A.
the local protein concentration. As the flexibility of adhesion molecules immobilized on surfaces has been shown to affect binding (29), the recombinant proteins were expressed as both covalent and non-covalent dimers by the selective incorporation of the hinge region of Fc (Fig. 1A). The purified recombinant proteins were nearly 95% pure, as judged from SDS-PAGE gels (Fig. 1B); treatment with PNGase confirmed the presence of N-linked sugars (data not shown). The dimeric nature of the fusion proteins was preserved in proteins lacking the intermolecular disulfides of the Fc hinge as shown by gel filtration (Fig. 1C). The different purified recombinant proteins bound with comparable affinities to the 1 μm-blue-dyed polystyrene protein A microspheres employed in the bead aggregation assay, as shown by SDS-PAGE (Fig. 1D).

The Extracellular Region of N-CAM Mediates Aggregation of Coated Beads—The bead aggregation assay entailed first binding 1–3 μg of purified recombinant proteins to 10 μl of a 1% solution of 1-μm blue-dyed protein A microspheres. The protein-coated beads were washed, resuspended, and dispersed in 500 μl of 10 mg/ml BSA by brief sonication, and then allowed to aggregate. The extent of aggregation was determined at specific time points by the appearance of 2–4 μm aggregates, as measured by a Coulter Counter. A recombinant protein incorporating the extracellular region of N-CAM (N-CAM-Fc, Fig.
mediated bead aggregation in a concentration-dependent manner (Fig. 2A). Uncoated beads failed to aggregate (data not shown). Furthermore, the monovalent Fab fragment of a polyclonal antibody that recognizes the cytoplasmic region of N-CAM 140 did not significantly affect bead aggregation (data not shown). The rate of aggregation of N-CAM coated beads increased with temperature (Fig. 2C) and was largely insensitive to changes in ionic strength (data not shown) suggesting that the protein-protein interaction was predominantly hydrophobic in nature. The extent of bead aggregation was independent of whether the Fc dimers were covalent or non-covalent (Fig. 1A, data not shown). To establish whether the dimeric nature of the recombinant proteins contributed to the strength of the homophilic binding, a heterodimeric N-CAM construct was prepared. Co-transfection of 293 cells with a plasmid encoding the N-CAM-Fc fusion protein and another encoding only the Fc domain resulted in the formation of both N-CAM-Fc and Fc homodimers, as well as N-CAM-Fc heterodimer (Fig. 1A). The production of heterodimers was maximized by using a 4:1 ratio of Fc:N-CAM-Fc plasmids in the transfection, and purified using an N-CAM affinity column (24). This preparation of heterodimeric protein was >90% pure, as judged by SDS-PAGE (data not shown). The relative rates of N-CAM-mediated aggregation determined at three loadings of the homo- and heterodimeric proteins (7, 14, and 30 pmol of N-CAM), were essentially identical. The aggregation data collected at 14 pmol loading is shown in Fig. 2D. A subsequent Western blot of the aggregating beads confirmed an equivalent loading of N-CAM onto the beads (Fig. 2E). This finding suggests that, in the bead aggregation assay, homophilic binding was not dependent on the formation of lateral or cis dimers.

**Ig1 Is Necessary for N-CAM Binding**—To investigate the role of the first Ig domain in N-CAM homophilic binding, we initially determined the ability of a recombinant protein lacking the first Ig domain (ΔIg1, Fig. 1A) to mediate bead aggregation. N-CAM-Fc and ΔIg1 bound to the beads with similar affinities (Fig. 1D), however, ΔIg1 was totally ineffective at mediating bead aggregation at comparable loadings to the wild type protein (50 pmol, Fig. 3A). This finding is consistent with Ig1 being essential for homophilic interactions of N-CAM.

The ability of Ig1-specific antibodies to block N-CAM-mediated bead binding was also determined. The monovalent Fab fragments of a polyclonal antibody raised against purified bacterial Ig1 (αIg1) inhibited N-CAM-mediated bead aggregation in a concentration-dependent manner (Fig. 3B). Complete inhibition was achieved at concentrations greater than 100 μg/ml. In addition, a monoclonal antibody that recognizes the first Ig domain of chick N-CAM also demonstrated concentration dependent inhibition, with 100% inhibition at 30 μg/ml (Fig. 3C).

Previously, we had demonstrated that a single point mutation of a surface-exposed hydrophobic residue in Ig1 (F19S) significantly reduced the reciprocal Ig12 binding affinity in solution without affecting the protein folding or stability (3). The crystal structures of dimeric Ig12 and Ig123 identified the intercalation of Phe19 into a pocket on the surface of Ig2 as a
prominent feature of the interaction (19, 20). Here, we have employed this point mutation to probe the role of the Ig12 interaction in N-CAM-mediated bead aggregation. An N-CAM-Fc fusion protein that incorporated the F19S mutation (Fig. 1A) was expressed in 293 cells at levels comparable to the wild-type sequence (~50 pmol of protein loading on 10 μl of beads). B, aggregation of N-CAM-Fc-coated beads (typically 20 pmol of protein on 10 μl of beads) was inhibited in a concentration-dependent manner by Fab fragments of a polyclonal antibody against the Ig1 domain of N-CAM, aIg1. The ability of antibodies to block N-CAM-mediated bead aggregation is reported as % inhibition. C, monoclonal antibody against the Ig1 domain was able to completely inhibit bead aggregation mediated by the extracellular region of N-CAM (same experimental details as for B). D, mutation of phenylalanine to serine at the defined Ig12 binding site (F19S) was sufficient to prevent N-CAM-mediated bead aggregation. The % aggregation for F19S N-CAM is reported relative to wild-type at protein loadings of ~10 and ~15 pmol.

In combination, these findings imply that not only is the N-terminal Ig domain necessary for N-CAM homophilic interactions, but that it mediates binding through a reciprocal interaction with Ig2.

Ig12 Interactions Are Required for N-CAM-mediated Cell-Cell Binding—To determine the importance of the Ig12 binding in N-CAM interactions on cells, the transmembrane isoform of human N-CAM, N-CAM 140 (Fig. 1A), was introduced into the N-CAM-null background of mouse L cells. Transfected cells were selected with G418 and immunopanned to sort for high N-CAM expression. Stably transfected L cells expressing N-CAM 140 underwent Ca2+-independent aggregation, as compared with cells transfected with the empty vector (Fig. 4A). In addition, the aggregation of transfected cells was blocked by preincubation with the aIg1 Fab shown to inhibit N-CAM-mediated bead aggregation (Fig. 4B). Addition of this Fab to cells transfected with vector only had no effect on aggregation (data not shown). The Fab fragment of a monoclonal antibody whose epitope lies only in chicken Ig3 (mAb1) did not significantly perturb aggregation of transfected cell, serving as a negative control (Fig. 4B). A second cell line stably expressing N-CAM incorporating the Ig12 distabilizing point mutation, F19S N-CAM 140, was generated as described above, and shown by FACS analysis to have similar cell surface expression of N-CAM to the cells expressing wild type N-CAM (data not shown). These cells also failed to undergo Ca2+-independent cell-cell aggregation (Fig. 4A).

The complementary observations in N-CAM-mediated bead aggregation and N-CAM-dependent cell-cell aggregation suggest that the nature of the interaction is similar in both systems. To assess whether the Ig1:Ig2 interaction is required for primary cell aggregation, we examined the ability of the αN-CAM antibodies to inhibit Ca2+-independent cell-cell aggregation of embryonic chick brain cells. Limited trypsin treatment of E8 chick brains yields a predominantly neural/neural progenitor cell population that has previously been shown to aggregate in an N-CAM dependent manner (26). The Ca2+-independent aggregation of E8 brain cells was inhibited by increasing concentrations of αN-CAM Fab to a maximum level of ~50% inhibition (Fig. 5A). Similarly, the aIg1 Fab inhibited cell-cell aggregation (Fig. 5B). The ability of aIg1 to inhibit the aggregation of N-CAM expressing primary cells is consistent with the notion that Ig1 interactions are involved in N-CAM homophilic binding in these cells.

Ig3 Is Not Directly Involved in N-CAM Homophilic Binding—Previous studies have suggested that the third Ig domain, Ig3, plays a critical role in N-CAM homophilic binding. A number of Ig3-specific reagents have been reported to inhibit cell-cell aggregation including antibodies, recombinant proteins, and peptides, and it has been proposed that self-association of the Ig3 domains is central to N-CAM binding (15–18). However, we and others (3, 19) have been unable to demonstrate self-association of the isolated Ig3 domain in solution. To evaluate the contribution of Ig3 to N-CAM homophilic binding, we produced recombinant Fc fusion proteins corresponding to just the N-terminal domain, Ig1, the N-terminal two domains, Ig12, and the N-terminal three domains, Ig123 (Fig. 1A), and compared the ability of these proteins to mediate bead aggregation.
On the blue-dyed protein A microspheres these shorter proteins failed to mediate significant aggregation (data not shown). However, at equivalent protein loading, Ig12 and Ig123 were able to mediate aggregation of carboxylate protein A beads. No aggregation was observed for beads coated with Ig1 (Fig. 6, A and B). These observations were consistent with interactions between Ig1 and Ig2 being essential for binding, and suggested that no additional interactions were mediated by Ig3.

The finding that the Ig12 and Ig123 fusion proteins mediated equivalent bead aggregation is consistent with earlier results from sedimentation equilibrium experiments, where identical $K_d$ values were determined for the dimerization of Ig12 and Ig123 (3, 21). However, it is possible that the proposed self-association of Ig3 requires that the domain be presented in the context of the native molecule. To investigate this possibility, we successively deleted the fifth, $\Delta 5$, fourth, $\Delta 45$, and third domains, $\Delta 345$, from the wild-type (full-length N-CAM extra-cellular region) fusion protein (i.e. each construct retains Ig1, Ig2, Fn1, and Fn2, see Fig. 1A) and determined their abilities to mediate bead aggregation. In each of these proteins, the deleted domain or domains were replaced with the dipeptide Lys-Leu (see “Experimental Procedures”). Deletion of Ig5 resulted in ~60% loss of bead aggregation relative to the wild-type protein. However, the subsequent deletion of Ig4 and Ig3 did not further affect the ability of these proteins to mediate aggregation (Fig. 6C). Given the essentially equal bead aggregation mediated by the $\Delta 5$, $\Delta 45$, and $\Delta 345$ proteins, it is unlikely that Ig3 self-associates when presented in the context of the native protein.

Additionally, we investigated the susceptibility of N-CAM-mediated bead aggregation to perturbations by Ig3-specific reagents. Monovalent Fab fragments from two Ig3-specific polyclonal antibodies, $\alpha$Ig3-A and $\alpha$Ig3-B, failed to affect bead binding at concentrations up to 500 $\mu$g/ml (Fig. 6D). Similarly, concentrations up to 1 mg/ml of two bacterially expressed Ig3 proteins corresponding to an N-terminally extended Ig3 domain, Ig3 (3), or Ig3 lacking the first $\beta$ strand, $\Delta$Ig3 (16) were ineffective in inhibiting bead aggregation. Furthermore, two Ig3-derived peptides reported to affect cell-cell binding (P5 and P10) were unable to modulate N-CAM-mediated bead binding (17) (Fig. 6D). The P5 peptide was synthesized with both free and derivatized terminal residues, however both forms were inactive in this assay. This inability of Ig3-specific reagents to perturb N-CAM homophilic binding on beads argues against possible intermolecular interactions of Ig3 with other extracellular domains of N-CAM.

In contrast to the lack of inhibition observed with the polyclonal $\alpha$Ig3 antibodies, the Fab fragment from the Ig3-specific monoclonal antibody, mAb1, demonstrated concentration-dependent, saturable inhibition of N-CAM-mediated bead aggregation. Furthermore, the maximum extent of inhibition achieved with mAb1 Fab was ~85% (Fig. 6F). Similar inhibition was observed with both the Fab and the intact antibody, excluding the possibility that nonspecific proteolysis during the generation of the Fab fragment reduced its efficacy (data not shown). Despite the failure of the $\alpha$Ig3 antibodies to directly affect N-CAM-mediated bead binding, one of the polyclonal antibodies was able to compete with mAb1 binding. N-CAM-Fc-coated beads were incubated with either $\alpha$Ig3-A or $\alpha$Ig3-B prior to addition of mAb1, and then allowed to aggregate. Only Ig3-A blocked the binding of mAb1, as seen by the reduced level of inhibition (Fig. 6F). This suggests that $\alpha$Ig3-A and mAb1 recognize neighboring epitopes.

The ability of $\alpha$Ig3 and recombinant Ig3 to affect N-CAM homophilic interactions on cells was studied in the N-CAM transfected L cells. At concentrations up to 500 $\mu$g/ml neither the Fab nor the recombinant Ig3 were able to significantly affect cell-cell aggregation. The effect of the chicken-specific monoclonal, mAb1, on aggregation of cells expressing hN-CAM is shown to demonstrate the extent of nonspecific inhibition (Fig. 7). The inability of $\alpha$Ig3 or recombinant Ig3 to affect N-CAM-mediated aggregation of transfected cells was similar to that observed in the bead assay and served to validate the bead assay.

The potential role of Ig3 in N-CAM homophilic binding was further investigated in E8 chick brain cells. As was seen in the transfected L cells, recombinant Ig3 had no significant effect on primary cell aggregation up to 1 mg/ml (Fig. 8A). Interestingly, the truncated form of recombinant Ig3 lacking the first $\beta$-strand (Ig3d) did show weak inhibition at high concentrations (1 mg/ml). This form of Ig3 has been previously reported to inhibit bead and cell aggregation (18). However, the Ig3 peptide reported by Rao, et al. to incorporate the proposed homophilic binding site and to inhibit N-CAM binding (P5) (16, 17) exhibited no activity in this assay (Fig. 8A). In contrast to bead and transfected cell experiments, the polyclonal $\alpha$Ig3-A that competed with mAb1 binding (see Fig. 6F) was as effective as $\alpha$N-CAM in inhibiting brain cell aggregation, while $\alpha$Ig3-B had no effect. In addition, the monoclonal mAb1 demonstrated weaker inhibition (~50–60% of that observed with $\alpha$N-CAM, Fig. 8A). Western blot analyses of the trypsin-dissociated brain cells revealed a trypsin-sensitive site in N-CAM. This site lies within a disulfide loop of an Ig domain, as digestion was visible only on reduced SDS-PAGE gels, and is highly sensitive; digestion was observed after only 15 min of treatment with 0.00007% trypsin at 37 °C. However, this digestion does not appear to affect N-CAM-mediated aggregation, as single brain cells dissociated by a collagenase/ hyaluronidase treatment which left the N-CAM intact, showed comparable sensitivity to Ig3 and mAb1 (data not shown).

The ability of $\alpha$Ig3-A and mAb1 to affect primary cell aggregation was also investigated in retinal cells from E10 chick. Single cells dissociated from retina with collagenase and hyaluronidase displayed intact N-CAM and aggregated robustly. The extent of aggregation attributable to N-CAM was similar to that observed in brain cells, as $\alpha$N-CAM inhibited ~50% of the aggregation (Fig. 8B). However, these cells displayed different sensitivities to the Ig3-specific antibodies. In retinal cell aggregation, the polyclonals $\alpha$Ig3-A and $\alpha$Ig3-B were totally
ineffective at inhibiting cell aggregation (as was seen in the bead and transfected cell assays), while the monoclonal mAb1 inhibited weakly (Fig. 8B). This difference between retinal and brain cell aggregation was not due to the reduced PSA level on retinal cells, as treatment of brain cells with EndoN did not affect the findings (data not shown).

**Fig. 6.** The third immunoglobulin domain, Ig3, is not essential for N-CAM-mediated bead aggregation. A, ability of recombinant Fc fusion proteins corresponding to the first (Ig1), the first two (Ig12), and the first three (Ig123) Ig domains to mediate bead binding was measured using 1 µm carboxylate protein A beads. Aggregation was measured by the number of particles in the 2–4 µm range, and the results are reported as aggregation relative to Ig123. B, equivalent binding of the fusion proteins to the carboxylate beads was established by Western blot analysis. C, ability of recombinant proteins corresponding to a deletion of Ig5 (Δ5), a deletion of Ig4 and 5 (Δ45), and a deletion of Ig3, 4, and 5 (Δ345) to mediate bead aggregation was compared with that of the wild-type sequence. Equal molar amounts of each protein were added to the beads, and their aggregation was determined relative to the wild-type protein. D, N-CAM-Fc-mediated bead aggregation was insensitive to the Fab fragment from a polyclonal antibody that recognizes the third Ig domain, Ig3, to bacterially expressed Ig3 protein and a truncated Ig3 lacking the first β-strand, Ig3s, and to synthetic peptides derived from Ig3, P5, and P10. The inhibition from 100 µg/ml αIg1 was defined as 100%, and results are reported as % inhibition. E, Fab fragment from a monoclonal antibody that recognizes chicken Ig3 (mAb1) was able to inhibit ~85% of the N-CAM-Fc-mediated bead aggregation. F, preincubation of N-CAM-coated beads with αIg3 partially blocks the ability of mAb1 to inhibition aggregation.

**Fig. 7.** N-CAM-transfected L cell aggregation is insensitive to Ig3-specific reagents. The N-CAM-dependent aggregation of stably transfected L cells is not significantly inhibited by αIg3 Fab at 500 µg/ml or recombinant Ig3 at 500 µg/ml. The monoclonal antibody, mAb1, serves as a negative control as it does not recognize human N-CAM. Cell aggregation experiments were performed as described in the legend to Fig. 3.

The deletion of Ig5 from the Fc fusion protein resulted in a dramatic reduction in its ability to mediate bead aggregation (see Fig. 6C). Here, we have established that the Ig1: Ig2 reciprocal binding is essential for binding; however, it was possible that Ig5 and/or Ig4 were required for a subsequent binding event. Domain-specific antibodies αIg4 and αIg5 failed to inhibit bead binding at 1 mg/ml (data not shown), but these findings were not definitive. To directly address the possibility of a secondary binding event involving additional domains, we performed a pseudo-heterophilic binding assay using wild-type N-CAM-Fc and the F19S protein. As the only difference between these proteins is the single point mutation at the Ig1: Ig2 binding site (F19S, see Fig. 3D), it was reasonable to assume that the F19S protein would be competent in any secondary binding event. Each protein was incubated separately with beads, washed extensively to remove unbound protein, and dispersed by sonication. N-CAM-Fc-coated beads were then mixed with equivalent concentrations of either F19S-coated or mock-treated beads and allowed to aggregate. The extents of aggregation measured in these mixed bead experiments were indistinguishable (Fig. 9A). An identical result was obtained when a 3-fold higher concentration of F19S compared with N-CAM-Fc-coated beads was used in this mixed bead experiment (data not shown). The failure to detect any additional binding with F19S coated over mock-treated
beads argued that any secondary interaction involving the Ig12 dimeric complex was significantly weaker than the Ig12 reciprocal interaction.

The Membrane Proximal FnIII Domain Is Not Required for N-CAM Homophilic Binding—An alternative explanation for the loss of binding activity on deletion of Ig5 is that the interacting region of the molecule needs to be positioned a precise distance from the surface of the bead in order to achieve optimal binding. To address this possibility, we deleted the membrane proximal FnIII domain from the N-CAM-Fc fusion protein (ΔFn2, Fig. 1A). FnIII and Ig domains are similar in size (19, 30), and compared the activity of ΔFn2 relative to the wild-type protein. In this assay the ΔFn2 was as effective as the entire extracellular region in mediating aggregation (Fig. 9B). Thus, in the context of the bead binding assay, the reduction in the distance of the interacting region from the bead surface due to the deletion of a single FnIII or presumably Ig domain does not compromise binding. Furthermore, it appears that the membrane proximal FnIII domain is not required for N-CAM homophilic binding.

To further define the minimal domains required for homophilic binding, we produced a recombinant Fc fusion protein containing just the 5 Ig domains (Ig1–5, Fig. 1A). This protein was expressed at moderate levels in the 293 EBNA cells, was readily purified on a protein A column, and appeared folded based on its CD spectrum. Although Ig1–5-mediated bead aggregation, the extent of aggregation was variable and did not show a simple dependence on protein loading on the beads (data not shown). This variability was observed with 3 different protein constructs (Ig1–5 residues Leu1–Val1480 Fc both with and without the 11 residue Fc hinge region, and residues Leu1–Pro181 Fc without the hinge region), suggesting that it was an inherent property of Ig1–5.

The Quaternary Structure of the Extracellular Region of N-CAM Affects Homophilic Binding—The dramatic loss in binding seen on deletion of Ig5 presented a dilemma. The inability of αg5 to perturb binding suggested that Ig5 itself is not directly involved in homophilic interactions. Moreover, the comparable activities of ΔFn2 and wild-type proteins implied that the distance from the bead surface was not critical. One possible explanation may lie in the precise structure of the recombinant proteins. We used PCR to generate coding sequences with appropriate 5′ and 3′-restriction endonuclease sites to allow for directional cloning into the Fc containing plasmid. This allowed any region to be deleted from the fusion proteins, but resulted in the incorporation of two additional codons at the deletion site. Specifically, in the ΔΔ, ΔΔΔ, ΔΔΔΔ fusion proteins, the deleted domains have been effectively replaced by the dipeptide Lys-Leu. The introduction of additional amino acids between domains may disrupt interdomain interactions. To test for this possibility, we determined the effects of N-CAM binding of introducing a 4 amino acid spacer (Ala-Ser-Gly) between Ig1 and Ig2, 1∧2 (Fig. 1A). The presence of 4 additional amino acids between Ig1 and Ig2 abrogated N-CAM binding (Fig. 10A), while positioning the additional residues between Ig2 and Ig3 reduced bead binding to ~50% of the wild-type protein (Fig. 10A). As was observed for the wild-type protein, 2∧3-mediated bead aggregation was fully inhibited by 100 μg/ml αg1, and was insensitive to polyclonal antibodies raised against Ig2, Ig4, Ig5, or against the cytoplasmic domain of N-CAM 140, αδ (Fig. 10B). In addition, the 2∧3 aggregation was inhibited ~90% by mAb1 (Fig. 10C), and was unaffected by P5 peptide (1 mg/ml) or recombinant Ig1 (data not shown and Fig. 10C). In contrast to the wild type protein, concentration-dependent, saturable inhibition of 2∧3 aggregation was observed with both the Ig3-
specific polyclonal, αIg3-A, and recombinant Ig3, but not with αIg3-B (Fig. 10C). αIg3-A completely inhibited 2×3 aggregation, while the maximum inhibition achieved by Ig3 was 35%. Thus, inserting the four amino acids between Ig1 and Ig2 abrogated N-CAM binding. Furthermore, the same insertion between Ig2 and Ig3 reduced binding and made it susceptible to perturbants that did not affect the wild-type molecule.

DISCUSSION

Defining the mechanisms by which cell adhesion molecules mediate cell-cell binding has been remarkably difficult. This is partly attributable to the relatively low affinities of the adhesive interactions, but also to the complexity of the cell membrane, where it is difficult to isolate homophilic binding from heterophilic interactions and consequential cell-signaling events. In this study, we have utilized a bead binding assay with recombinant proteins to investigate homophilic interactions of N-CAM in the absence of interacting proteins, and have complemented these studies with experiments performed in transfected and primary cells. A similar approach was used in studies on C-cadherin aggregation, where a correlation was observed between bead and cell-based experiments (4). We have obtained a qualitatively similar description of N-CAM homophilic binding from bead and cell experiments, indicating that this approach is also useful in characterizing Ig CAM interactions.

The extracellular region of N-CAM-mediated concentration- and temperature-dependent aggregation when bound to protein A beads that was sensitive to αN-CAM antibodies. In this bead assay, no differences were detected in the extent of aggregations between mono- and divalent constructs (i.e. heterodimeric and homodimeric N-CAM, Fig. 1A). Although it has been proposed that lateral interactions between adhesion molecules on the same cell contribute to the efficacy of binding, the current finding does not support a requirement for such interactions. In addition, the influence of the disulfide bonds in the Fc hinge region on flexibility and N-CAM binding was addressed. No difference in bead aggregation was detected between covalent and noncovalent dimers (i.e. with or without the Fc hinge region, Fig. 1A). This suggests that the linker sequences between the N-CAM domains and the Fc portion of the fusion protein (11/12 amino acids) provided sufficient flexibility in both constructs to facilitate binding.

The cumulative data from the bead and cell experiments, including domain deletion and antibody inhibition experiments, indicate that the first 2 Ig domains are necessary for N-CAM homophilic binding. In addition, in bead and transfected cell experiments, binding was abrogated by the F19S point mutation previously shown to disrupt the reciprocal Ig12 binding in solution (3). These results suggest that the interaction of N-CAM on beads and in transfected cells is the same as that observed in solution. We have previously reported that the F19S mutation did not affect N-CAM-mediated aggregation of transfected COS-7 cells (3). It is possible that COS-7, but not L cells, express a heterophilic ligand for N-CAM, which binds independently of F19S mutation.

The insertion of 4 amino acids between Ig1 and Ig2 completely abrogated bead binding. The inserted sequence of small, relatively hydrophilic residues was designed to introduce significant conformational freedom between the Ig domains, while not facilitating the type of intramolecular interactions seen in L1/axonin-1 (31, 32). The sequence was inserted between Lys193 and Leu194, the two residues identified in the crystal structure as a linker between Ig1 and Ig2 (19). This placement would increase the distance between interacting residues on Ig1 and Ig2 and thereby effectively disrupt the network of interdomain hydrogen bonds and salt bridges suggested from the crystal structure to orient the two domains. Thus, the loss of bead binding on insertion of these four residues is consistent with a loss of cooperativity in Ig12 binding, where the affinity has been reduced from the ~100 μM measured for reciprocal binding to the ~3 mM determined for single domain interactions in solution (3, 21). Mutation of two glutamic acid residues, Glu111 and Glu146 (19), results in a similar loss of Ig12 binding. In the crystal structure, these residues form salt bridges that are expected to orient Ig1 with respect to Ig2. The abrogation of binding by increasing the conformational freedom between Ig1 and Ig2, either through the insertion of additional residues or through point mutations, further supports the proposal that this reciprocal interaction is essential for N-CAM homophilic binding. Although the polyclonal antibody raised against bacterial Ig2, αIg2, did not affect binding (data not shown), the Ig2 sequence is highly conserved across species and is therefore assumed to be not very antigenic (33). This is consistent with the poor reactivity of αIg2 toward the native N-CAM (18).

The bead and cell aggregation studies presented here do not support a role for Ig3 in the adhesive interaction of N-CAM.
homophilic binding. We were unable to detect any binding attributable to Ig3 in two alternate approaches with domain deletions constructs. Specifically, the equivalent binding of Ig12 and Ig123, and of Δ45 and Δ345 argues against any involvement of Ig3. In addition, with a few exceptions discussed below, we failed to see detectible inhibition of N-CAM aggregation by reagents targeted to Ig3. The most notable exception was the inhibition observed with mAb1, which recognizes an epitope in near the N terminus of Ig3 (mAb1 recognizes recombinant Ig3 but not recombinant Ig3s). However, the data suggests that binding of this antibody perturbs the Ig1:Ig2 interaction rather than by directly blocking a binding event involving Ig3. The two deletion constructs Δ45 and Δ345 gave equivalent binding, suggesting that they bind via identical mechanisms; only Δ45 contains Ig3 and was susceptible to mAb1 inhibition. Thus, including Ig3 introduced the mAb1 epitope (which we suggest allows mAb1 to perturb the Ig1:Ig2 adhesive interaction) but did not contribute to the binding. This interpretation is supported by inhibition studies using the two polyclonal anti Ig3 antibodies. These antibodies were raised in parallel injections, yet demonstrated different specificities in inhibition experiments. While neither anti Ig3 antibodies inhibited N-CAM-Fc-mediated bead aggregation, anti Ig3-A was able to compete with mAb1 binding. These findings support the notion that mAb1 inhibition is attributable to the unique position of its epitope such that antibody binding affects the juxtaposed Ig12 interaction, and do not indicate an involvement of Ig3 in the adhesive interaction. Surprisingly, the mAb1 inhibition of cell aggregation appeared cell type-specific and less effective than in comparable bead experiments, suggesting that the monoclonal epitope may be differentially protected in cells. The second exception was the inhibition of the weaker 2:3-mediated aggregation by anti Ig3-A, but not by anti Ig3-B. We propose that the introduction of the four amino acid linker between Ig2 and Ig3 exposes an epitope/s, normally buried in the native protein, similarly positioned to the mAb1 epitope in that antibody binding affects the Ig12 interaction.

The conclusions from the bead aggregation experiments with recombinant fusion proteins were largely in agreement with transfected and primary cell experiments. The aggregation of transfected L cells and chick retinal cells were similarly insensitive to Ig3A and B, however, surprisingly, anti Ig3-A did inhibit the aggregation of primary brain cells. Retinal cells are a more homogenous population than total brain cells, and they exhibit a more robust NCAM-dependent aggregation. The presence of other proteins on the chick brain cells that influence NCAM aggregation and binding may explain the difference in the inhibitory effect of anti Ig3.

Although the reciprocal Ig12 interaction is necessary for N-CAM-mediated aggregation, the orientation of molecules and, in particular, their binding site presentation on cells can affect their recognition by apposing cells (2). It has been suggested that glycans orient molecules with respect to the cell membranes (34, 35), and it is possible that some or all of the glycans on N-CAM serve a similar function. In addition, cell surface presentation is influenced by the domain-domain orientations. Detailed structural information from several CAMs has revealed that the characteristics of the interface, including the length of the non-β linker, the number of hydrogen and salt bridges, and its hydrophobicity, influence the relative domain orientations (2). We have shown here that the disruption of the Ig2 Ig3 interface was detrimental (the additional 4 residues between Ig2 and Ig3 reduced binding by ~50%) although the adhesive Ig12 was not modified. Similar findings have been reported for other cell surface molecules. The crystal structure of the integrin LFA-1 I domain bound to the N-terminal 2 Ig domains of ICAM-1 identified specific interactions only between the I domain and the first Ig domain of ICAM-1 (36). However, mutations in the second Ig domain, predicted to affect the relative orientation of the two Ig domains, reduce binding in transfected COS cells (37). Thus, mutations that disrupt the presentation of binding sites on the cell surface are able to affect binding strength. An homology-based prediction of the structure of the adhesion molecule L1 has neighboring domains in contact (except for Ig2 and Ig3) and mapped many of the disease-associated mutations to the domain-domain interfaces (38). A similar homology based prediction on N-CAM suggests that its extracellular domains are also in contact, with short, 1–2 residue linker sequences between domains. Our data suggest that native domain-domain interfaces are required for optimal cell-cell binding mediated by homophilic interactions of N-CAM. One exception to this proposal is the interface between the 2 FnIII domains, as deletion of Fn2 did not affect binding. The Fn1 Fn2 boundary is the site of several splice variants, which range from the introduction of a single aag codon to the additional 35 amino acids found in the muscle-specific domain (MSD) (39). The existence of these splice variants, and the current finding that Fn2 is not required for bead aggregation, argues that the domain-domain interactions between Fn1 and Fn2 are not required for maximal homophilic binding. Interestingly, a recent report on the MSD isoform identified multiple O-linked sugars in the inserted sequence, and suggested that the sugars may function to provide rigidity to the insert (40).

The proposal that intimate domain-domain orientations influence the interactions of cell bound N-CAM appears to contradict the early EM studies of N-CAM on mica that identified a flexible hinge in the extracellular region (13, 14). Recent EM investigations of L1 indicated that non-native conformations may be observed due to interactions of proteins with the mica (31). Thus, the flexibility seen in the EM studies of N-CAM may be the result of a conformationally sensitive site that is perturbed by interactions with the mica. The site of flexibility in the EM images lies between Ig4 and Ig5, based on the structures of N-CAM Ig and FnIII domains (19). Supporting evidence for a conformationally sensitive site in this region comes from the observation of a proteolytic fragment from native N-CAM whose size is consistent with cleavage near the Ig4 Ig5 boundary (41). Furthermore, the recombinant fusion proteins lost adhesiveness after several freeze-thaw cycles without obvious signs of degradation. Recent surface force measurements on the interactions of immobilized N-CAM argued for the existence of a flexible hinge with a range of motion of 10 degrees (5). Flexibility of this order has been observed in the crystal structures of several cell adhesion molecules and is not inconsistent with the model proposed here for N-CAM binding.

On the basis of the force measurements of N-CAM interactions, a modified mechanism for homophilic binding was proposed (5). The authors observed an interaction that they attributed to the reciprocal Ig12 binding, in agreement with both our data and the published crystal structures (19, 20). In addition, a stronger interaction was detected that was attributed to an Ig3:Ig3 binding. We have found no evidence of such an interaction in solution or in our bead experiments, and similarly, Soroka et al. (19) reports that Ig3 was monomeric in their hands.

In addition, a novel zipper mechanism for N-CAM homophilic binding has been proposed based on the crystal structure of the first 3 Ig domains of rat N-CAM (19). In this model, the previously characterized Ig1-Ig2 interaction was reclassified as a cis interaction,
and three additional domain:domain interactions identified in the crystal were defined as adhesive trans interactions. This classification of interactions as cis or trans was based on the relative domain orientations in the crystal and thus, may be further redefined when the orientations of the remaining 5 extracellular domains are known. Furthermore, the fact that the Ig123 protein was reported to be dimeric not oligomeric in solution indicates that the proposed trans interactions are significantly weaker than the Ig1: Ig2 interaction. Moreover, our data show that Ig12 and Ig123 have the same binding constant.

Our data support a model for N-CAM homophilic binding in which the reciprocal binding of Ig12 provides the adhesive bond. However, the strength of this interaction can be modulated by the presentation of the binding sites on the cell surface, and is thus dependent on the domain orientations as determined by the domain-domain interfaces.

Acknowledgments—We thank Jamie Fritz, Kat Woodard, Katie Moiseff, and Michael Saunders, for expert technical assistance, Drs. Vince Mauro and Joe Gally for critical reading of this manuscript, and Dr. Jane Dyson for the P5 and P10 peptides.

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