

# Identification of a Peptide Sequence Involved in Homophilic Binding in the Neural Cell Adhesion Molecule NCAM

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**Abstract.** The neural cell adhesion molecule NCAM is capable of mediating cell-cell adhesion via homophilic interactions. In this study, three strategies have been combined to identify regions of NCAM that participate directly in NCAM-NCAM binding: analysis of domain deletion mutations, mapping of epitopes of monoclonal antibodies, and use of synthetic peptides to inhibit NCAM activity. Studies on L cells transfected with NCAM mutant cDNAs using cell aggregation and NCAM-covasphere binding assays indicate that the third immunoglobulin-like domain is involved in homophilic binding. The epitopes of four monoclonal antibodies that have been previously shown to affect cell-cell adhesion mediated by NCAM were also

mapped to domain 3. Overlapping hexapeptides were synthesized on plastic pins and assayed for binding with these monoclonal antibodies. One of them (PP) reacted specifically with the sequence KYSFNY. Synthetic oligopeptides containing the PP epitope were potent and specific inhibitors of NCAM binding activity. A substratum containing immobilized peptide conjugates also exhibited adhesiveness for neural retinal cells. Cell attachment was specifically inhibited by peptides that contained the PP-epitope and by anti-NCAM univalent antibodies. The shortest active peptide has the sequence KYSFNYDGSE, suggesting that this site is directly involved in NCAM homophilic interaction.

**C**ELL-CELL adhesive interactions are important contributors to the establishment of form and pattern during development (for reviews, see Rutishauser and Jessell, 1988; Takeichi, 1990; Reichardt and Tomaselli, 1991). A basic understanding of these complex interactions ultimately requires the isolation of adhesion molecules and the characterization of their mechanisms of action. The neural cell adhesion molecule NCAM<sup>1</sup> was one of the first adhesion molecules to be detected (Rutishauser et al., 1976; Brackenbury et al., 1977), isolated (Thiery et al., 1977), and characterized (Hoffman et al., 1982). NCAM is a relatively abundant glycoprotein present on both neural and non-neural cells, and is capable of mediating calcium-independent cell-cell adhesion (Rutishauser et al., 1982).

The structure of NCAM has been defined in considerable detail (Murray et al., 1986; Barthels et al., 1987; Cunningham et al., 1987; Barbas et al., 1988; Barton et al., 1988). This large membrane-associated glycoprotein belongs to the Ig superfamily of recognition molecules (Williams and Barclay, 1988). Its extracellular domain consists of five contiguous Ig-like domains, each with characteristic intrachain disulfide loops. NCAM is present in many polypeptide iso-

forms that are generated by tissue-specific splicing of RNA species derived from a single copy gene (for review, see Walsh and Doherty, 1991). The three most abundant forms, NCAM-180, NCAM-140, and NCAM-120, have similar amino-terminal extracellular domains but truncated carboxyl-terminal domains. While NCAM-180 and NCAM-140 are integral membrane proteins, NCAM-120 is attached to membranes via a glycosyl-phosphatidylinositol anchor (He et al., 1986; Hemperley et al., 1986). NCAM is further diversified by posttranslational modifications. A striking modification is the  $\alpha$ -2,8-linked polysialic acid, the amount of which is regulated during development and modulates cell-cell interactions (for reviews, see Edelman, 1983; Rutishauser, 1989).

NCAM appears to participate in cell-cell adhesion via homophilic interactions. The initial evidence for homophilic binding studies of lipid vesicles containing purified NCAM (Rutishauser et al., 1982; Hoffman and Edelman, 1983). More recently, the transfection of NCAM cDNA into fibroblasts to induce NCAM-dependent cell-cell binding (Edelman et al., 1987; Pizzey et al., 1989) has further supported this hypothesis. However, NCAM-mediated adhesion may be more complex than a simple homophilic ligation. It is known that NCAM also exhibits binding affinity for heparan sulfate (Cole et al., 1986b; Cole and Glaser, 1986), which can augment NCAM-mediated cell-cell binding (Cole et al., 1986a).

1. *Abbreviations used in this paper:* EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; KLH, keyhole limpet hemocyanin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; NCAM, neural cell adhesion molecule; PCR, polymerase chain reaction.

In defining the role and interrelationship of NCAM binding activities, it is valuable to identify the exact location of their respective functional sites and to elucidate the mechanism of binding. One adhesion-dependent region, corresponding to the heparin binding domain, has been mapped to the second Ig-like domain (Cole et al., 1986b; Frelinger and Rutishauser, 1986), and more recently to a 17-amino acid segment within this domain (Cole and Akeson, 1989; Reyes et al., 1990). However, the location of homophilic binding sites has been only generally defined. On the basis of mAb mapping, a site has been proposed to reside within a region corresponding to domains 3 and 4 (Frelinger and Rutishauser, 1986; Cunningham et al., 1987). To more precisely define NCAM homophilic binding sites, we have used three different strategies, including analysis of domain deletion mutants, mapping of mAb epitopes, and use of synthetic oligopeptides to inhibit NCAM homophilic interaction. Results presented in this report suggest that a sequence of 10 amino acids (KYSFNYDGE) located in domain 3 is directly involved in NCAM homophilic binding.

## Materials and Methods

### Materials

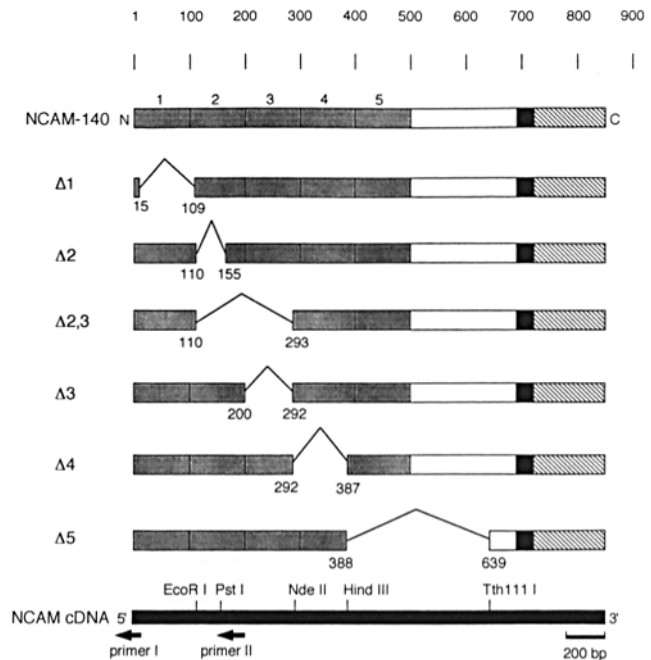
Prederivatized plastic pins and polypropylene trays were obtained from Cambridge Research Biochemicals (Valley Stream, NY). Active esters of 9-fluorenylmethoxy-carbonyl amino acids were obtained from MilliGen (Millipore Corp., Bedford, MA), phenylacetamidomethyl polystyrene resins were from Applied Biosystems Inc. (Foster City, CA), and *t*-butoxycarbonyl amino acids were from IAF Biochemicals (Montreal, Quebec, Canada). Covaspheres were obtained from Duke Scientific Corp. (Palo Alto, CA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) were purchased from Pierce Chemical Co. (Rockford, IL). Other reagents used were of highest chemical grade.

### Construction of Deletion Mutations in NCAM cDNA

pEC1402 that contains the entire coding region of chick NCAM-140 cDNA (Edelman et al., 1987) was kindly provided by Dr. B. Murray (University of California, Irvine, CA). A 1.8-kb HindIII fragment and a 2.8-kb EcoRI fragment were isolated from pEC1402 and inserted in pBluescript SKII(+) for deletion and DNA sequencing. Deletions were made using the convenient restriction sites, such as EcoRI, PstI, NdeI, HindIII, and Tth111 I (Fig. 1). To delete the heparin-binding site in domain 2 ( $\Delta 2$ -NCAM), the 2.8-kb EcoRI fragment was digested with PstI and HindIII to release a 0.7-kb fragment that was used to replace the 0.9-kb EcoRI-to-HindIII fragment in the pEC1402 vector. To delete domain 4 ( $\Delta 4$ -NCAM), the cDNA was digested with Nde I, filled in with Klenow fragment to generate blunt end, and then ligated to the EcoRI site using EcoRI linker. To delete domain 5 ( $\Delta 5$ -NCAM), the cDNA was digested with Tth111 I, filled in to generate blunt end, and then ligated to the HindIII site using HindIII linker. Both  $\Delta 1$ -NCAM and  $\Delta 3$ -NCAM were constructed using the primers: 5'-GAA-TTCGCAACGCTGATC-3'(I), 5'-ACATATGTGCTCTGCCTGGCAGC-3'(II), and the M13 universal primer, in polymerase chain reactions (PCR). Primer I contained an EcoRI site at its 5' end and the polymerase chain reaction (PCR) product was used to replace the fragment upstream of the internal EcoRI site to construct  $\Delta 1$ -NCAM. Primer II contained the NdeI site at its 5' end and the PCR product was used to replace the EcoRI to NdeI fragment to generate  $\Delta 3$ -NCAM. All deletion mutations were verified by double-strand DNA sequencing (Chen and Seeburg, 1985) using the dideoxy chain termination method (Sanger et al., 1977). The NCAM mutants were all cloned into the expression vector pEC, which used the SV-40 early promoter to drive NCAM expression.

### Cell Transformation

Mouse L (*tk*<sup>-</sup>) cells were grown with  $\alpha$ -MEM medium containing 10% FCS in 10-cm dishes until subconfluence. Cells were collected and plated on 10-cm dishes (at 1:10 dilution) 1 d before transformation. The cells were



**Figure 1.** NCAM deletion mutant constructs. Deletion mutants were constructed using wild-type cDNA coding for NCAM-140. The resulting NCAM mutant products were schematically represented, with the five homologous Ig-like domains shown by stipples, the transmembrane region by a solid bar, and the cytoplasmic domain by stripes. The numbering of amino acids is in accordance with Cunningham et al. (1987). Numbers adjacent to each deletion indicate amino acids flanking the deletion and present in the mutant protein. Mutant cDNAs were cloned into the pEC vector for transfection into L cells. NCAM cDNA and the restriction sites used in the construction of these deletion mutations are shown at the bottom. The two arrows indicate the complementary sites of the two primers used to generate PCR fragments.

transfected with 20  $\mu$ g of NCAM plasmid DNA using the calcium phosphate precipitation method (Sambrook et al., 1989). 2 d after transformation, cells expressing NCAM were selected with a rabbit anti-NCAM polyclonal antibody using the cell panning method (Wysocki and Sato, 1978). Microbiological-grade Petri dishes (10 cm diam) were coated with 10 ml of rabbit anti-NCAM IgG at 5  $\mu$ g/ml for 40 min and washed five times with PBS and once with 1% fetal bovine serum in PBS. Cell suspension (3 ml) was added to each dish and allowed to adhere to the IgG-coated surface for 60 min at 4°C. The unbound cells were removed by gentle washing, while the bound cells were transferred to a tissue culture-grade dish. 2 wk later the cells were selected again using the same procedure to isolate the stable NCAM-expressing cells. The bound cells were further subcloned in 96-well dishes using the limited dilution method.

### Immunofluorescence Staining of NCAM

Cells were seeded sparsely on coverslips 24 h before staining. Cells were washed once with PBS and then fixed with 100% methanol at  $-20^{\circ}\text{C}$  for 2 min. Coverslips were incubated for 5 min at room temperature with a blocking solution of 1% BSA in PBS. Coverslips were then incubated with 100  $\mu$ l of 1% BSA and 5  $\mu$ g/ml rabbit anti-NCAM polyclonal antibody for 1 h. The cells were then washed five times with PBS before incubation with biotinylated goat anti-rabbit IgG for 1 h. Cells were again washed five times and then incubated with streptavidin-conjugated Texas Red. Slides were observed using a Wild Leitz Orthoplan fluorescence microscope (Wild Leitz, Toronto, Canada). When cells were stained with anti-NCAM mAb, rhodamine-conjugated, affinity-purified F(ab')<sub>2</sub> fragments of goat anti-mouse IgG were used as the second antibody.

### Electrophoresis and Immunoblotting

Cells ( $1 \times 10^6$ ) were plated on dishes 24 h before harvest. Cells were col-

lected in PBS containing 4 mM EDTA. After centrifugation at 1,000 rpm for 3 min, the cell pellet was washed once with PBS and lysed in a solution containing 2% SDS, 8 M urea, 10 mM Tris (pH 8.0), 1 mM EDTA, 2 mM PMSF, 5 µg/ml trypsin inhibitor, 10 µg/ml aprotinin, and 10 µg/ml DNase I. Undissolved material was removed by centrifugation at 10,000 rpm for 2 min. Aliquots of the soluble fraction (equivalent to  $\sim 2 \times 10^5$  cells) were electrophoresed on a 7.5% SDS-polyacrylamide gel (Laemmli, 1970) and then electroblotted onto nitrocellulose filter (Towbin et al., 1979). The blot was indirectly stained with a rabbit polyclonal antiserum directed against NCAM and an alkaline phosphatase-conjugated second antibody.

### Quantification of NCAM Expression in Transformants

The relative levels of NCAM expression in L cell transformants were determined by using an ELISA as described by Doherty et al. (1989). Approximately 20,000 cells were seeded in 96-well plates and grown for 18 h. After removal of the culture media, cells were fixed with prechilled methanol ( $-20^\circ\text{C}$ ) for 10–15 min. The wells were incubated with 1% BSA for 60 min to block nonspecific protein-binding sites. Anti-NCAM mAb (100 µl at 2 µg/ml) was added to the wells and incubated at  $20^\circ\text{C}$  for 60 min. After three washes with 1% BSA in PBS, the cells were further incubated with 100 µl of anti-mouse horse peroxidase-conjugated F(ab')<sub>2</sub> (1:1,000 dilution) for 60 min. Cultures were washed four times with PBS and twice with water. Color development was carried out by incubation with 100 µl of 0.05% 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) and 0.02% (vol/vol) H<sub>2</sub>O<sub>2</sub> in citric acid buffer for 60 min. The OD at 405 nm was monitored with a Titertek Multiscan microtiter plate reader (Flow Laboratories, Toronto, Canada).

### Solid-Phase Peptide Synthesis

Overlapping hexapeptides were synthesized on polyethylene pins as described by Geysen et al. (1987). The peptides were assembled on the pins in the C to N terminus direction using 9-fluorenylmethoxy-carbonyl amino acids (Carpino, 1987). Larger quantities of oligopeptides were synthesized by classical solid-phase methods (Erickson and Merrifield, 1976; Steward and Young, 1984) on phenylacetamidomethyl resin supports using *t*-butoxycarbonyl amino acids. Synthesis was carried out on an automated peptide synthesizer (model 430; Applied Biosystems, Inc.). Side chain protecting groups were removed and the peptides were cleaved from the support using anhydrous hydrogen fluoride. The peptides were extracted from the resin with 10% aqueous acetic acid, lyophilized, and analyzed for purity by reverse-phase HPLC and for composition by amino acid analysis. Peptides were desalted on a P2 gel filtration column equilibrated in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> and lyophilized.

### ELISA for Peptide-conjugated Plastic Pins

Peptides coupled to solid-support plastic pins were incubated in a blocking solution containing 2% (wt/vol) BSA, 0.1% (vol/vol) Tween-20, and 0.05% (wt/vol) sodium azide in PBS for 30 min at room temperature with gentle rocking. The pins were then incubated overnight in microtiter plates containing 130 µl per well of mAb (2–5 µg/ml in blocking solution) at  $4^\circ\text{C}$ . They were washed four times, 10 min each, in a dish pan containing PBS and 0.05% Tween-20. This was followed by incubation for 1 h in microtiter wells containing 130 µl of a goat anti-mouse IgG antibody conjugated with peroxidase or alkaline phosphatase on a platform shaker. Pins were washed four times, 10 min each, and the once in the substrate buffer. The pins were then transferred to a microtiter plate containing 100 µl of substrate per well. Color development was monitored at 405 nm using a microtiter plate reader.

### Aggregation of Brain Vesicles

Brain vesicles were prepared from 14-d-old chick embryos as described (Hoffman et al., 1982), and their rate of aggregation was determined in a rotation-suspension assay using a particle counter (Rutishauser et al., 1985). Briefly, fresh membrane vesicles suspended in PBS were rotated at 90 rpm on a rotary shaker at  $25^\circ\text{C}$ , with aliquots taken at 0, 3, and 6 min. The aliquots were fixed in 1% glutaraldehyde before counting with a ZBI analyzer (Coulter Electronics, Hialeah, FL).

### Cell Aggregation Assay

NCAM transformants were plated on dishes 20 h before assay to ensure that they were in their exponential growth phase. Aggregation assay was carried

out according to Pizzey et al. (1989) with minor modifications. Cells were collected in PBS containing 2 mM EDTA and centrifuged at 500 rpm for 5 min. Cells were washed once with PBS and a suspension of single cells ( $\sim 3 \times 10^6$  cells/ml) was prepared in  $\alpha$ -MEM medium containing 1% FBS. 200-µl aliquots were incubated in plastic tubes at  $37^\circ\text{C}$  for 40 min, followed by four cycles of up and down rotation on a Labquake shaker (8 cycles/min) to ensure sampling from a homogeneous population. Single cells were counted using a hemacytometer.

When neural retinal cells were used in this assay, cells were isolated from 10-d-old chick embryos using the method of Brackenbury et al. (1981). Retinal cells were dissociated in Mg<sup>2+</sup>/Ca<sup>2+</sup>-free HBSS containing 0.005% (wt/vol) trypsin and 2 mM EDTA. The cells were then centrifuged through a step gradient of BSA before resuspension at  $\sim 5 \times 10^6$  cells/ml in PBS containing 50 µg/ml of DNase I. 0.2-ml aliquots were rotated on a platform shaker at 150 rpm at  $37^\circ\text{C}$  and the percentage of cell aggregation was monitored at regular time intervals.

### Binding of NCAM-conjugated Covaspheres to Cells

Green or red MX covaspheres (100 µl) were briefly sonicated before the addition of 20 µg of chick brain NCAM and the final volume was adjusted to 200 µl with PBS. After incubation at room temperature for 75 min, the covaspheres were centrifuged at 10,000 g for 10 min at  $4^\circ\text{C}$ . The pellet was resuspended in 1 ml of PBS containing 1% BSA to block the unreactive sites on the covaspheres. The covaspheres were centrifuged and resuspended in 100 µl PBS. To assay for the ability of the NCAM-covaspheres in cell binding, NCAM-transformed cells were seeded sparsely on coverslips 20 h before the assay. In case of neural retinal cells, freshly prepared cells were allowed to adhere to coverslips for 30 min at  $37^\circ\text{C}$ . Coverslips were then washed once with PBS and incubated with 100 µl of NCAM-covaspheres (at 1:20 dilution of stock) for 30–40 min at room temperature on a platform shaker. After washing several times with PBS, cells were observed using epifluorescence microscopy. Cells with more than five covaspheres attached on the surface were scored as positive cells and generally 100–200 cells were scored in each experiment.

### Cell-Substratum Adhesion Assay

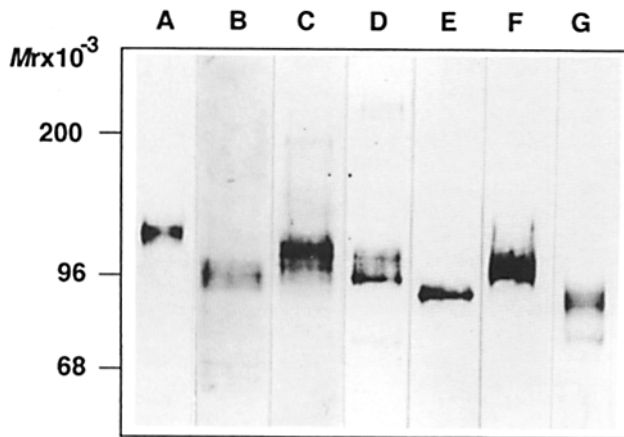
The plastic surface of Petri dishes was coated with nitrocellulose solubilized in methanol, according to the method of Lagenaur and Lemmon (1987), and the dishes were air dried. P5 was conjugated to keyhole limpet hemocyanin (KLH) using EDC (Yamada et al., 1981). The 2-ml reaction mixture contained 4 mg P5 and 10 mg KLH and the reaction was carried out for a period of 4 h at room temperature. The P5-KLH conjugate was separated from the uncoupled peptide on a P2 column. P11 (CEKYSFNYDGSE) was conjugated to KLH via the *cys* residue added to its amino terminus using MBS (Lerner et al., 1981). Coupling was carried out overnight at room temperature and the uncoupled peptide was removed by dialysis. To allow the peptide conjugates to adsorb to the nitrocellulose surface, 50-µl aliquots of P5- or P11-KLH (100 µg/ml) were placed on 1-cm-diam spots previously marked on the plastic surface for 1.5 h. Excess binding sites were blocked by 1% (wt/vol) BSA and the dish was washed three times with PBS.

Retinal cells were isolated from day 10 chick embryos and resuspended in leucine-free medium containing 0.2% (wt/vol) BSA. Cells were metabolically labeled with [<sup>3</sup>H]leucine (25 µCi/ml) for 2 h at  $37^\circ\text{C}$ . Labeled cells were washed with medium containing 10% FBS and 50-µl aliquots were placed on the peptide-KLH spots. Dishes were incubated for 2 h at  $37^\circ\text{C}$  for cell attachment. In competition experiments, 25-µl aliquots of competitor (2 mg/ml) were placed on the peptide-KLH spots for 5 min before the addition of the 25 µl of labeled cells at twofold cell concentration. At the end of the incubation period, dishes were flooded with PBS to remove the unbound cells, followed by three washes. Control spots with adsorbed KLH were included in each dish to monitor the consistency of the washing procedure and all assays were carried out in triplicate. The bound cells were solubilized by rinsing each spot twice with 50 µl of 2% (wt/vol) SDS and the combined lysed material was counted in a scintillation counter.

## Results

### Expression of Deletion Mutants of NCAM in L Cell Transformants

Our strategy to identify domains that contribute to the NCAM homophilic binding site was to express NCAM pro-



**Figure 2.** NCAM expression in L cell transformants. NCAM transformants were collected and subjected to electrophoretic analysis. Proteins were blotted onto nitrocellulose filter and immunostained using an anti-NCAM rabbit antiserum. Molecular weight standards are indicated on the left. Molecular sizes of mutant NCAM proteins were estimated. Lane A, wild-type NCAM (sd form) with 135 kD; lane B,  $\Delta$ 1-NCAM with 95 kD; lane C,  $\Delta$ 2-NCAM with 125 kD; lane D,  $\Delta$ 3-NCAM with 94 kD; lane E,  $\Delta$ 2,3-NCAM with 87 kD; lane F,  $\Delta$ 4-NCAM with 116 kD; and lane G,  $\Delta$ 5-NCAM with 85 kD.

teins carrying deletion mutations and then determine which mutations would abolish NCAM homophilic interaction. A series of deletion mutations was generated in the cDNA of NCAM-140 (Fig. 1). Transformation vectors carrying these mutant inserts were used to transfect L cells. Cotransformation was initially performed using the pSVneo vector (Southern and Berg, 1982) and G418-resistant colonies were isolated and cloned. About 20% of these colonies expressed NCAM, with a transformation efficiency of  $\sim 1 \times 10^{-5}$ . Cells were also transformed with the pEC vector without the NCAM cDNA insert to serve as controls. Since most of the NCAM-positive clones were not stable in their expression of NCAM, they required frequent reselection by the cell panning method (Wysocki and Sato, 1978). Cells expressing NCAM were selected by several cycles of cell panning on a substratum coated with rabbit anti-NCAM IgG, followed by subcloning in 96-well plates. Clonal lines that had been passaged for 8–10 wk were routinely reselected by cell panning for high expression cells. Transformants expressing non-functional NCAM mutants were more stable and did not require frequent reselection.

L cell transformants were subjected to immunoblot analysis to identify the form of NCAM expressed (Fig. 2). The wild-type NCAM migrated as a broad band with an apparent molecular size of 135–140 kD, similar to what is expected for NCAM-140. The sizes of the other NCAM deletion mutants expressed in transformants were also in agreement with their predicted values. The relative levels of NCAM in transformed L cells were also quantified by ELISA and the results are summarized in Table I. All of the NCAM transformants showed a five- to eightfold higher signal than the control transformant. Since NCAM was not detected in control cells by immunoblot analysis, the weak signals obtained with these cells probably represent nonspecific trapping of the antibody. To examine the cellular distribution of NCAM, transformed cells were fixed and immunostained with a rabbit an-

**Table I.** Expression of NCAM in L Cell Transformants

NCAM construct	Relative level of NCAM expression (OD <sub>405</sub> )
pEC	0.057 ± 0.015
Wild-type NCAM	0.269 ± 0.015
$\Delta$ 1-NCAM	0.277 ± 0.032
$\Delta$ 2-NCAM	0.261 ± 0.026
$\Delta$ 3-NCAM	0.298 ± 0.015
$\Delta$ 2,3-NCAM	0.394 ± 0.037
$\Delta$ 4-NCAM	0.371 ± 0.028
$\Delta$ 5-NCAM	0.429 ± 0.011

ELISA was carried out according to the methods of Doherty et al. (1989). Anti-NCAM mAbs 5E and 9A (Watanabe et al., 1986) were used as primary antibodies. Color development was carried out for 60 min and the OD at 405 nm was read using a multititer plate reader. Values represent the mean ± SD of three separate assays.

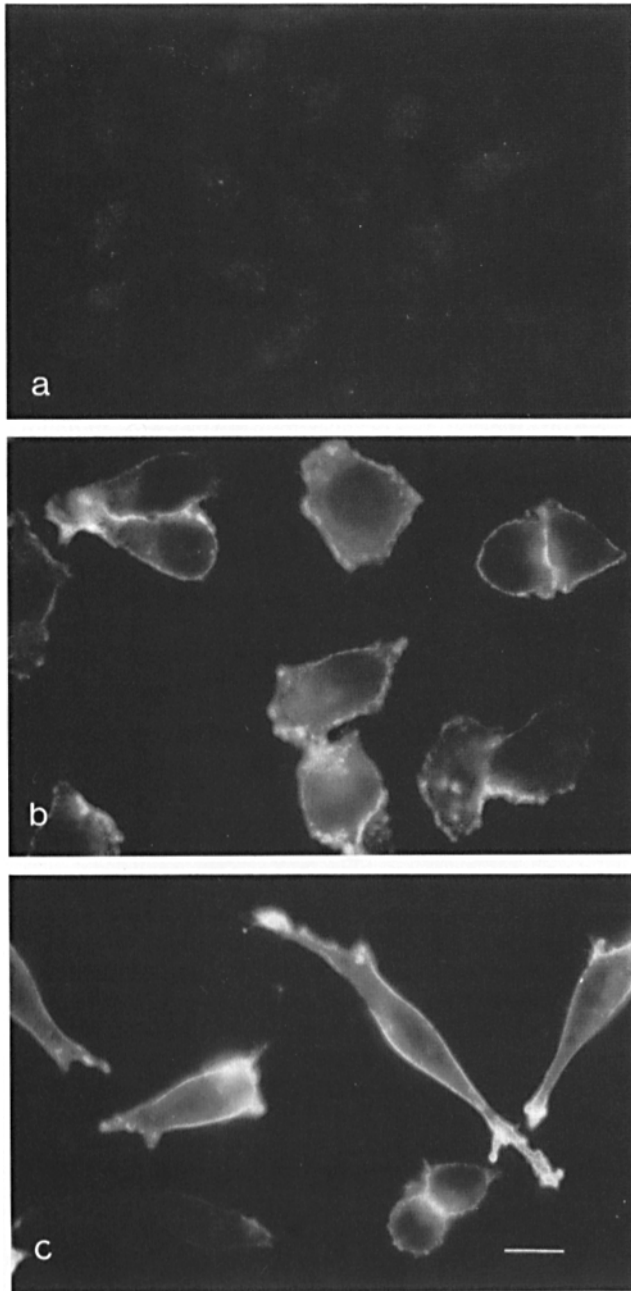
tiserum. Most of the cells showed strong fluorescence labeling associated with the plasma membrane (Fig. 3). These data indicate that both wild-type and mutant NCAM proteins were transported properly to the plasma membrane of transformed cells and stably anchored there.

### Aggregation of Transformed Cells

L cell transformants expressing wild-type NCAM exhibited a mutual adhesiveness in culture, giving rise to compact colonies with cell–cell contacts. On the other hand, control transformants retained the L cell morphology and rarely engaged in cell–cell contacts. In cell adhesion assays, with NCAM transformants subjected to rotation at 60–120 rpm, only small aggregates were formed with no significant difference between NCAM-positive and control cells. However, when cells were allowed to aggregate without external shear forces as described by Pizzey et al. (1989), a clear difference could be observed between the control transformant and cells expressing NCAM, with the latter forming much larger aggregates (Fig. 4). Quantitative results obtained with assays using this procedure are summarized in Table II. Over half of the cells expressing wild-type NCAM were recruited into large aggregates, while only  $\sim 15\%$  of control cells formed small aggregates. Cells expressing NCAMs with deletions in domain 1 ( $\Delta$ 1-NCAM), domain 2 ( $\Delta$ 2-NCAM), domain 4 ( $\Delta$ 4-NCAM), or domain 5 ( $\Delta$ 5-NCAM) were also capable of forming aggregates, although to a lesser degree than intact NCAM transformants. Nevertheless, the percentages of cell aggregation elicited by these NCAM mutants were significantly higher than that of the control transformant. In contrast, transformants expressing NCAMs with deletions in domains 2 and 3 ( $\Delta$ 2,3-NCAM) or domain 3 alone ( $\Delta$ 3-NCAM) lost their ability to form aggregates completely. From these results, it would appear that the third Ig-like domain of NCAM is particularly important in NCAM-mediated cell–cell adhesion.

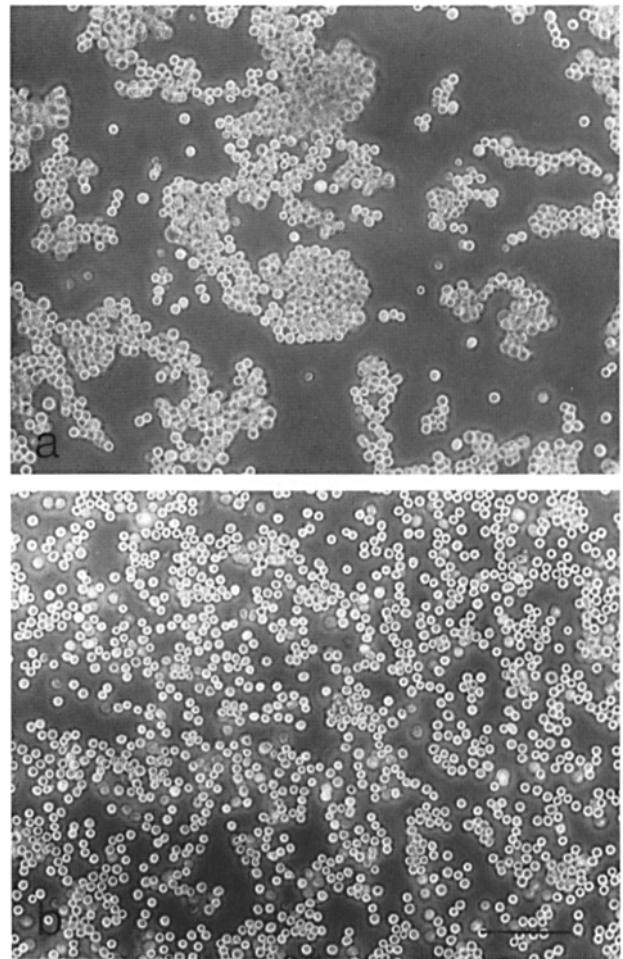
### Binding of NCAM-conjugated Covaspheres to Transformed L Cells

In these studies, purified NCAM conjugated to covaspheres was tested for binding to cells at room temperature. Under these conditions, uptake of NCAM-covaspheres by L cells or control transformants was negligible for up to 60 min of incubation, and binding of NCAM-covaspheres to these cells was minimal (Fig. 5). These results indicate that there is lit-



**Figure 3.** Epifluorescence micrographs of L cell transformants. Transformed cells were cultured on glass coverslips and fixed with methanol. Cells were stained with rabbit anti-NCAM antiserum. (a) Cells transformed with the pEC vector; (b) cells transformed with the wild-type NCAM construct; and (c) cells transformed with the  $\Delta 4$ -NCAM construct. Bar = 10  $\mu\text{m}$ .

the nonspecific attachment of covaspheres to cells. With the use of NCAM transformants, this assay served as a useful *in vitro* test for the homophilic binding activity of NCAM. When cells expressing wild-type NCAM were examined, specific binding of NCAM-covaspheres could be observed within 10–15 min. Samples were routinely incubated for 30 min and then observed by fluorescence microscopy. Contours of cells expressing NCAM were conspicuously decorated by the fluorescent spheres (Fig. 5). This binding was abolished when cells were preincubated with anti-NCAM Fab, but not with preimmune rabbit Fab.



**Figure 4.** Aggregate formation of L cell transformants. Transformed cells were collected and assayed for their ability to form aggregates in still medium at  $5 \times 10^6$  cells/ml. 0.2-ml aliquots were incubated at 37°C for 40 min and then rotated up and down four times on a shaker. Samples were observed under the light microscope. (a) Cells transformed with the wild-type NCAM construct and (b) cells transformed with the control vector. Bar = 100  $\mu\text{m}$ .

Cells expressing mutant NCAMs were also assayed for their ability to bind NCAM-covaspheres. The result for the  $\Delta 4$ -NCAM transformant is shown in Fig. 5c and is representative of  $\Delta 1$ -,  $\Delta 2$ -, and  $\Delta 5$ -NCAM transformants (Table II). However, cells expressing  $\Delta 2,3$ -NCAM or  $\Delta 3$ -NCAM were unable to bind NCAM-covaspheres. As with the cell aggregation data, these results suggest that NCAM homophilic binding is associated with Ig domain 3.

#### ***Immunoreactivity of Monoclonal Antibodies with NCAM Mutants***

A limitation of deletion analysis is that the three-dimensional structure of the protein may undergo substantial changes, and effects on the binding activity of NCAM may not be attributable to the actual deletion. Additional approaches are therefore necessary to ascertain results derived from the NCAM deletion mutants. The number of mAbs raised against chicken NCAM have been found to exert either positive or negative effects on NCAM-mediated cell adhesion (Watanabe et al., 1986). Of these, mAbs PP and 12B in-



**Table II. Intercellular Adhesion and Binding of NCAM-Covaspheres to Cells Expressing Mutant NCAMs**

NCAM construct	Cell aggregation*	Binding of NCAM-covaspheres† (% positive cells)
	%	
pEC	15 ± 5	9 ± 1
Wild-type NCAM	54 ± 12	57 ± 3
Δ1-NCAM	38 ± 8	43 ± 13
Δ2-NCAM	40 ± 9	44 ± 6
Δ3-NCAM	13 ± 8§	10 ± 4
Δ2,3-NCAM	13 ± 2§	7 ± 2
Δ4-NCAM	34 ± 4	71 ± 7
Δ5-NCAM	38 ± 3	46 ± 7

\* Cells were dispersed and resuspended at  $2 \times 10^6$  cells/ml. Aliquots were allowed to aggregate in still medium at 37°C for 40 min and then rotated up and down four times before counting. Values represent the mean percentage of cells in aggregates ± SD of three to five experiments.

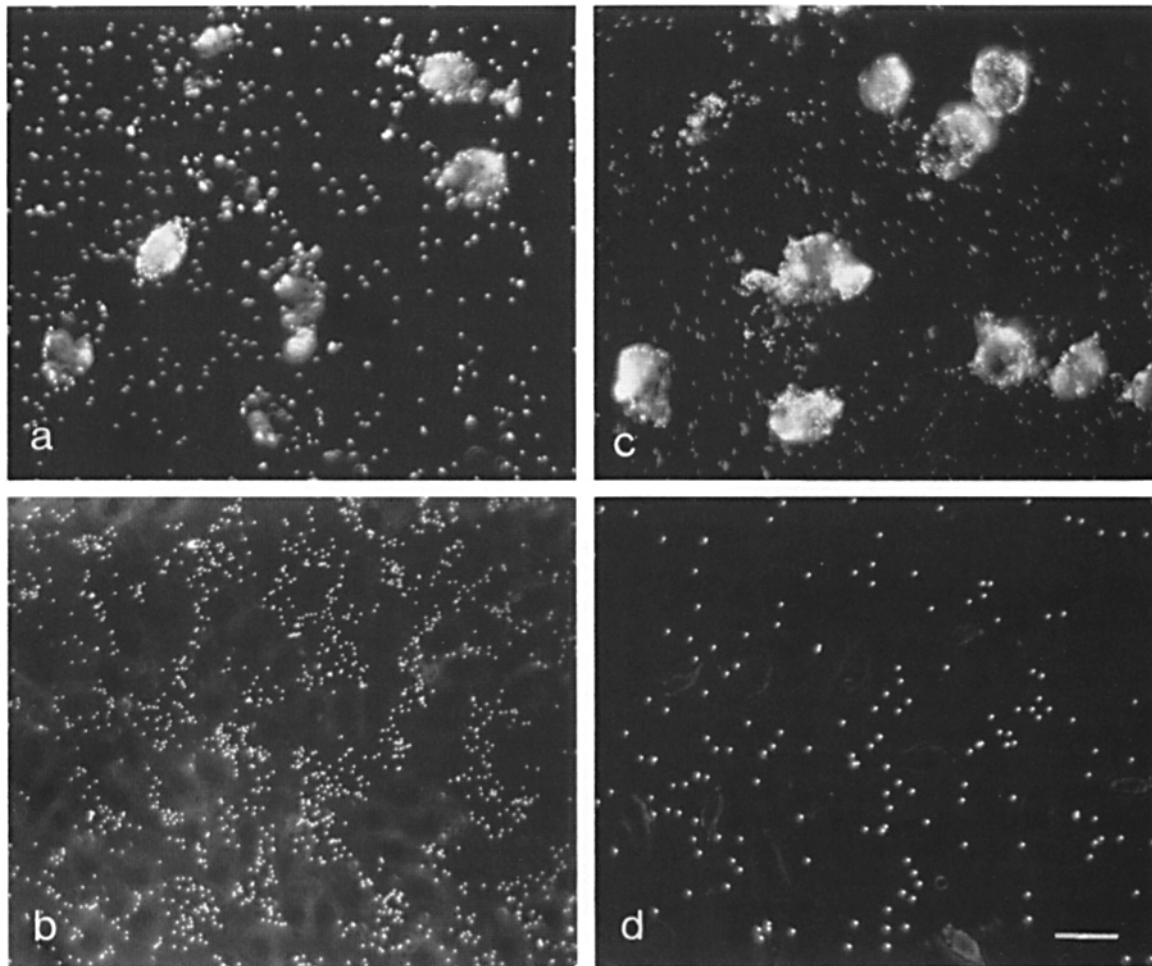
† Binding of NCAM-conjugated covaspheres to transformed cells was carried out at room temperature for 20 min as described in Materials and Methods. Cells with more than five covaspheres attached on the surface were scored as positive cells; results represent the mean ± SD of three independent experiments.

§ Results obtained with cells transformed with the Δ3-NCAM or Δ2,3-NCAM construct do not show a significant difference from the control ( $P > 0.05$ ), while results derived from the other transformants are all statistically different from the control transformant, with  $P < 0.001$ .

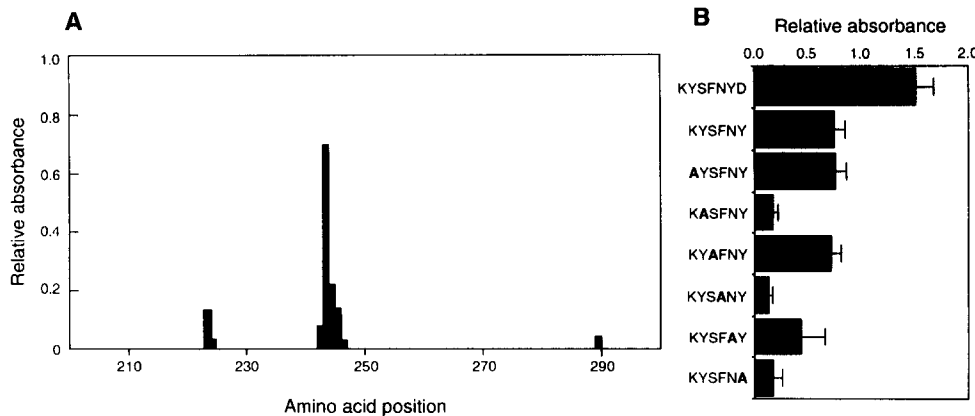
hibited cell adhesion, while mAbs 9A and 11B enhanced adhesion. Identification of epitopes recognized by these mAbs should provide an additional method to locate sites of NCAM binding function. Cells expressing different mutant NCAM proteins were used to map the epitope locations for these four mAbs, using both cell panning and immunofluorescence assays. Cells were also tested against rabbit polyclonal anti-NCAM antibodies as a positive control, and against mAb-30B which recognizes the cytoplasmic domain of NCAM-180 (Watanabe et al., 1986) as a negative control. Cells expressing either Δ2,3-NCAM or Δ3-NCAM did not react with any of the four anti-NCAM mAbs in either assay, while the other mutant NCAM proteins were recognized by all four mAbs (data not shown). These results indicate that the epitopes of these four mAbs reside in Ig domain 3.

### Mapping of the mAb PP Epitope

Assuming that the epitopes recognized by these four mAbs are in physical proximity to the homophilic binding site in domain 3, it would be useful to determine their exact locations in the protein sequence of this domain. The fact that these four mAbs are able to react with denatured NCAM



**Figure 5.** Binding of NCAM-conjugated covaspheres to L cell transformants. Coverslips were washed once with PBS and then incubated with 200  $\mu$ l of NCAM-conjugated covaspheres for 20 min at room temperature. Cells were gently washed three times with PBS before observation by epifluorescence microscopy. As a control, NCAM transformants were incubated with anti-NCAM Fab (0.5 mg/ml) for 20 min and then washed once with PBS before incubation with NCAM-covaspheres. (a) Cells transformed with the wild-type NCAM construct; (b) cells transformed with the Δ2,3-NCAM construct; (c) cells transformed with the Δ4-NCAM construct; and (d) cells in a pre-coated with anti-NCAM Fab. Bar = 20  $\mu$ m.



**Figure 6.** Binding of mAb-PP to NCAM overlapping hexapeptides. Hexapeptides were assembled on polyethylene pins as described by Geysen et al. (1987). Pins were incubated with PP-IgG (5  $\mu$ g/ml) in microtiter wells and the relative amounts of IgG bound for each pin was assayed by ELISA. The x axis in A shows the corresponding position of the NH<sub>2</sub>-terminal amino acid of each hexapeptide in the intact NCAM molecule (Cunningham et al., 1987). Each vertical bar represents a measure

of antibody binding (absorbance at 405 nm) to the corresponding hexapeptide. This histogram in B shows the binding of PP-IgG to different peptide analogues and the values are expressed as mean  $\pm$  SD of three determinations.

fragments in immunoblots (Watanabe et al., 1986) suggests that they may be able to recognize a linear sequence of amino acids. To identify such sequences, the strategy of Geysen et al. (1987) was adopted, using overlapping hexapeptides covering the NCAM amino acid sequence from Leu-208 to Met-300 (Cunningham et al., 1987). Thus, each successive peptide contained the last five amino acids of the previous one plus the next amino acid in the sequence. These hexapeptides were synthesized on plastic pins, and the binding of anti-NCAM mAbs to the pins was assayed by ELISA. The only positive signals obtained were with mAb-PP. The histogram in Fig. 6 A depicts the relative intensities of ELISA signals obtained with mAb-PP. The hexapeptide sequence KYSFNY reproducibly reacted most strongly with mAb-PP, while neighboring sequences were only weakly reactive. These results thus define the boundaries of the PP epitope to between Lys-243 and Tyr-248.

To determine the relative contribution of these six amino acids in antibody binding, peptide analogues with single amino acid substitutions were synthesized on plastic pins. The relative ELISA intensities obtained with these peptide analogues are shown in Fig. 6 B. Substitutions of Tyr-244, Phe-246, or Tyr-248 with Ala reduced the reactivity of the epitope by >70%. On the other hand, substitutions of Lys-243 or Ser-245 had negligible effects on antibody binding. Substitution of Asn-247 with Ala showed an intermediate effect. Interestingly, the addition of Asp-249 to the COOH terminus of the hexapeptide led to a twofold increase in IgG

binding (Fig. 6 B), although the immunoreactivity of the sequence YSFNYD was relatively low (Fig. 6 A). This suggests that the addition of one amino acid between Tyr-248 and the plastic pin might have allowed the peptide to react more readily with the PP-IgG.

#### Effects of Synthetic Peptides on the Aggregation of Brain Membrane Vesicles

To determine whether the epitope of mAb-PP is actually a part of the homophilic binding site, oligopeptides were synthesized and tested for their ability to compete for NCAM-mediated adhesion. Initially, four oligopeptides (P5, P6, P7, and P10) covering the PP epitope and/or its adjacent sequences were synthesized and tested for their ability to interfere with brain membrane vesicle aggregation over assay periods of 3 and 6 min. P5 contained 21 amino acids, starting with Lys-243 at its amino terminus, while P6 lacked the amino-terminal PP epitope sequence covered by P5 and started with Asp-249 (see Fig. 10). P10 contained the sequence between Lys-243 and Glu-252, while P7 contained a proximal sequence, spanning the sequence between Thr-229 and Lys-243. As a control, a peptide P5S that contained a scrambled P5 sequence was included in the assay.

Chick embryonic brain membrane vesicles containing the high sialic acid form of NCAM aggregated rapidly at 25°C, and this adhesion was inhibited specifically by the presence of Fab derived from either rabbit anti-NCAM antibodies or

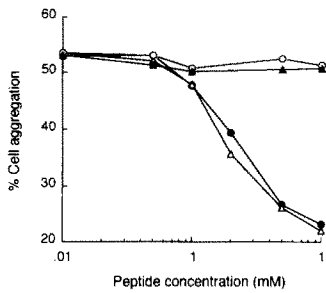
**Table III. Effect of Synthetic Peptides on Brain Membrane Vesicle Adhesion**

Peptide	Sequence	Aggregation of vesicles (%)*			
		3 min	% inhibition	6 min	% inhibition
None		39 $\pm$ 3	–	53 $\pm$ 4	–
P5	KYSFNYDGSELIKKVDKSDE	1 $\pm$ 2	97	0 $\pm$ 0	100
P5S	EDSKYVKIIKLYSGDENFSDK	41 $\pm$ 3	0	55 $\pm$ 5	0
P6	DGSELIKKVDKSDE	37 $\pm$ 4	5	52 $\pm$ 5	2
P7	TKDGEPIEQEDNEEK	31 $\pm$ 3	21	47 $\pm$ 5	11
P10	KYSFNYDGSE	6 $\pm$ 3	85	22 $\pm$ 7	58
Anti-NCAM polyclonal Fab <sup>‡</sup>		8 $\pm$ 1	79	15 $\pm$ 2	72
mAb-PP Fab <sup>§</sup>		19 $\pm$ 3	51	29 $\pm$ 2	45

\* Brain membrane vesicles from embryonic day 14 chicks were rotated at 90 rpm at 25°C, and the percent decrease in particle count was determined at 3 and 6 min. In competition experiments, peptides were added at 0.8 mg/ml. Data are presented as mean  $\pm$  SD of four to eight determinations.

<sup>‡</sup> 0.5 mg/ml Fab.

<sup>§</sup> 0.1 mg/ml Fab.



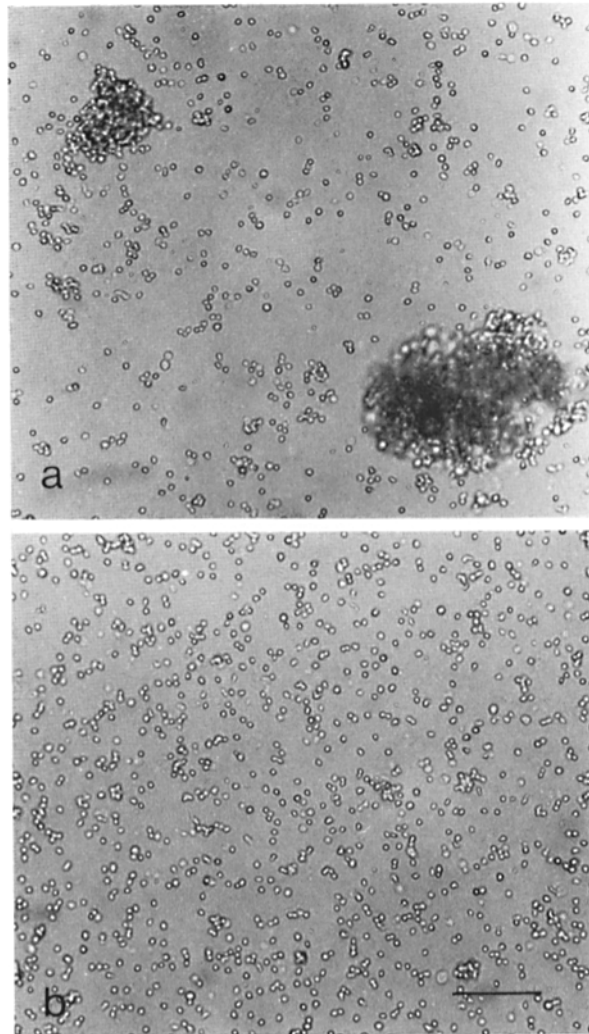
**Figure 7.** Dose effects of synthetic peptides on retinal cell aggregation. Neural retinal cells were prepared from 10-d-old chick embryos. Aggregation of retinal cells was carried out in the presence of different concentrations of P5 (●), P5S (○), P10 (△), or P6 (▲) at 37°C for 30 min. Values represent the average of two to three separate assays.

mAb-PP (Table III). Remarkably, the presence of P5 produced an even more dramatic inhibition than either antibody, with an almost complete block of adhesion. P10 also was a potent inhibitor, although at the later assay point (6 min) its effectiveness decreased to levels similar to that of antibodies. In contrast, P5S, P6, and P7 had no effect at all in this assay. Since the only difference between P5 and P6 is the six additional amino acids at the amino terminus of P5, it is likely that this amino-terminal segment of P5 harbors an NCAM binding site related to cell adhesion.

### Effect of Synthetic Peptides on Neural Retinal Cell Aggregation

Neural retinal cells from day 10 chick embryos exhibit NCAM-mediated cell-cell adhesion, which can be effectively inhibited by anti-NCAM Fab (Rutishauser et al., 1982). Whereas brain membrane vesicles contained the high sialic acid form of NCAM, neural retinal cells express the low sialic acid form. It was therefore of interest to determine whether P5 and P10 could inhibit neural retinal cell reaggregation. Neural retinal cells were rotated in the presence of different concentrations of P5 and P10, and both peptides were equally effective in inhibiting aggregate formation, with 50% inhibition achieved at ~0.2 mM (Fig. 7). Effects of the control peptides P5S and P6 were negligible even at 1 mM. Control cells without peptide, as well as cells incubated with P5S or P6, underwent rapid reassociation, forming large aggregates within 20 min. In contrast, aggregation of cells incubated with P5 was strongly inhibited (Fig. 8). In addition to P5S and P6, several additional control peptides of different sizes were included in this assay. P1 and P2 were sequences derived from ovalbumin, while P4 contained the homophilic binding site of the cell adhesion molecule gp80 of *Dicystostelium discoideum* and was previously shown to be a potent inhibitor of gp80 function (Kamboj et al., 1989). Although the number of cells recruited into aggregates increased gradually over time in the P5- or P10-containing samples, only small aggregates were observed at 60 min. Similar results were obtained with anti-NCAM Fab, except that the Fab had a more persistent effect than P5 (Table IV). On the other hand, P6, P7, and the other control peptides did not exert significant inhibitory effects on neural retinal cell adhesion (Table IV).

The other known binding site of NCAM, associated with heparin binding, resides in domain 2 and is also implicated in NCAM-mediated cell adhesion (Cole et al., 1986a; Frelinger and Rutishauser, 1986; Cole and Akeson, 1989). For comparison, neural retinal cells were also assayed for



**Figure 8.** Phase micrographs showing cell aggregates formed in the presence of synthetic peptides. Neural retinal cells were prepared and aggregated as described in the legend to Fig. 7. Cell samples were observed under a light microscope after 30 min of rotation. (a) Cells aggregated in the presence of P6 (1 mg/ml), and (b) cells aggregated in the presence of P5 (1 mg/ml). Bar = 100  $\mu$ m.

adhesion in the presence of a peptide (P3) that spans the identified heparin binding site. P3 inhibited retinal cell adhesion less effectively than P5 or P10 (Table IV). Since P3 and P10 blocked two separate sites on NCAM, they were tested together to determine whether there was any synergistic effect. It was observed that P3 and P10 were not additive in their effects, with an extent of inhibition similar to that of P10 alone.

### Inhibition of Binding of NCAM-conjugated Covaspheres to Cells by Synthetic Peptides

To further demonstrate that P5 and P10 indeed competed for the homophilic binding site on NCAM, the binding of NCAM-covaspheres to NCAM-transformed L cells was carried out in the presence of different peptides. P5 completely abolished the binding of NCAM-covaspheres to cells expressing wild-type NCAM, while P6 had no significant



Table IV. Effect of Synthetic Peptides on Adhesion among Neural Retinal Cells

Peptide	Sequence	Aggregation of cells (%) <sup>*</sup>			
		30 min	% inhibition	60 min	% inhibition
None		49 ± 5	0	70 ± 6	0
P1	IINFEKLTIEWTSSNVMEER	47 ± 2	4	65 ± 2	7
P2	ISQAVHKAHAINE	43 ± 1	12	66 ± 2	6
P3	KHKGRDVILKKDVR	32 ± 6 <sup>‡</sup>	35	48 ± 12 <sup>‡</sup>	31
P4	SGYKLVNDS	45 ± 1	8	69 ± 2	1
P5	KYSFNVDGSELIKKVDSDE	22 ± 9	55	45 ± 4	36
P5S	EDSKYVKIKLYSGDENFSDK	53 ± 1	0	67 ± 6	4
P6	DGSELIKKVDSDE	53 ± 4	0	69 ± 1	1
P7	TKDGEPIEQEDNEEK	46 ± 5	6	68 ± 6	3
P10	KYSFNVDGSE	24 ± 7	51	46 ± 4	34
P3 + P10 <sup>§</sup>		22 ± 3	55	42 ± 2	40
Anti-NCAM Fab <sup>  </sup>		16 ± 6	67	24 ± 1	66

<sup>\*</sup> Neural retinal cells were isolated from chick day 10 embryos and reassociated in the presence of 1 mg/ml of test peptide. Samples were rotated at 150 rpm at 37°C and aggregation was monitored at 30 and 60 min. Data represent the mean ± SD of 3–10 experiments. The percentage of inhibition was calculated against the control sample without added peptide.

<sup>‡</sup> Results using P3, P5, P10, P3 + P10, and anti-NCAM Fab as competitor are statistically different from the control sample ( $P < 0.005$ ).

<sup>§</sup> Retinal cells were reassociated in the presence of both P3 (1 mg/ml) and P10 (0.5 mg/ml).

<sup>||</sup> Cells were reassociated in the presence of 0.5 mg/ml of Fab derived from a rabbit antiserum directed against NCAM.

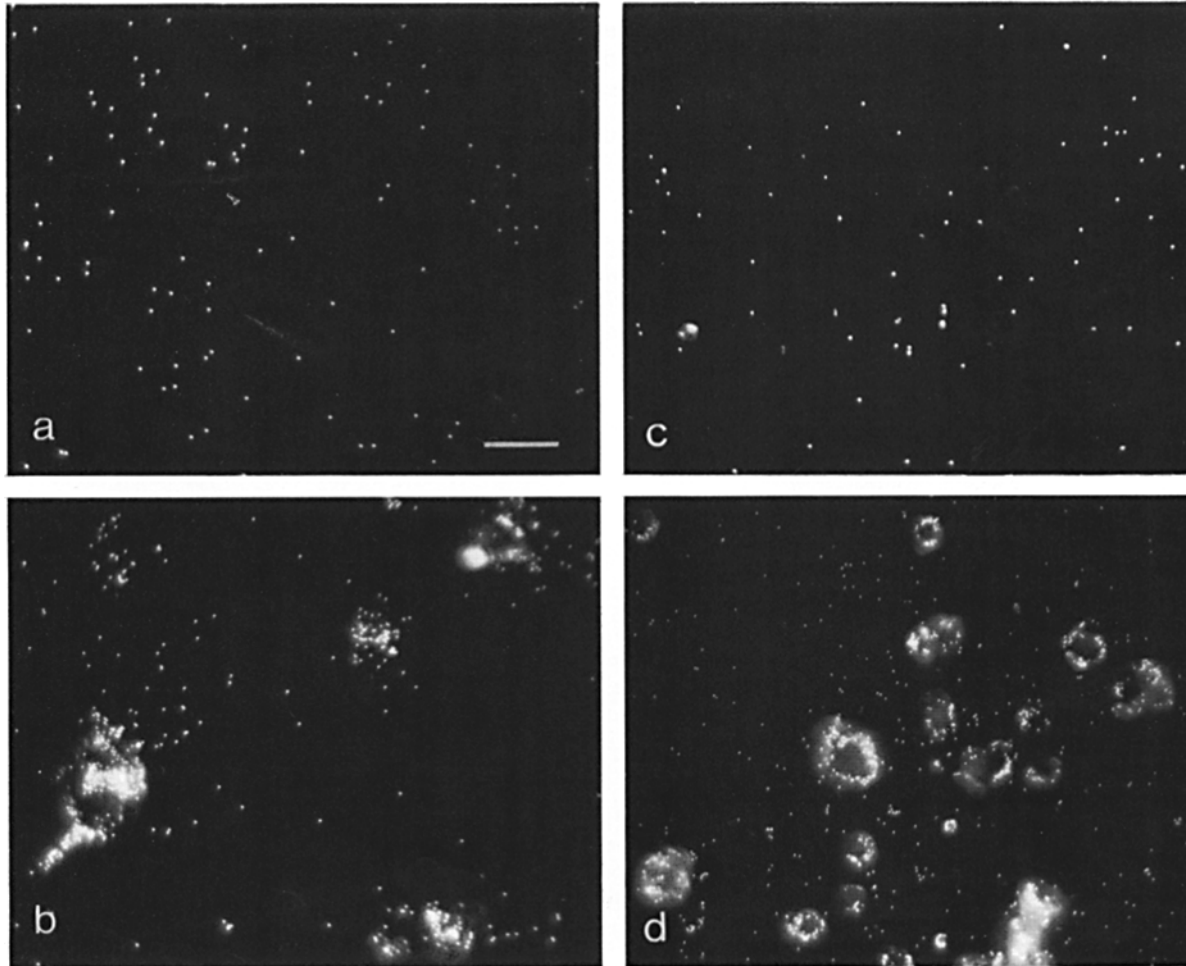


Figure 9. Inhibition of NCAM-conjugated covasphere binding to cells. NCAM transformed cells and neural retinal cells were assayed for their ability to bind NCAM-covaspheres in the presence of either P5 or P6 (1 mg/ml). Binding was carried out at room temperature for 20 min and coverslips were viewed by epifluorescence microscopy. (a, b) NCAM-covasphere binding to wild-type NCAM-transformed L cells in the presence of P5 and P6, respectively; (c, d) NCAM-covasphere binding to neural retinal cells derived from day 10 chick embryos in the presence of P5 and P6, respectively. Bar = 20  $\mu$ m.

**Table V. Effect of Synthetic Peptides on the Binding of NCAM-conjugated Covaspheres to Cells**

Peptide	Sequence	% cells with bound covaspheres	
		Transformed L cells*	Neural retinal cells†
None		55.3 ± 6.1	46.3 ± 5.8
P3	KHKGRDVILKKDVR	56.1 ± 6.0	43.5 ± 2.9
P5	KYSFNVDGSELIKKVDSDE	1.4 ± 2.2	3.0 ± 2.0
P5S	EDSKYVKIKLYSGDENFSDK	56.4 ± 7.8	45.8 ± 5.6
P6	DGSELIKKVDSDE	52.4 ± 7.9	41.6 ± 4.8
P7	TKDGEPIEQEDNEEK	54.5 ± 5.4	42.1 ± 4.8
P10	KYSFNVDGSE	1.4 ± 1.9	2.1 ± 0.4

\* Transformed cells expressing wild-type NCAM were cultured on coverslips and incubated with NCAM-conjugated covaspheres for 30 min at room temperature. Cells were observed using epifluorescence microscopy and the percentage of positive cells was estimated as described in Materials and Methods. Values represent the mean ± SD of three experiments.

† Neural retinal cells were isolated from chick day 10 embryos, attached to coverslips, and then assayed for covasphere binding. Values represent the mean ± SD of three experiments.

effects (Fig. 9). Similar results were obtained with transformed cells expressing Δ4-NCAM (data not shown). When the shorter peptide P10 was examined, it was again found to be a potent inhibitor and the binding of NCAM-covaspheres to cells was reduced to background level. The control peptides P5S and P7 did not affect covasphere binding (Table V). Thus the binding site identified in the cell aggregation studies also appears to be required for NCAM-NCAM binding. Interestingly, P3, which contains the heparin binding domain, had little effect in this assay. Thus, in this assay the heparin binding site does not appear to be necessary for NCAM homophilic binding.

Similar results were obtained when the same experiments were carried out using NCAM-covaspheres and retinal cells (Fig. 9 and Table V). Both P5 and P10 inhibited the binding of NCAM-covaspheres to retinal cells. P3 and the other control peptides failed to exert negative effects on NCAM-covasphere binding to cells.

### Attachment of Neural Retinal Cells to Peptide Substrates

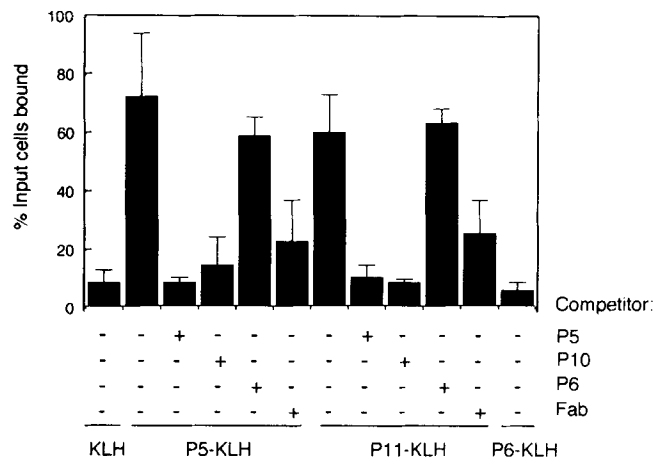
While P10 clearly delineates an NCAM binding site, the question remains as to whether it participates directly in homophilic binding. To test this possibility, the binding activity of synthetic peptides was investigated using a cell-substratum adhesion assay. Peptides P5 and P11 were conjugated to KLH using EDC and MBS, respectively, and adsorbed to nitrocellulose-coated Petri dishes. P11 (CEKYSE-NYDGSE) overlaps with P10, spanning the sequence between Glu-242 and Glu-252, with an additional Cys residue added at its amino terminus for the purpose of conjugation. Neural retinal cells labeled with [<sup>3</sup>H]leucine were placed in these dishes and assayed for attachment to the substratum. Over 60% of input cells attached to the immobilized P5-KLH or P11-KLH (Fig. 10). In contrast, immobilized KLH alone or P6-KLH gave <10% attachment of input cells.

The specificity of the substrate attachment activity of P5 and P11 was further supported by competition studies using free peptides. In both cases, attachment of retinal cells to the peptide substrates was reduced to background levels by P5 or P10, while P6 had no effect (Fig. 10). To demonstrate the involvement of NCAM, retinal cells were precoated with anti-NCAM Fab before the attachment assay. Attachment of

these cells to P5-KLH or P11-KLH substratum was greatly reduced (Fig. 10), indicating that the attachment of retinal cells to the dish was dependent on the interaction of NCAM with either P5 or P11.

### Discussion

These experiments have led to the identification of an amino acid sequence in NCAM that is capable of mediating homophilic NCAM-NCAM binding. Our initial approach



**Figure 10.** Binding of neural retinal cells to peptide-KLH conjugates. Neural retinal cells were prepared from 10-d-old chick embryos and then metabolically labeled with [<sup>3</sup>H]leucine (25 μCi/ml) for 2 h at 37°C in a leucine-free medium. Petri dishes were coated with nitrocellulose solubilized in methanol. Aliquots of peptide-KLH conjugates were placed on marked spots on the plastic surface and allowed to adsorb to the nitrocellulose for 1.5 h. Cells (50 μl) were placed on these spots and incubated for 2 h at 37°C. In competition studies, 25-μl aliquots of P5 or P10 (2 mg/ml) were placed on these spots for 5 min and then 25 μl of cells, containing ~3.5 × 10<sup>5</sup> cells, was placed on each spot. Alternatively, retinal cells were precoated with anti-NCAM Fab (0.5 mg/ml) for 30 min. After washing once to remove unbound Fab, cells were deposited on the Petri dish for the attachment assay. At the end of the incubation period, unbound cells were gently removed by rinsing the dishes with PBS. The amount of bound cells was estimated by solubilizing the cells on each spot with SDS and then counting in a scintillation counter. The data represent the mean of four experiments.

				P10			
				P5	P6		
CHICK	216		P7				268
CHICK		CDADGFPEPTMTWTKDGEPIEQEDN- <b>EKYSFN</b> YD <b>GSE</b> LIKKVKDSDEAEYIC					
XENOPUS		CDADGFPDPEISWLKKGEPIDG- <b>EKISFN</b> EDQ <b>SE</b> MTIHHVEKDDAEYSC					
HUMAN		CDAEGFPEPTMSWTKDGEQIEQEE- <b>DDEKYIF</b> SDD <b>S</b> QLTIKKVKDNDEAEYIC					
RAT		CDADGFPEPTMSWTKDGEPIEENEEDDE <b>KHIF</b> SDD <b>S</b> SELTIRNVKDNDEAEYVC					
MOUSE		CDADGFPEPTMSWTKDGEPIEENEEDDE <b>KHIF</b> SDD <b>S</b> SELTIRNVKDNDEAEYVC					

**Figure 11.** Alignment of the chick NCAM homophilic binding site with sequences of other species. The Ig-like domain 3 sequence (from Cys-216 to Cys-268) of chick NCAM (Hemperley et al., 1986; Cunningham et al., 1987) was aligned with sequences of the same

region from *Xenopus* (Krieg et al., 1989), human (Dickson et al., 1987), rat (Small et al., 1987), and mouse (Barthels et al., 1987). The chick decapeptide sequence involved in homophilic interaction and identical residues present in the other vertebrate NCAM sequences are shown in bold type. The solid lines represent the position of the peptide sequences for P5, P6, P7, and P10.

involved the construction of a series of NCAM deletion mutants for transfection into noncohesive mouse L cells. Quantitative cell reaggregation assays indicated that a high percentage of cells expressing the wild-type form of NCAM-140 were capable of forming large aggregates in the absence of shear forces, consistent with a homophilic adhesion mechanism. Transformants expressing  $\Delta 2,3$ -NCAM or  $\Delta 3$ -NCAM were unable to form aggregates, suggesting that sequences within domain 3 are intimately involved in the adhesion process. In contrast, transformants expressing NCAM mutants with domains 1, 2, 4, or 5 deleted were capable of aggregation, indicating that these four domains are less important in homophilic interactions. Similarly, the specific binding of NCAM-covaspheres to NCAM-transformed cells was also found to be dependent on the presence of domain 3, as only transformants expressing  $\Delta 2,3$ -NCAM or  $\Delta 3$ -NCAM lost their ability to bind NCAM-covaspheres. Together, these results provide strong evidence that a homophilic binding site is present in Ig domain 3 of NCAM.

Cells expressing  $\Delta 4$ - or  $\Delta 5$ -NCAM did demonstrate somewhat lower levels of adhesion than the intact molecule. As NCAM-NCAM dimers appear to involve extended molecules with a hingelike structure (Hall and Rutishauser, 1987; Becker et al., 1989), the deletion of segments distal to domain 3 could shorten the distance of the homophilic binding site from the plasma membrane, rendering it less accessible to NCAM on adjacent cells. The deletion of either domain 1 or 2 also had only a small negative effect on cell aggregation, despite the fact that the heparin binding site in domain 2 is thought to contribute to the stability of NCAM-mediated cell-cell adhesion (Cole et al., 1986a; Frelinger and Rutishauser, 1986; Reyes et al., 1990). Moreover, the deletion of domain 2 did not have a significant effect on the binding of NCAM-covaspheres to cells expressing this mutant NCAM, and the binding of covaspheres to cells expressing wild-type NCAM was not affected by the peptide P3, which contains the heparin binding domain. These results suggest that our transfected cell model lacks the competence to exhibit the effects of heparan sulfate on NCAM binding, possibly because L cells do not express the appropriate type or amount of heparan sulfate. Such a deficit could well explain why aggregation of NCAM-transformed cells is much more sensitive to shear forces than retinal cells. In any case, our results suggest that NCAM-NCAM binding can occur through domain 3 to domain 3 interactions.

Our second approach was to map the epitopes of four mAbs that have been shown to affect NCAM-mediated adhesion (Watanabe et al., 1986). All four of the mAb epitopes have been mapped to domain 3, consistent with the previous report that these sites lie between the heparin binding do-

main and the glycosylation sites of domains 4 and 5 (Frelinger and Rutishauser, 1986). These mAbs were assayed against a series of overlapping hexapeptides assembled on plastic pins in order to map their respective epitopes. mAb-PP was the only one that recognized a linear hexapeptide sequence, reacting strongly with the sequence KYSFNY. The specificity of this epitope is further supported by the finding that substitution of any one of the three aromatic amino acids with Ala drastically diminishes its reactivity with mAb-PP. These results also suggest that aromatic interactions (Burley and Petsko, 1985), as well as the formation of hydrogen bonds, may be important for PP binding to its epitope.

The ability of mAb-PP to inhibit NCAM-mediated cell-cell adhesion suggests that its epitope is part of or in proximity to the homophilic binding site. Indeed, synthetic oligopeptides containing the PP-epitope exhibit strong inhibitory effects on NCAM activity in several in vitro assays. The observation that the neighboring sequence (P6) was not inhibitory further demonstrates that the PP epitope forms an integral part of the homophilic binding site. The shorter active peptide sequence within P5 is that of P10, with its first six amino acids constituting part of the PP epitope. P10 was just as effective as p5 in inhibiting retinal cell aggregation and the binding of NCAM-covaspheres to retinal cells or NCAM transformants. However, it should be noted that P10 was less potent in the membrane vesicle assay, particularly during later times in the assay, consistent with a lower affinity than P5 for NCAM. Also, the efficacy of these peptides seems to vary somewhat between the vesicle assay and the retinal cell aggregation assay. The reason for this is not clear, but it could reflect differences in the components present on the membrane surface, the different forms of NCAM expressed in brain and retinal cells, or differences in assay conditions, such as shear force. Nevertheless, both P5 and P10 show consistent inhibitory effects in all three assays. Together, these findings further narrow down the active region to the decapeptide sequence KYSFNYD**GSE**.

A simple cell-substratum adhesion assay has been used to confirm the biological activity of the synthetic peptides. When either P5-KLH or P11-KLH was immobilized on nitrocellulose coated dishes, retinal cells attached efficiently to these surfaces. This cell-substratum interaction appeared to be highly specific since retinal cells did not attach to KLH or P6-KLH. Cell attachment to P5-KLH and P11-KLH was also inhibited by free P5 or P10 peptides, but not by P6. Binding of anti-NCAM Fab to cells before the assay blocked cell attachment to these peptide conjugates, indicating that the peptides were interacting directly with NCAM on the cell surface.

A homology search among NCAM sequences reveals that the first five Ig-like domains exhibit a very high degree of sequence homology among chick, human, rat, and mouse, with >80% sequence identity. Surprisingly, the chick homophilic binding site is one of the few less conserved regions (Fig. 11). The decapeptide sequence shows 70% sequence identity with the same region in *Xenopus* NCAM, but only 50% sequence identity with human, mouse, and rat NCAM. On the other hand, the mouse and rat sequences are identical and show 80% sequence identity with the human sequence. All five species share four common amino acid residues at this site: Lys-243, Phe-246, Asp-249, and Ser-251. The sequence divergence and the presence of three aromatic amino acid residues in this region probably contribute to its antigenicity and thus the success of our immunological approach in identifying it as a homophilic binding site. Our results do not preclude the contribution of other sequences within the more conserved regions of domain 3 to homophilic binding. However, it is evident that perturbation of binding that involves the KYSFNVDGSE sequence is sufficient to disrupt homophilic interaction.

The implication of the sequence divergence is not yet clear, but it could account for species differences in NCAM-mediated adhesion. That is, the binding of brain membrane vesicles from different species to a layer of chick retinal cells, while always inhibited by anti-chick NCAM Fab, is quantitatively less efficient than the chick-chick combination (Hall and Rutishauser, 1985). That chick NCAM can bind at all to mouse NCAM possibly reflects the fact that other sites, such as the domain 2 heparin binding region, also support interaction. Moreover, as discussed below, certain key features of the homophilic binding site may be highly conserved among all species.

A salient feature of the chick NCAM homophilic binding site is the alternation of hydrophilic and hydrophobic residues, which is typical of a  $\beta$ -sheet structure. Secondary structure predictions also show that this region has a high probability for  $\beta$ -sheet structure, with its carboxyl-terminal residues adopting a  $\beta$ -turn (Chou and Fasman, 1978). By analogy with the known crystal structure of Ig domains, this region corresponds to the C' strand of the  $\beta$ -barrel structure (Santoni et al., 1988; Williams and Barclay, 1988). Since the homophilic binding site is rich in terms of polar and charged residues, it may be exposed on the surface of the molecule, thus facilitating interactions with other NCAM molecules.

While this sequence shows little structural resemblance to the HAV-containing cell adhesion sequence of cadherins (Blaschuk et al., 1990), it appears to share some structural features with the homophilic binding site of gp80, a cell adhesion molecule known to mediate  $\text{Ca}^{2+}$ -independent cell-cell adhesion in *Dictyostelium discoideum* (Siu et al., 1985, 1987; Kamboj et al., 1988). gp80 shows limited sequence and structural similarities with NCAM and Ig, suggesting that it may also belong to the Ig superfamily (Matsunaga and Mori, 1987; Siu and Kamboj, 1990). The gp80 homophilic binding site consists of the octapeptide sequence YKLVNDS, which is also predicted to assume a  $\beta$ -sheet conformation (Kamboj et al., 1989). The two charged residues (Lys and Asp) have been shown to play a vital role in the initial electrostatic interactions between two apposing gp80 molecules, and it is of interest to speculate that the charged residues at similar positions (Lys-243 and Asp-249) in the NCAM homophilic binding site may also play a central

role. That these two charged residues are among the four amino acids that remain invariant in the five vertebrate species (Fig. 11) is also consistent with a key role for them in homophilic binding. Thus, despite the divergence in sequence among the species discussed above, some potentially critical features of the binding site appear to be conserved.

Our studies suggest a domain 3 to domain 3 interaction in NCAM-NCAM binding, which appears to be independent of other molecular interactions. However, our results are also consistent with an auxiliary but independent role for a heparin binding site in domain 2. The interactions centered at the homophilic binding site may represent the first step of NCAM-NCAM binding, leading to conformational changes and molecular interactions involving other parts of NCAM and possibly with other molecules as well (Cole and Akeson, 1989; Kadmon et al., 1990).

Our data cannot distinguish between a parallel or anti-parallel alignment of the third domains of two molecules on apposing surfaces. A more detailed analysis will be required to determine the location of the binding site for the KYSFNVDGSE sequence and, in particular, whether this sequence binds with the identical sequence or with a different site in domain 3. In the case of the *Dictyostelium* cell adhesion molecule gp80, there is evidence that its homophilic binding site interacts in an anti-parallel manner with the same sequence of another gp80 molecule (Kamboj et al., 1989). Should it be the same for NCAM, it is conceivable that the aromatic side-chains of Tyr-244, Phe-246, and Tyr-248 may undergo stacking (Burley and Petsko, 1985), which would in turn further stabilize the interactions between NCAM molecules on apposing surfaces.

Finally, it must be emphasized that NCAM is a large multi-domain, multi-function structure, apparently capable of heterophilic as well as homophilic interactions, and of attenuating as well as promoting cell-cell interactions. Thus, while delineation of the domain 3 binding site is an important advance in defining the homophilic binding properties of the molecule, much additional study will be required to understand its full role at the cell surface.

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