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Structural requirements for neural cell adhesion molecule–heparin interaction

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Two biological domains have been identified in the amino terminal region of the neural cell adhesion molecule (NCAM): a homophilic-binding domain, responsible for NCAM-NCAM interactions, and a heparin-binding domain (HBD). It is not known whether these two domains exist as distinct structural entities in the NCAM molecule. To approach this question, we have further defined the relationship between NCAM-heparin binding and cell adhesion. A putative HBD consisting of two clusters of basic amino acid residues located close to each other in the linear amino acid sequence of NCAM has previously been identified. Synthetic peptides corresponding to this domain were shown to bind both heparin and retinal cells. Here we report the construction of NCAM cDNAs with targeted mutations in the HBD. Mouse fibroblast cells transfected with the mutant cDNAs express NCAM polypeptides with altered HBD (NCAM-102 and NCAM-104) or deleted HBD (HBD⁻) at levels similar to those of wild-type NCAM. Mutant NCAM polypeptides purified from transfected cell lines have substantially reduced binding to heparin and fail to promote chick retinal cell attachment. Furthermore, whereas a synthetic peptide that contains both basic amino acid clusters inhibits retinal-cell adhesion to NCAM-coated dishes, synthetic peptides in which either one of the two basic regions is altered to contain only neutral amino acids do not inhibit this adhesion. These results confirm that this region of the NCAM polypeptide does indeed mediate not only the large majority of NCAM's affinity for heparin but also a significant portion of the cell-adhesion-mediating capability of NCAM.

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Introduction

An increasing number of cell-surface molecules have been identified which appear to mediate cell-cell or cell-extracellular matrix interactions. The cadherins are a small family of related polypeptides that mediate calcium-dependent cell adhesion (Takeichi, 1988). The integrins act as transmembrane receptors, interacting with cytoskeletal elements intracellularly and with extracellular matrix proteins such as fibronectin and laminin extracellularly (Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1988). Members of the immunoglobulin supergene family such as neural cell adhesion molecule (NCAM) and L1 have been shown to mediate calcium-independent adhesion of neural cells (Edelman, 1988; Rutishauser and Jessell, 1988). At the level of mechanism, transfection experiments have strongly suggested that cadherin-mediated adhesion is via homophilic interaction: that is, cadherin molecules on one cell bind to cadherin molecules on a second cell to mediate adhesion of the cells. However, the precise portions of the cadherin molecules that mediate these molecular interactions are not known. Analysis of the mechanisms of integrin interaction with extracellular ligands is much more advanced, significantly through the discovery of the key role of the arg-gly-asp RGD sequence (Pierschbacher and Ruoslahti, 1984). Many members of the integrin family recognize RGD containing primary sequences within extracellular polypeptides but maintain specificity of interaction, apparently because of the varying conformation of the RGD region within the context of each polypeptide chain (Ruoslahti and Pierschbacher, 1987). In addition, fibronectin and laminin themselves may interact with cells through other non-RGD–recognizing receptors and via their affinities for other cell-surface constituents such as glycosaminoglycans and glycolipids (Mugnai *et al.*, 1988a).

Both L1 and NCAM have been proposed to mediate adhesion via a homophilic interaction mechanism (Edelman, 1988). However, in independent experiments, a heparin-sensitive component of neural adhesion was identified

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and later shown to be due to NCAM polypeptides (Cole and Glaser, 1986). These observations raised the possibilities that 1) the initial observations of homophilic NCAM-NCAM interaction perhaps could be due to copurification of a heparan sulfate proteoglycan (HSPG) with the isolated NCAM polypeptides used for the experiments or 2) that NCAM molecules on one cell could interact with multiple surface components on a second cell. Thus, when cDNAs for NCAM were isolated, it was interesting to note that a potential heparin-binding domain (HBD) could be readily identified based on clusters of basic amino acids within the predicted linear amino acid sequence (Barthels *et al.*, 1987; Cunningham *et al.*, 1987; Small *et al.*, 1987). Synthetic peptides containing this candidate HBD sequence were tested and shown to have both heparin-binding capacity and also the ability to inhibit cell-NCAM adhesion (Cole and Akeson, 1989).

The possible physiological importance of the NCAM HBD is illustrated by a comparison with molecular mechanisms of cell-cell and cell-matrix interactions in other systems. The heparin-binding domain of fibronectin promotes neurite outgrowth when used to coat culture dishes (Rogers, 1985; Mugnai *et al.*, 1988b). A laminin-HSPG complex has been shown to enhance neurite outgrowth in a similar assay (Lander et al., 1985; Davis *et al.*, 1987; Sandrock and Matthew, 1987), and the heparin-binding region of laminin is involved in neural crest migration (Perris *et al.*, 1989). The role of myelin-associated glycoprotein in neuron-glial interactions may also depend on HSPGs, although the amino acid sequence involved in heparin-HSPG binding has not been identified (Poltorak *et al.*, 1987). A consensus HBD sequence for heparin-binding proteins has been proposed by Cardin and Weintraub (1989). In addition to fibronectin (Hynes, 1985), other extracellular matrix molecules known to have roles in cell-matrix adhesion, including vitronectin (Suzuki *et al.*, 1985) and thrombospondin (Dixit *et al.*, 1986; Lawler and Hynes, 1986), also have regions that are homologous to the HBD consensus sequence. These findings indicate the general importance of HBD-mediated modes of cell adhesion.

In the present study we have used NCAM polypeptides with normal and mutated HBDs and synthetic peptides representing normal and mutated NCAM HBD regions to analyze the structural requirements for NCAM-heparin binding. Here we report that alterations in the sequence of the HBD substantially reduce the ability of intact NCAM polypeptides to bind to heparin and to promote retinal cell attachment and that alterations in the sequence of synthetic peptides mimicking this region eliminate the ability of those peptides to compete with cell-NCAM adhesion.

Results

Mouse fibroblast L cells were used as recipients for transfected NCAM cDNAs placed under the control of (RSV LTR) promoter. These cDNAs were produced by in vitro mutagenesis protocols, as previously described (Reyes and Akeson, 1988), and encode NCAM polypeptides with amino acid substitutions in the HBD, as shown in Figure 1. In NCAM-102 and NCAM-104, two and four of the six arginines/lysines in the HBD, respectively, are replaced by neutral amino acids, whereas in HBD$^-$, the entire HBD is deleted. L-cell lines resulting from cotransfection with these NCAM vectors and a neomycin resistance plasmid were isolated by growth in G418-containing media followed by dilution cloning. The cell lines and the corresponding NCAM polypeptide they are predicted to express are listed in Table 1.

To determine whether the altered NCAM polypeptides were processed and expressed on the cell surface in an appropriate fashion, we tested cell lines by immunofluorescence and immunoblot assays. When treated with polyclonal anti-NCAM, cell lines 102, 104, 4.2, and L3.4 showed immunofluorescence, whereas untransfected L cells did not, thus confirming

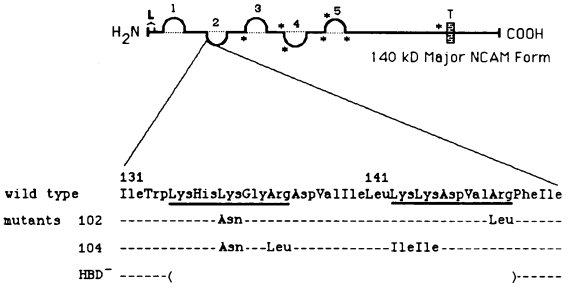

Figure 1. **Amino acid sequence of NCAM HBD mutants.** The putative heparin-binding domain is located in immunoglobulin-like loop 2 in the extracellular portion of the NCAM molecule. The leader sequence (L), transmembrane region (T), and possible glycosylation sites (*) are also indicated. The two clusters of basic amino acid residues in the HBD are underlined. The amino acid sequences of the HBD mutants are shown below the wild-type sequence. Dashes indicate identity of the mutant sequence with wild type. Parentheses indicate the boundaries of the deletion in HBD$^-$. Amino acids are numbered in reference to their position in the proposed mature 140-kDa rat NCAM sequence (Small *et al.*, 1987).

Table 1. Number of NCAM molecules present at the cell surface of transfected cells

Cell line	NCAM polypeptide	NCAM molecules/cell
102	NCAM-102	4.2 ± 2.4
104	NCAM-104	22.3 ± 5.0
4.2	HBD$^-$	20.8 ± 1.8
L3.4	wild type	15.0 ± 3.8

The number of NCAM molecules expressed on the surface of transfected cells was determined as described previously (Akeson *et al.*, 1988). The numbers reported are the averages ($\times 10^{-4}$) of four or more determinations ± SD.

the cell-surface presence of NCAM polypeptides in the transfected cells (Figure 2). Furthermore, the level of NCAM expression in the cell lines expressing mutant NCAMs was determined by a quantitative antibody-binding assay and found to be similar to those of L3.4 cells transfected with wild-type NCAM (Table 1). For comparison, rat neuronal cell lines express 5–15 $\times$ 10^5 NCAM molecules per cell (Akeson *et al.*, 1988).

To test the molecular size of the polypeptides produced, immunoblotting assays were carried out on solubilized and electrophoresed cell homogenates. An NCAM band with M_r of 140 kDa and of similar size to NCAM from L3.4 cells was observed with cell lines 102 and 104 (Figure 3).

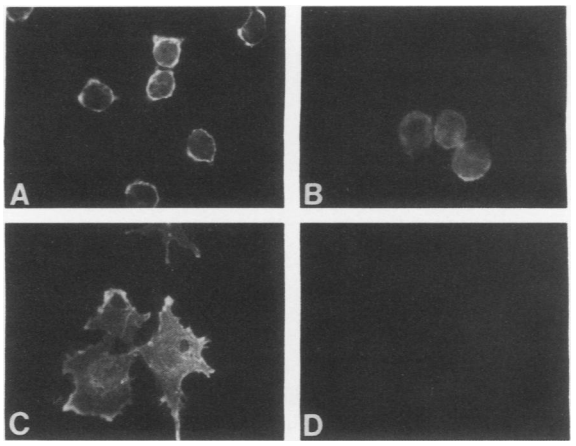

Figure 2. **Immunofluorescence staining of NCAM-transfected L cells.** Cells plated on coverslips were reacted with a rabbit polyclonal antiserum against total adult rat brain NCAM. After washing, coverslips were incubated with rhodamine-labeled goat anti-rabbit serum. Photographs were taken under rhodamine optics for 5 s. Negatives were developed under identical conditions. (A) Substitution mutant line 104. (B) Deletion mutant line 4.2. (C) Wild-type NCAM line L3.4. (D) Untransfected L cells.

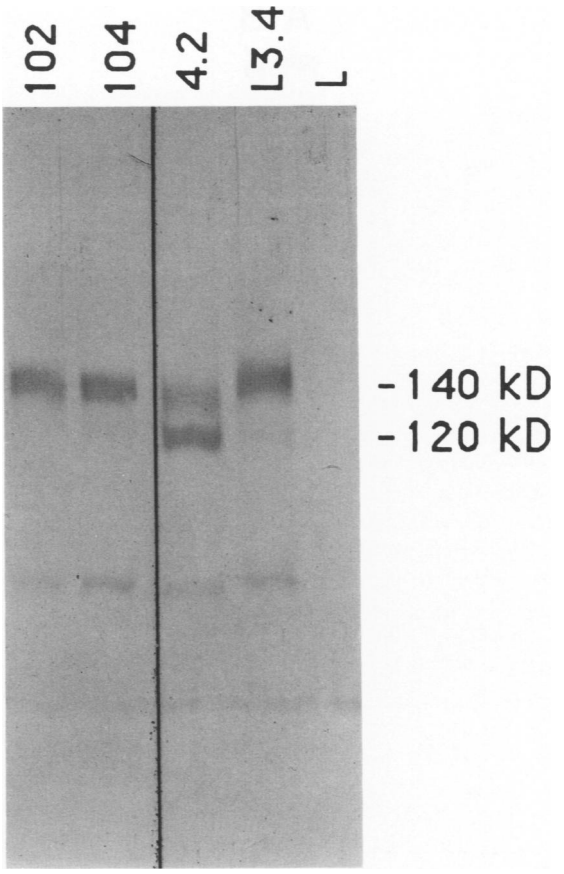

Figure 3. **Immunoblot analysis of NCAM-transfected L cells.** Cells were incubated in buffer containing 1% NP-40 and protease inhibitors. Aliquots of the soluble fraction (equivalent to 5 $\times$ 10^4 cells per lane) were run on a 6% SDS-polyacrylamide gel then transferred to nitrocellulose. The blot was reacted with a rabbit polyclonal antiserum against total adult rat brain NCAM, then with biotinylated goat anti-rabbit IgG. Bands were visualized using horseradish peroxidase and H_2O_2. A nonspecific band at ~80 kDa is observed in all lanes. Molecular sizes were determined by comparison with standards (not shown).

These results are consistent with the production of full-length NCAM polypeptide. The deletion mutant cell line 4.2 also expressed a major band at roughly 140 kDa and, in addition, a band at ~120 kDa. To determine the relationship between these bands, we analyzed the forms of NCAM present at the cell surface of 4.2 cells by the use of lactoperoxidase-catalyzed iodination and immunoprecipitation. Immunoprecipitates of lactoperoxidase-labeled 4.2 cells gave a single band that had an M_r similar to the band obtained from R5-6 cells, another L-cell line that has been transfected with wild-type NCAM cDNA (Figure 4). (The HBD-polypeptide expressed in line 4.2 lacks 14 of the 858 amino acids of rat 140-kDa NCAM, but this difference

Figure 4. **Identification of NCAM forms present at the cell surface.** Transfected L-cell lines containing the HBD deletion mutant (line 4.2, lane A) or wild-type 140-kDa NCAM polypeptide (line R5-6, lane B) were surface-iodinated using standard methods of lactoperoxidase-catalyzed iodination of live cells. Cells were then washed, solubilized in buffer containing 1% NP40 and protease inhibitors, and the insoluble material separated by centrifugation. Aliquots of the detergent soluble material were immunoprecipitated with polyclonal anti-NCAM sera and *Staphylococcus aureus* and then electrophoresed on an 8% SDS-polyacrylamide gel. Gel lanes are aligned by the slight radioactive artifact at the top of the gel, which is also visible at the top of the photograph. The M_r of these bands is 135–140 kDa (standards in parallel lanes not shown).

could not be reproducibly detected on 8% acrylamide gels.) The 120-kDa band observed in immunoblots of 4.2 cells was not observed in these immunoprecipitates of surface-labeled cells. This result indicates the 120-kDa band is not present at the cell surface. It may be an intermediate form undergoing glycosylation or other processing events. The 120-kDa band has also been observed in immunoblots of cell lines transfected with other NCAM cDNAs coding for altered HBDs (different from those coding for NCAM-102 and NCAM-104) and is therefore not specific for the HBD deletion construct (data not shown).

As an initial test of the effects of mutagenesis of the HBD on NCAM function, NCAM polypeptides were isolated from cell lines L3.4, 102, 104, and 4.2. Isolated wild-type rat NCAM bound significant amounts of ^{125}I-heparin, although less than that bound by NCAM isolated from chicken brain tissue (Figure 4). ^{125}I-heparin binding by both NCAM-102 and HBD$^-$ polypeptides was significantly reduced. The NCAM-104 had binding that was less than that of the control bovine serum albumin (BSA)-coated wells. The binding of ^{125}I-heparin by wild-type and mutant NCAMs was also inhibitable by unlabeled heparin to levels that were statistically indistinguishable from binding of heparin to BSA (data not shown). The specificity of heparin binding in these assays has also been demonstrated in previous studies (Cole *et al.,* 1985).

Having established that the HBD region was responsible for the heparin-binding capacity of the NCAM polypeptide, we next tested the influence of this region on the adhesion of cells to the NCAM polypeptide. We have previously shown that retinal cells will attach to NCAM bound to nitrocellulose-coated petri dishes (Cole and Akeson, 1989). The use of this cell-substratum adhesion assay allows the analysis

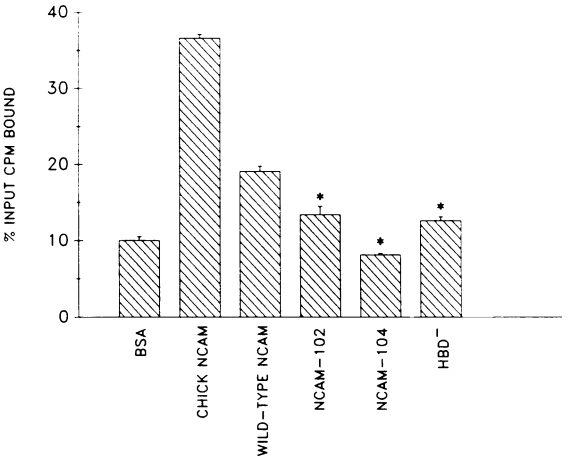

Figure 5. **Binding of ^{125}I-heparin to NCAMs isolated from transfected cell lines.** Immunopurified embryonic chick NCAM (10 μg/ml) or NCAMs purified from the transfected cell lines (10 μg/ml) were incubated with 20 000 cpm of ^{125}I-heparin as described in Materials and methods. The mixture was then applied to nitrocellulose in a dot-blot apparatus and binding was quantified by cutting out and counting individual spots. BSA (20 μg/ml) was used to estimate nonspecific heparin binding. The mean ± SD of 5 experiments, conducted with the use of duplicate samples, is shown. *, values significantly different from wild-type NCAM ($p < 0.001$).

of cell adhesion via the binding of HSPG on cells to an NCAM substratum, because heparin itself and an antibody to HSPG both inhibit this adhesion (Cole *et al.*, 1985). Thus, the effect of amino acid changes in the NCAM sequence or the ability of NCAM to mediate cell adhesion through this mechanism could be tested. When isolated wild-type rat NCAM was used as a substratum, the extent of retinal cell adhesion was similar to a chick NCAM substratum (Figure 6). However, dissociated retinal cells only exhibited minimal attachment to NCAM-102 or HBD$^-$ polypeptides.

Previous experiments had also demonstrated that the adhesion of chick retinal cells to NCAM-coated dishes could be partially inhibited by synthetic peptides corresponding to the HBD region (Cole and Akeson, 1989). To test further the role of the HBD in NCAM function, we tested synthetic peptides in which the arginines/lysines of one of the two basic amino acid clusters that comprise the HBD were altered to neutral amino acids (Table 2). The rationale for altering only one cluster of residues was to determine whether one or two binding sites were present within the sequence. If multiple binding sites are present, one would expect to observe only a partial loss of binding activity by changing only one of the two basic amino acid regions. Significant inhibition of adhesion of cells to NCAM-coated plates was observed with peptide HBD-2, corresponding to the wild-type NCAM sequence, but not with peptide SCR, which has an identical composition to peptide HBD-2 but a randomized sequence such that the basic amino acid residues are no longer clustered (Figure 7). Peptides HBD-3 and HBD-4, each of which has one basic amino acid cluster altered, did not inhibit cell-NCAM adhesion.

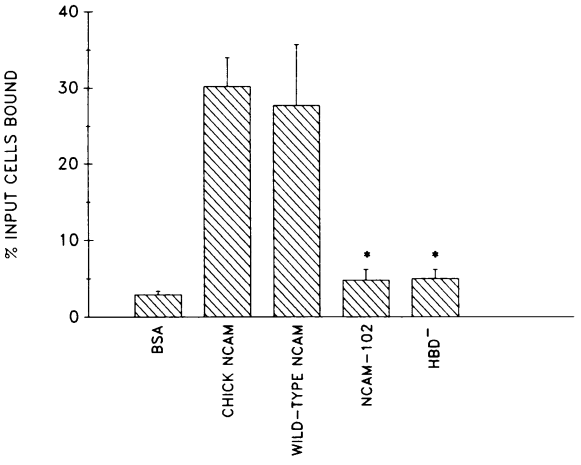

***Figure 6.* Cell-substratum adhesion to NCAM forms.** Petri dishes (35 mm) were coated with nitrocellulose as described in Materials and methods, and then a 20-mm area of the dish was incubated with 10 μg of purified NCAM isolated from the various transfected cell lines. Remaining protein-binding sites on the nitrocellulose were then blocked by incubating with EBSS, 1% BSA. Cell adhesion was determined by incubating the dishes for 1 h with [^{3}H]leucine-labeled embryonic chick day 10 retinal cells. These cells contain primarily neurons, and light microscopic analysis of attached cells indicates that primarily neurons bind to the NCAM substratum. Cell adhesion was quantified by dissolving bound cells in PBS, 1% Triton X-100 and determining the percentage of input cells bound. The mean ± SD of 3 experiments, using duplicate samples, is shown. *, values significantly different from wild-type NCAM ($p < 0.005$).

Table 2. HBD Synthetic Peptides

Peptide	Amino acid sequence
HBD-2	H_2N-Ile-Trp-**Lys-His-Lys**-Gly-**Arg**-Asp-Val-Ile-Leu-**Lys-Lys**-Asp-Val-**Arg**-Phe-Tyr-Cys-COOH
SCR	H_2N-**Lys**-Ile-**Arg**-Phe-**His**-Asp-Val-Ile-**Lys**-Trp-Asp-**Lys**-Tyr-Gly-**Arg**-Leu-Val-**Lys**-Cys-COOH
HBD-3	H_2N-Ile-Trp-Ile-His-Asn-Gly-Leu-Asp-Val-Ile-Leu-**Lys-Lys**-Asp-Val-**Arg**-Phe-Tyr-Cys-COOH
HBD-4	H_2N-Ile-Trp-**Lys-His-Lys**-Gly-**Arg**-Asp-Val-Ile-Leu-Ile-Ile-Asp-Val-Leu-Phe-Tyr-Cys-COOH

HBD-2 represents the wild-type HBD sequence, with HBD-3 and HBD-4 having a basic domain altered to uncharged residues. Basic amino acids are indicated in bold type. The carboxy terminal tyrosine residue was included for iodination and the cysteine for coupling.

Discussion

The amino acid sequence of the HBD is completely conserved in rat, mouse, and chicken NCAM (Barthels *et al.*, 1987; Cunningham *et al.*, 1987; Small *et al.*, 1987), suggesting that the conclusions reached in this study apply generally to NCAMs from many species. Although our results indicate that the HBD studied here comprises a major portion of the heparin-binding capacity of rat NCAM, it is difficult to state unequivocally that there are no other HBDs within NCAM because the assay conditions used here may not entirely reflect the heparin-binding potential of NCAM in vivo. For example, the binding of wild-type rat NCAM to ^{125}I-heparin was less than that of chicken NCAM. The chicken NCAM was isolated from chick brain, whereas the rat NCAM was isolated from transfected cultured fibroblastic cells. It is possible that posttranslational modifications of the rat NCAM

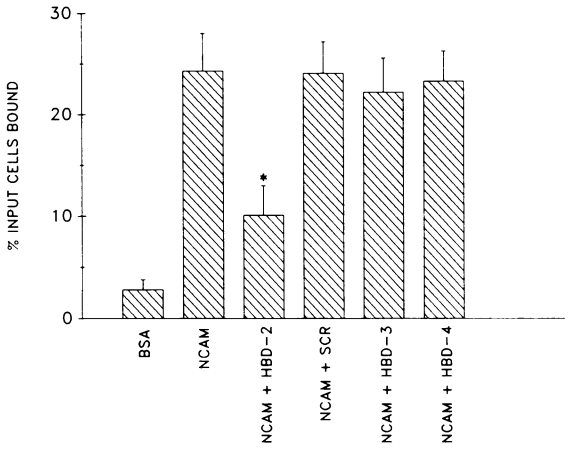

***Figure 7.* Effect of amino acid substitutions on activity of HBD-2 synthetic peptide.** Nitrocellulose-coated petri dishes were incubated with 20 μg of purified chick NCAM, and retinal cell adhesion was measured as described in Figure 6. To assess the role of the two basic amino acid regions in the function of the HBD, the various synthetic peptides (50 μg/ml) were added to the assay medium during the adhesion assay. The amino acid sequences of the four peptides are shown in Table 2. The mean ± SD of 4 experiments, conducted in duplicate, is shown. *, values significantly different from control ($p < 0.001$).

polypeptide either were not made or were made inappropriately in these fibroblasts. Furthermore, carbohydrate structural variants of HSPGs, the proposed natural ligand for the NCAM HBD, may have greater affinity for NCAM sequences than does the heparin sulfate used in the in vitro assay (Cole and Burg, 1989). Nevertheless, it seems clear that the HBD analyzed here represents the major heparin-binding site within the NCAM polypeptide and may well include all the heparin-binding capability of NCAM. Scatchard analysis of NCAM-heparin binding provides further evidence that only a single heparin-binding site exists in NCAM (Nybroe *et al.,* 1989).

The NCAM HBD contains two clusters of basic amino acid residues, each cluster consisting of three lysines/arginines within a linear sequence of five amino acids. The linear arrangement of basic residues within the NCAM HBD fits the consensus sequence for heparin-binding domains proposed by Cardin and Weintraub (1989). In one model of heparin-HBD interaction suggested by these workers, basic residues of the HBD are clustered together when displayed in the helical wheel model used to represent three-dimensional relationships of amino acids contained within α helical regions of polypeptides (Schiffer and Edmunson, 1967). This positively charged region acts as the initial attachment site for the negatively charged heparin molecule (Cardin and Weintraub, 1989). Although the three-dimensional structure of the portion of NCAM containing the HBD has not been reported, immunoglobulin supergene family members can be modeled on the repetitive, anti-parallel, β-pleated sheet structure of immunoglobulin domains (Williams and Barclay, 1988). Modeling the second Ig-like domain, which contains the HBD, on the known structure of an immunoglobulin domain places the HBD in proposed β-pleated sheet C and the transition between sheet C and sheet D. The three-dimensional structure of the transition zone between sheets is less well characterized. Modeling the HBD linear sequence in the helical wheel representation used for α helices results in the segregation of K^{135}, K^{142}, and R^{146} to one side of the helix (Figure 8). These three critical residues are located in either of the two basic clusters in the linear sequence of the NCAM HBD. In NCAM-102 and NCAM-104, two of these three residues have been altered, whereas in HBD^-, the entire region of high-positive-charge density is absent. This model would then account for the decreased binding affinity of the mutant NCAM polypeptides for heparin (Figure 5), although it is not clear why NCAM-102 and HBD^- bound small amounts of heparin. However, it cannot be ruled out that small amounts of

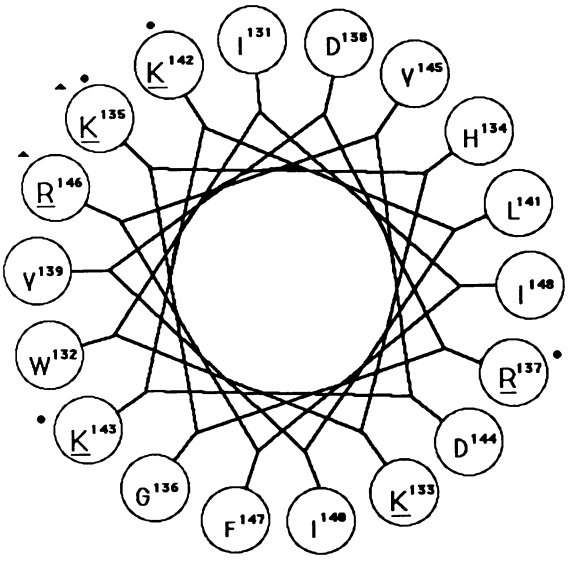

***Figure 8.* Helical wheel projection of NCAM HBD.** NCAM amino acids 131 to 148 are diagrammed according to the method of Schiffler and Edmunson (1967). Basic amino acids are underlined. Residues altered in NCAM-102 and NCAM-104 are indicated by ▲ and ●, respectively.

fibronectin, below the limits of detection by immunoblotting, could still be present in the rat NCAM preparations and thus could contribute to this heparin binding (see Materials and methods).

The adhesion of retinal cells to dishes coated with various forms of NCAM was determined (Figure 6). Retinal cells adhered to a similar extent to chick brain and wild-type rat NCAM, whereas NCAM-102 and HBD^- failed to promote cell adhesion. Because retinal cells do not attach to chick fibronectin (G. Cole, unpublished data), any contaminating fibronectin in the NCAM preparations would probably not contribute to this adhesion assay. It is possible that mutations in the HBD could result in conformational changes in other regions of the NCAM molecule that are involved in cell adhesion. However, at least in the case of NCAM-102, where only two amino acids were changed, this is probably not the case.

HBD-2, a synthetic peptide representing wild-type HBD, partially inhibits cell adhesion to NCAM-coated dishes, but HBD-3 and HBD-4, each of which has alterations in either one of the two basic clusters, do not interfere with NCAM-cell adhesion (Figure 7). The results indicate that an intact HBD is necessary for peptide biological activity and suggests that only a single binding site is formed by the two clusters of basic amino acids, in agreement with the features of the α helical model discussed above. The partial inhibition of cell adhesion observed here could be interpreted to suggest that other regions of the molecule may be involved in adhesion. It is also possible that higher peptide concentrations, or longer peptides more accurately modeling the three-dimensional structure of the Ig-like domain 2 and the HBD, may be much more active in inhibiting NCAM-NCAM and cell-cell adhesion.

The importance of the HBD in mediating NCAM function is thus strengthened by our observations that NCAM-102 and HBD^- cannot promote retinal cell adhesion and that the altered HBD synthetic peptides are not capable of perturbing cell adhesion to an NCAM substratum. These data raise the intriguing question of whether the HBD comprises the complete and exclusive region of the NCAM polypeptide mediating adhesion. At present we cannot unequivocally determine the extent to which NCAM-NCAM interaction (and the consequent cell-cell adhesion) is dependent on the HBD. Recent studies have demonstrated a lack of aggregation between NCAM-coated latex beads, unless micromolar amounts of heparin are included in the assay medium (Kadmon *et al.,* 1990). These data can be interpreted either as a requirement for heparin binding to the HBD region in NCAM to activate homophilic binding or, alternatively, that HSPG-NCAM interactions are solely responsible for NCAM-mediated cell interactions. However, kinetic analyses of NCAM-heparin binding and NCAM homophilic interaction have also given conflicting results. One study suggests that NCAM-NCAM binding is not inhibitable by added soluble heparin, although the ionic strength of the incubation buffer used to measure NCAM-heparin binding may have been too high to permit NCAM-heparin binding in vitro (Moran and Bock, 1988). Another study suggests that NCAM-heparin binding occurs more rapidly than NCAM-NCAM binding (Nybroe *et al.,* 1989). This latter result agrees with a previously proposed model in which NCAM-heparin binding promotes a conformational change in NCAM that leads to homophilic binding (Cole and Glaser, 1986).

An independent approach to defining the region of the NCAM polypeptide that mediates adhesion is to map the binding sites of monoclonal anti-NCAM antibodies that inhibit cell-cell adhesion. Such experiments suggest the adhesion-mediating portion of the molecule maps near immunoglobulin-like domain 2, consistent with a major role for the HBD. Two monoclonal antibodies—5E and 79B, which block cell-cell adhesion—also compete for the binding of mAb B1A3, which inhibits NCAM-heparin binding (Frelinger and Rutishauser, 1986; Cole and Glaser, 1986). These data therefore provide additional evidence for the integral role of the HBD in NCAM function.

Thus, additional experiments will be necessary to determine whether NCAM mediates cell-cell adhesion only via the HBD or whether an independent homophilic binding domain exists in NCAM. In the latter case, site-directed mutagenesis of the putative homophilic binding domain should perturb cell-cell adhesion but not NCAM-heparin binding. The presence of multiple binding sites for independent ligands has been described for other adhesion molecules like fibronectin, laminin, and tenascin (Martin and Timpl, 1987; Obara *et al.,* 1988; Erickson and Bourdon, 1989; Kouzi-Koliakos *et al.,* 1989). The results described here do strongly indicate that one major mechanism by which NCAM has the potential to mediate cell-cell adhesion is the HBD in immunoglobulin-like loop 2.

Materials and methods

DNA constructions and transfection of L cells

A 2.7-kb *Xmn* I-*Kpn* I fragment of pR18, a full-length cDNA clone for the 140 kDa form of rat NCAM, contains the entire coding region and 143 bp of 5′ untranslated region (Small *et al.,* 1987). This fragment was inserted into the expression vector pRSV18 (the gift of Dr. Bruce Aronow). pRSV18 was derived from pRSVcat (Gorman *et al.,* 1982) by inserting the multiple cloning site of pUC18 downstream of the RSV LTR sequence and removing both the CAT coding sequence and the small t intron. To generate NCAM HBD mutants, the 2.7-kb *Xmn* I-*Kpn* I fragment was subcloned into M13mp19 to give M13-NCAM. Substitution mutants were constructed as previously described (Reyes and Akeson, 1988). The deletion mutant was constructed by the use of the 25mer 5′-TGGTAGTAGACCAAGTATCAGGATA-3′, which, when annealed to M13-NCAM, loops out the coding region for amino acids 133 to 146 of NCAM, thus deleting the entire HBD (Figure 1). All mutations were verified by DNA sequencing with the use of the dideoxy chain termination method (Reyes and Akeson, 1988). A 1-kb *Bgl* II fragment spanning the region of mutation was isolated from each mutant and used to replace the corresponding region in the wild-type pRSV18-NCAM construct. Mouse L cells (5×10^5 cells per plate) were co-transfected with 1 μg pRSV18-NCAM mutant DNA and 20 ng pKOneo (VanDoren *et al.,* 1984) by the use of the calcium phosphate precipitation method (Sambrook *et al.,* 1989). Transfected cells were selected by resistance to 0.4 mg/ml G418 in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and maintained under selection. Cell lines were dilution cloned before analysis. To ensure that the transfection protocol did not cause nonspecific induction of the endogenous NCAM gene in L cells, a batch of L cells was transfected with pKOneo alone. These cells were then tested in the immunofluorescence and immunoblot assays described below and shown not to express NCAM (data not shown).

Immunoassays

Methods for cell-surface immunofluorescence, lactoperoxidase-catalyzed iodination of live cells, and immunoprecipitation have been reported previously (Akeson *et al.,* 1988). The quantitative determination of NCAM cell-surface molecules per cell is performed by determining antibody excess conditions for the first and second antibodies (in this case the second reagent was ^{125}I-protein A), using a constant number of cells, as described previously in detail (Lessard *et al.,* 1979; Rodman and Akeson, 1981; Akeson *et al.,* 1988). Binding assays are then performed using a 5- to 10-fold excess of monoclonal antibody 3F4, which reacts with all NCAM forms, and a 3- to 5-fold excess of ^{125}I-protein A and quantified by gamma counting.

Immunoblot analysis

Cells (1×10^6) were plated on dishes ~20 h before harvest. Cells were washed with 10 mM tris(hydroxymethyl)-aminomethane HCl (Tris-HCl), pH 7.45, 0.14 M NaCl, then incubated in buffer containing 1% NP-40, 10 mM Tris-HCl, pH 7.45, 0.14 M NaCl, 10 μg/ml aprotinin, and 0.1 mM PMSF. Lysates were spun briefly to remove insoluble debris. Aliquots of the soluble fraction (equivalent to 5×10^4 cells) were run on a 6% sodium dodecyl sulfate (SDS)-polyacrylamide gel, then electroblotted onto nitrocellulose. The blot was reacted with a rabbit polyclonal antiserum against total adult rat brain NCAM (Akeson *et al.,* 1988), then with biotinylated goat anti-rabbit IgG. Bands were visualized using horseradish peroxidase and H_2O_2. Molecular sizes were determined by comparison with standards (not shown).

Heparin-binding assays

The ability of the variant forms of NCAM to bind heparin was determined by measuring the binding of radio-iodinated heparin to immunopurified NCAM. NCAM was immunopurified from adult chick brain by the use of the B_1A_3 monoclonal antibody as previously described (Cole and Glaser, 1986). This NCAM was used as standard for heparin binding because it has been shown previously to bind heparin (Cole and Glaser, 1986). The rat NCAM forms were immunopurified from transfected L cell lines with the use of the 3F4 monoclonal antibody (Akeson *et al.,* 1988). In pilot experiments, significant heparin binding was observed with all NCAM forms. Because fibroblasts synthesize fibronectin, which is capable of binding heparin, we carried out Western blotting analysis of the purified NCAM samples. These demonstrated that fibronectin was present as a minor contaminant (data not shown). Thus, in subsequent NCAM isolations, the eluted NCAM was passed over a gelatin Sepharose column to remove fibronectin. The absence of fibronectin and presence of NCAM in this column-purified preparation was confirmed by Western blotting.

Binding of heparin to various forms of NCAM was carried out as described previously (Cole *et al.,* 1985), except ^{125}I-heparin was used. Heparin was conjugated to fluoresceinamine and radio-iodinated as described by Smith and Knauer (1987). A specific activity of ~4000 cpm/ng heparin was obtained. Aliquots of NCAM (10 μg/ml) or BSA (20 μg/ml) were incubated in hypotonic buffer (phosphate-buffered saline [PBS] diluted 1:10) with 20 000 cpm ^{125}I-heparin at 37°C for 1 h. The mixture was then blotted onto nitrocellulose with the use of a dot-blot apparatus, allowed to flow through by gravity, and then washed three times with the incubation buffer. ^{125}I-heparin binding was then quantified by γ counting. The effect of an excess of unlabeled heparin on binding was also determined by the addition of 50 μg/ml of heparin during the incubation period.

Cell adhesion assays

The ability of NCAMs isolated from the transfected cell lines to mediate cell adhesion, or of various synthetic peptides containing altered HBDs to perturb retinal cell adhesion to immunopurified NCAM, was determined. We employed a modification of the adhesion assay of Lagenaur and Lemmon (1987), as described previously (Cole and Akeson, 1989). Briefly, 35-mm petri dishes were coated with nitrocellulose and the entire surface area incubated with 20 μg of embryonic chick NCAM to assess synthetic peptide effects. To measure adhesion to the mutated NCAM forms, only a 20-mm diameter region of the petri dish was incubated with 10 μg of the purified NCAM. This modification was employed because of limiting amounts of these NCAM polypeptides. Excess protein binding sites were then blocked with Earle's balanced salts solution (EBSS) containing 1% BSA. Metabolically labeled embryonic day 10 chick retinal cells were then added to the NCAM-coated dishes for 1 h at 37°C. The effect of the different HBD peptides (HBD-2, HBD-3, HBD-4, and scrambled [SCR]) on cell attachment was determined by the addition of 50 μg/ml of peptide to the assay medium during the incubation period. After the incubation period, unbound cells were removed by vacuum aspiration, bound cells were dissolved in 1% Triton X-100, and bound radioactivity was quantitated by liquid scintillation counting.

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