Topography of N-CAM Structural and Functional Determinants.
II. Placement of Monoclonal Antibody Epitopes

Andrew Lawrence Frelinger III and Urs Rutishauser
Neuroscience Program, Department of Developmental Genetics and Anatomy,
Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Abstract. The accompanying report (Watanabe, M., A. L. Frelinger III, and U. Rutishauser, 1986, J. Cell Biol., 103:1721-1727) describes a set of monoclonal antibodies (mAbs) directed against N-CAM epitopes representing the known major structural and functional domains of the molecule. In this study, we have generated and separated a variety of peptide fragments from N-CAM, and then used their size and reactivity with each antibody to position the antigenic sites along the peptide chain. This epitope map, together with the biological properties of the antibodies and previous studies on N-CAM, have been used to construct a topographical model for the molecule in the cell membrane.

Materials and Methods

The present study is based on a diverse set of mAbs whose biological activity and epitopes have been characterized extensively in the preceding paper (27). This paper focuses on the placement of these epitopes in the linear sequence of the polypeptide chain, and the construction of a detailed model for N-CAM that takes into account the known structural and functional features of the molecule.

Preparation of N-CAM-160

The 160-kD N-CAM polypeptide, the largest of the three major N-CAM proteins, was immunoaffinity-purified from 14-d chick embryo brain by established procedures (15) using mAb 4I). As previously reported (27), this antibody reacts with the 160-kD form of N-CAM but not with the 130- or 110-kD forms, suggesting that it recognizes an epitope in the carboxyl-terminal 30-kD portion of N-CAM-160. The majority of the N-CAM purified was glycosylated N-CAM-160 (molecular mass range, 200–230 kD; 16) with some smaller fragments presumably derived from the carboxyl-terminal portion of the molecule.

Cleavage of FrI and N-CAM-160

To improve the reproducibility of cleavage patterns obtained with enzymes, N-CAM-160 or FrI (2 mg/ml) were first denatured by boiling for 3 min in the presence of 0.5 % SDS (for V-8 protease) or NP-40 (trypsin). Polypeptide fragments were generated by treatment with V-8 protease (1:150, 1:750, or 1:3750 wt/wt enzyme/N-CAM for 30 min at 37°C; Sigma Chemical Co., St. Louis, MO), trypsin (1:300, 1:1500, or 1:7500 wt/wt for 30 min at 37°C; Sigma Chemical Co.), or cleavage with saturated cyanogen bromide (CNBr) in 70% formic acid in 2 h at room temperature followed by lyophilization. The digests were separated by SDS PAGE, and immunoblotted with each antibody.

Preceding studies on the structure of the neural cell adhesion molecule (N-CAM) (23) have led to the proposal that the overall structure of the molecule is a single polypeptide chain divided into three regions (3): an amino-terminal 65-kD domain that includes the homophilic cell–cell binding site (24), a central region containing the large and unusual polysialic acid (PSA) moieties (5, 15), and a membrane-associated region extending to the carboxyl terminus (3). Further analyses demonstrated that the molecule spans the cell membrane and is synthesized in three predominant polypeptide forms (15, 22) that differ in the size of their cytoplasmic extension (9). The N-CAM gene has been identified in both mouse (12) and chicken (20), and a partial sequence of the chicken gene has been reported (13).

1. Abbreviations used in this paper: Endo-F, endoneuraminidase F; N-CAM, neural cell adhesion molecule; PSA, polysialic acid.
Results

To position mAb epitopes along the N-CAM polypeptide chain, we tested each mAb for its ability to recognize different subpopulations of proteolytic and CNBr fragments of N-CAM and Frl. Assignment of epitope position was based on the following: groups of antibodies that recognized the same set of larger fragments, the use of epitopes present in more than one set to order these peptides, and then a final adjustment of relative locations according to antibody staining of a variety of smaller fragments. All tests for antibody reactivity were carried out by immunoblotting methods after the fragments were separated by SDS PAGE. The mAbs were initially screened for reaction with N-CAM that had been boiled in SDS (27), and all also recognized epitopes associated with the fractionated peptides.

The use of SDS PAGE to separate the fragments has also provided an estimate of polypeptide chain length, which is an important parameter in the construction of the epitope map. The presence of carbohydrate on a fragment, although in many cases representing a useful epitope, introduces error into the assignment of polypeptide length. To assess the magnitude of this uncertainty, the enzyme endoneuraminidase F (endo-F) (27) was used to remove all N-linked carbohydrate from several of the fragments (because of their rarity and small size, it is assumed that O-linked oligosaccharides have little if any impact on the estimation of N-CAM peptide size). With the exception of the PSA-containing fragment, which has been considered separately in the accompanying paper (27), fragments that had originally been recognized by antibodies directed against carbohydrate decreased in apparent molecular mass by ~3 kD after treatment with endo-F. Fragments reacting with two of these mAbs decreased in size by 6 kD (data not shown). In the following epitope analysis, fragments have not been routinely treated with endo-F to retain information about the distribution of carbohydrate-dependent antigens. Instead, on the basis of the consistent effect observed with endo-F, the apparent length of the fragment polypeptides has been decreased by 0, 3, or 6 kD, according to the number of different carbohydrate epitopes detected.

The information and logic used to construct an epitope map from a large number of fragments and antibodies is difficult to present directly from the immunoblots of fractionated peptides. Therefore, presentation of the results will begin with the SDS PAGE and immunoblot information, progress to a tabular summary of the most important fragments, and then conclude with alignment of only those peptides that are essential to the placement of epitopes. For simplicity, the results will be presented separately for peptides generated from the 65-kD amino-terminal Frl fragment of N-CAM, and then for peptides generated from intact N-CAM–160, which is the largest of the naturally occurring N-CAM polypeptides.

Recognition by mAbs of Fragments Generated from Frl

The 65-kD Frl fragment of N-CAM, which has been shown to contain the N-CAM amino terminus (3), was shown in the accompanying paper to react with mAbs 10A, 11B, 9A, 12B, PP, 79B, 5E, 104, and CHO, but not other mAbs, was eliminated and the apparent molecular mass decreased by ~6–59 kD. This is consistent with the previous finding that mAbs 104 and CHO are carbohydrate-dependent (27), and that only a small portion of total N-CAM glycosylation is associated with this fragment (3).

The trypsin and V-8 protease fragments of the glycosylated (65-kD) form were separated by electrophoresis, transferred to nitrocellulose, and then reacted with each mAb. Staining of these fragments with the different mAbs revealed three distinct patterns of reactivity (Fig. 1), with a few important variations (Figs. 2 and 3). In Fig. 1, A represents staining with mAb 12B and also exemplifies the pattern obtained with 10A, 9A, 11B, and PP. The similarity of these patterns is demonstrated by a comparison of A with B, which was produced by reaction with 11B. In contrast, mAbs 79B and 5E both stained a very different group of fragments (C), and fragments recognized by CHO and 104 represented the third major pattern (D).

Similarities and differences in the staining of selected fragments of Frl are summarized in Table I. Peptides recognized by the same subset of mAbs are grouped together: group I (Fig. 1, A–D) includes large fragments that reacted with many mAbs, group II fragments (Fig. 1 C) reacted only with 5E and 79B, group III (Fig. 1, A and B) represents peptides

![Figure 1](image-url)
Table I. Detection of mAb Epitopes on Peptide Fragments of Fr I

<table>
<thead>
<tr>
<th>Group</th>
<th>Peptide</th>
<th>5E</th>
<th>79B</th>
<th>PP</th>
<th>12B</th>
<th>9A</th>
<th>11B</th>
<th>10A</th>
<th>104</th>
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Data derived from Figs. 1-3. The peptide names indicate the enzymes used for cleavage (T, trypsin; V, V-8 protease) followed by the estimated polypeptide size in kilodaltons and, where applicable, the size of the glycosylated peptide in parenthesis. Antibodies 105, 30B, and 4D did not recognize any peptides derived from FrI.

Epitopes on Fragments of N-CAM–160

The patterns of mAb staining obtained with fragments derived from N-CAM–160 are shown in Fig. 4. mAbs that recognize similar fragments are represented by a single panel. As would be expected, epitopes representing the FrI region (5E, 79B, PP, 12B, 9A, 11B, 10A, and CHO; 104 was not tested) are again represented by three major patterns with minor variations. However, these patterns, while reproducible (compare 5E with 79B, and PP with 12B) are quite different from the corresponding patterns derived from FrI. Although the differences are in part due to a slight increase in acrylamide concentration, the major factor seems to be that N-CAM–160 is more rapidly degraded than FrI. In the case of CHO, additional fragments are detected in the CNBr digest of N-CAM–160 that are also recognized by mAbs against epitopes not contained within FrI. Thus, unlike the polypeptide-dependent FrI epitopes, the carbohydrate-dependent CHO antigen appears to be repeated in other regions. In fact, as demonstrated in the final map, this epitope occurs at at least three positions along the N-CAM polypeptide chain.

Since the NH2-terminal region of N-CAM has already been characterized with peptides derived from FrI, the fragments obtained from N-CAM–160 are primarily considered with respect to the central and carboxyl-terminal portions of the polypeptide. These regions are recognized by mAbs 105, 30B, 4D, CHO, and 20A (another carbohydrate-dependent antigen distinct from the CHO epitope). Whereas staining with CHO, 105, and 4D each gave a distinct fragment pattern, 20A and 30B produced very similar patterns (Fig. 4). However, 20A and 30B differ in that 20A recognizes all forms of N-CAM (data not shown) and the 30B epitope is confined to N-CAM–160 (27).

When the N-CAM–160 fragments were arranged according to the presence of epitopes (Table II), eight groups could be distinguished. The first two sets correspond to groups I and II from digests of FrI (Table I). The third group provides...
additional information about the Fr1 region in that it is a fragment containing the 5E but not the 79B epitope. Group IV, V, and VI peptides contain part of the central, carbohydrate-rich region of the polypeptide as identified by the 105 epitope, which has been shown (27) to be associated with the 36-kD PSA-containing CNBr fragment (14). The differences between groups IV, V, and VI allow discrimination of epitopes 105 and CHO from 20A, 30B, and 4D. Moreover, group V also reacts with mAbs 30B and 4D, which are specific for the intracellular domain of N-CAM-160. Group VII is recognized only by 20A and 30B and, together with the 4D-specific group VIII, serves to distinguish among these epitopes.

**Construction of a Linear Map of Epitopes**

The groups of peptides recognized by subsets of antibodies as presented in Tables I and II indicate that certain epitopes are closely linked to others. To place individual epitopes along the polypeptide chain, it remains to order these linkage groups, establish the sequence of the epitopes within a group, and, finally, to position them according to the size of overlapping fragments. The fragments characterized in Figs. 1–4 account for all of the epitopes described in the accompanying paper (27), and provide sufficient information to place them with considerable resolution along the polypeptide chain. The rationale behind this map, based on representative fragments from each group in Tables I and II, to-
### Table II. mAb Epitopes Associated with Peptide Fragments of N-CAM-160

<table>
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Data derived from Fig. 4. The peptide names indicate the method used for cleavage (T, trypsin; V, V-8 protease; C, cyanogen bromide) followed by the estimated polypeptide size in kilodaltons and, where applicable, the size of the glycosylated peptide in parenthesis.

Together with their estimated polypeptide lengths from Figs. 1-4, is displayed in Fig. 5 and described below. The nomenclature used for fragments is described in the legend of Table II.

The amino-terminal 59-kD portion of the molecule is represented by fragment Frl and epitopes 5E, 79B, PP, 12B, 9A, 11B, 10A, and CHO. The key fragments in establishing the overall structure within this region are the three Frl-derived peptides V40, T34(37), and T18. V40 only contains epitopes 5E and 79B; T34(37) contains all Frl epitopes except 5E, 79B and CHO; while T18 (and also T27) reacted with PP as well as with 5E and 79B, and therefore provides both a link between V40 and T34(37) and places PP at one end of Frl. Additional resolution in the placement of Frl determinants is achieved through peptides V23 and T41 (44). V23 contains only 5E and because of its large size relative to T18, places this epitope on the amino terminal side of 79B. T41(44) has all Frl epitopes except CHO, and therefore restricts this epitope to one end of Frl. In fact, the size and position of the other CHO-minus peptides described above indicate that this epitope lies within a short distance from either the amino or carboxyl terminus of Frl. Because the Frl fragment staining pattern obtained for CHO was identical to that for 104 (Fig. 1) except for the absence of T47(50) and T41(44) (Fig. 3), and contained no fragments uniquely shared with 5E or 79B, CHO has been assigned to the end of Frl that contains 104.

Two aspects of the Frl epitope map therefore remain undefined: the relative order of 12B, 9A, 11B, 10A, and 104, which, however, are confined to a region of <17 kD, and orientation of the map with respect to the amino terminus. We initially assigned both the 104 and CHO carbohydrate-dependent epitopes to the carboxyl terminus on the basis of previous studies that indicate that most, if not all, N-CAM carbohydrate is associated with the central region of the N-CAM polypeptide chain (3, 14). This placement is supported by fragment T23 (Table I), which contains epitopes PP, 12A, 9A, 11B, and 10A, but not 104 or CHO. Moreover, it has recently been established that the 25-kD heparin-binding fragment (1), which contains the 5E and 79B epitopes (27), has the same NH₂-terminal amino acid sequence as the intact molecule (Cole, G., and L. Glaser, personal communication). With the establishment of this orientation, the approximate distances of the Frl epitopes from the amino terminus of N-CAM are: 5E at 22 kD, 79B at 23 kD, PP at 40 kD, the 12B to 104 complex within 41–58 kD, and CHO near the 59-kD Frl terminus.

The central portion of N-CAM, which contains the PSA residues and extends to the cell membrane, is represented by epitopes CHO, 105, and 20A. Of these, only 105 is not a carbohydrate-dependent antigen, and as mentioned above, CHO is present on at least three different sites and cannot be used to identify overlapping peptides between this region and Frl. The overall placement of these epitopes is based on fragments C36(65–130) and C25(31). C36(65–130) contains PSA (2), CHO, and 105, whereas C25(31) has 105, CHO, 20A, 30B, and 4D. Because peptide V25 only reacted with mAb 105, and fragment V18(21) only contained 20A and 30B, it follows that PSA and CHO both must lie near one end of C36(65–130) and 105 at the other (see Fig. 5). Similarly, the size of V18(21) argues that 105 is immediately followed by another CHO epitope. The data would also allow CHO to lie in a small intracellular region between 30B and 4D, a possibility that seems unlikely in view of the carbohydrate nature of this epitope. Placement of the 20A epitope near the NH₂-terminal end of V18(21) and near CHO is based on the fact that this mAb reacts with N-CAM–110.

The cytoplasmic domain of N-CAM–160 has two markers,
Figure 5. Mapping of mAb epitopes along the N-CAM polypeptides. Peptide names as defined in Tables I and II are indicated at the margins of the figure. Only a minimal subset of the peptides used to construct the map (see text) are illustrated; other peptides listed in the two tables serve to confirm the assignments of epitope position. The estimated size of the N-CAM polypeptides and their peptide fragments, without carbohydrate, is represented by the length of each line, with the scale in kilodaltons at the top of the figure. Epitopes associated with each peptide are shown, with individual positions by vertical lines; several epitopes were located together between 40 and 60 kD, and are grouped within parentheses. Carbohydrate-dependent epitopes have been enclosed by boxes.

30B and 4D. As indicated above, fragments V18(21) places I05, CHO, and 20A at one end of C25(31) and, consequently, 30B followed by 4D at the other. The order and close proximity of 30B and 4D is further supported by the size of T21, which contains only 4D and therefore extends toward the carboxyl terminus of N-CAM-160. The orientation of the central and intracellular regions is indicated by the fact that 30B and 4D are located inside the cell (27) and the carboxyl termini of N-CAM-140 and N-CAM-160 have been shown to extend into the cell cytoplasm (9, 27).

Although no fragment was found that overlapped Fr1 and the central region, the position of this second group of epitopes could be estimated from the properties of the naturally occurring 110-kD N-CAM polypeptide. This form of the molecule contains all the epitopes except for 30B and 4D, and its size indicates that peptide C36(65-130) must begin within ~10 kD of the end of Fr1. With this placement, the length of the overlapping fragments described above would extend to within ~6 kD of the carboxyl terminus of N-CAM-160. The assignment of approximate epitope positions from the amino terminus would therefore be PSA and CHO at 70-80 kD, 105 at 106 kD, CHO again at 108 kD, 20A at 110 kD, 30B at 130 kD, and 4D at 132 kD.

Discussion

In the accompanying report we describe a group of mAbs that can discriminate among and perturb the known major structural and functional regions of N-CAM. The results presented in this paper serve to assign the antigenic epitopes recognized by these antibodies to positions along the polypeptide chain. The purpose of this discussion, after initial comments on the epitope map, is to combine these new findings with our existing knowledge about N-CAM in the form of a topographical model of the molecule in association with the cell membrane (Fig. 6).

mAb epitope mapping on peptide fragments has been used to establish overall landmarks for several large proteins (see the introduction). In this approach, the establishment of structure-function relationships has usually been based on a correlation between the biological effects of different mAbs and their ability to compete with each other in binding to the native intact protein (see 7, 18). While binding competition studies to native N-CAM were included in our characterization of anti-N-CAM mAbs, we have attempted to obtain additional information by preparation of a variety of overlapping peptides using SDS PAGE for peptide fractionation and size determination, and immunoblots to assess similarities and differences between the fragments. The major assumptions required in this method are that the antigenic determinants are still detectable after fragmentation of the protein, and that these epitopes are not irreversibly destroyed during SDS PAGE. To minimize such difficulties, the mAbs were screened for their ability to react with proteolytic

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160, N-CAM-130, and N-CAM-110 polypeptides differ in their carboxyl-terminal cytoplasmic extensions as illustrated by the lines at or near the cell membrane and near epitope 30B; the smaller amount of PSA on embryonic retinal or adult brain N-CAM is indicated by the lines through the multiple carbohydrate chains shown extending from the central region of the molecule. Nine PSA chains of equal length are depicted, but the actual distribution of the sugar among these chains may differ substantially. Also, although the location of the heparin-binding region is indicated, it is not known if all forms of N-CAM have this activity. This model is not precise in the folding of the polypeptide chain or the exact relative positions of all epitopes, but attempts to account for the overall size, shape, membrane disposition, and functions of the molecule in terms of its known biochemical properties.

The known or proposed molecular features and biological activities of N-CAM are presented schematically in Fig. 6, together with the epitopes that serve to locate and perturb them. In addition to the present map and earlier less-detailed versions of the molecule (see the introduction), this model incorporates several additional observations, as discussed below.

Although the epitope map did not provide a sequence for 12B, 9A, 11B, and 10A, the spatial relationships shown among these epitopes attempt to illustrate some of the results of the binding competition studies in the accompanying report. For example, mAb 9A interfered with binding of 11B and PP, and PP interfered with 79B as well as 9A and 11B. In a two-dimensional schematic illustration, however, it was not possible to represent all of the competition studies accurately. In any case, it is notable that the overall results of the binding competition studies, which were carried out on native, membrane-bound N-CAM, are completely consistent with the epitope map. Thus, the 10A to PP group, and the 5E and 79B group competed more strongly within themselves than with each other, and there was no interaction with 105.

An additional rationale for grouping of epitopes was their effect on cell–cell adhesion. Half of the mAbs tested had strong and reproducible effects on adhesion, and these could be divided into three categories: those whose Fab inhibited adhesion (PP and 12B), Fabs that caused a strong enhancement (9A and 11B), and those that inhibited but are located in the heparin binding region (5E and 79B). With respect to multiple site contribution to cell–cell binding activity, it is interesting that combinations of Fabs from the two different inhibitory groups (for example 12B and 5E), were more effective than either alone (27). Altogether, it appears that both binding activities and all of the mAbs described here that affect adhesion are concentrated in a single compact, highly antigenic domain derived from ~40 kD of contiguous polypeptide.

The existence of mAbs whose monovalent Fab fragments enhance adhesion was at first surprising. However, another alteration of N-CAM, the removal of PSA, has also been shown to enhance aggregation rates (2, 25). It is therefore possible that the attachment of Fab outside of an actual binding site might cause a conformational change that mimics the effects on adhesion produced by removal of PSA.

The fact that the site of cleavage to produce Frl coincides with the only portion of the map that did not yield an overlap-
The depiction of the central carbohydrate-rich domain is highly schematic, as there is relatively little known about the number and length of the unbranched alpha-2,8-linked sialic acid polymers. The illustration of nine chains of ~25 residues each reflects the evidence that there are three N-linked carbohydrate cores located in the sialic acid–containing peptide (14), each with the potential for one to three chains (6), and that the heavily sialylated form of the molecule from embryonic brain is 30% sialic acid by weight (15) or ~225 sialic acid residues. In the absence of data to suggest whether all possible chains exist or if there are differences in their length, the total amount of sialic acid has been shown in a uniform distribution. In contrast, N-CAM from embryonic retina or postnatal brain has only one-third of this amount of sialic acid (15, 21) (see dashed line in Fig. 6), and the distribution of the saccharide does not appear to be uniform (6).

The next feature depicted in the model is the membrane-associated region. The position of the membrane is defined by the inter- and intracellular location of epitopes described in the accompanying paper, the carboxyl terminus of N-CAM-110 that has been proposed to not have a significant intracellular component (dashed line at the membrane in Fig. 6) (9, 13), and the size of the largest (108-kD) fragment, Fr2, released from intact cells by limited proteolysis (3). This region is most accurately marked by epitope 20A and is also near 105 and the most carboxyl-terminal CHO determinant. It has been depicted tentatively as an extended region on the inner leaflet of the plasma membrane (dashed line at the membrane in Fig. 6) (9, 13), and the fact that transmembrane segments of proteins are often composed of a single alpha helix. An important assumption in the model is that the polypeptide chain traverses the cell membrane only once, or at least within a short stretch of polypeptide. In support of this feature is the fact that all epitopes mapped in the amino-terminal region (9). The most direct evidence, however, comes from the recent gene sequence analysis (13), which indicates that there is a single hydrophobic region in the COOH-terminal half of N-CAM, precisely at the site suggested by our studies.

The intracellular domains are the least well-described parts of N-CAM. The different polypeptide forms (160, 130, and 110 kD) are depicted as having a 50-kD, a 20-kD, and no cytoplasmic extension, respectively (see dashed lines in Fig. 6). Although it has been suggested that these N-CAMS are derived from a single gene (12, 19) produced from different messenger RNAs, nothing is known about the function of this portion of the polypeptide. However, one or more phosphorylation sites, at threonine and serine residues, have been reported to occur in each of the two intracellular domains (10, 26). An extended segment at 130 kD in N-CAM-160 is illustrated primarily to allow for the fact that the cytoplasmic portion of N-CAM-130 can exist and presumably function by itself and is therefore likely to represent a domain that folds independently of the 130–160-kD region. It is notable that the proposed extended segment also contains both the antigenic sites recognized by 30B and 4D.

In summary, the incorporation into an epitope map of information obtained in studies from several laboratories has led to the formulation of a more detailed topographical model for N-CAM. Although the model will need considerable refinement, it should serve as a basis for testing a variety of structure–function relationships, particularly through the use of the mAbs that mark and perturb its key features.

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