Mapping of Three Carbohydrate Attachment Sites in Embryonic and Adult Forms of the Neural Cell Adhesion Molecule

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ABSTRACT The sialic-rich carbohydrate moiety of the neural cell adhesion molecule (N-CAM) undergoes major structural changes during development and plays a significant role in altering the homophilic binding of the molecule. In order to understand the mechanism of these changes, a cyanogen bromide (CNBr) fragment that contained 90% of the sialic acid of N-CAM was isolated and characterized according to the number of carbohydrate attachment sites and reactivity with specific monoclonal antibodies. The CNBr sialopeptide migrated on SDS PAGE as a broad zone of M_r 42,000–60,000. Upon treatment with neuraminidase, it was converted to a single component of M_r 42,000, and subsequent, limited treatment with endoglycosidase F gave four evenly spaced components of M_r 35,000–42,000, suggesting that it contained three attachment sites for N-linked oligosaccharides. The fragment reacted with monoclonal antibody 15G8, which detects the sialic acid in embryonic N-CAM, and with a monoclonal antibody, anti-(N-CAM) No. 2. Treatment with neuraminidase or with endoglycosidase F destroyed reactivity with 15G8 but not with anti-(N-CAM) No. 2. A similar CNBr sialopeptide was obtained from adult N-CAM; it contained sialic acid, had three N-linked oligosaccharides and reacted with anti-(N-CAM) No. 2 but not with 15G8 monoclonal antibodies. A peptide fragment, Fr2, comprising the NH_2 terminal and middle regions of the molecule yielded a CNBr fragment closely similar to the fragment obtained from the whole molecule. The CNBr fragment from Fr2 reacted with monoclonal antibody anti-(N-CAM) No. 2. Fr1, comprising the NH_2 terminal region alone, failed to react. These data confirm that the majority of the sialic acid is localized in the middle region of the N-CAM molecule and support the hypothesis that embryonic to adult conversion of N-CAM is the result of differences in sialidase or sialytransferase activity.

The neural cell adhesion molecule (N-CAM)[1] is a cell surface glycoprotein (1–6) that mediates calcium-independent aggregation of neural cells in vitro (4, 5). N-CAM, isolated from embryonic chicken brain contains an unusually large amount of sialic acid, 30% by weight, in a polymeric form (6) with α 2–8 linkages (7). The molecule undergoes a transition perinatally from this highly negatively charged embryonic (E) form to several adult (A) forms (8, 9) containing only one-third the amount of sialic acid (8). This process has been termed E–A conversion (1, 2, 9, 10).

Earlier structural studies (8, 11) showed that embryonic and adult forms of N-CAM are biochemically similar in their amino acid and neutral sugar content, but differ in their sialic acid concentration (8). The E form migrates as a broad zone on SDS PAGE of apparent molecular weight from M_r 200,000 to M_r 250,000, whereas the A forms appear as two discrete bands of M_r 180,000 and 150,000 in the chicken with an additional band of M_r 120,000 in the mouse. Upon neuraminidase treatment, E and A forms are both reduced to two closely related polypeptides of M_r 140,000 and 170,000.

A scheme relating the various structural regions of the molecule is shown in Fig. 1 (11), and is useful for understanding the rationale followed in the current work for localizing

[1] *Abbreviations used in this paper:* CNBr, cyanogen bromide; E–A conversion, embryonic to adult conversion of N-CAM; Endo F, endo-β-*N*-acetylglucosaminidase F; Fr1, fragment 1; Fr2, fragment 2; N-CAM, neural cell adhesion molecule.

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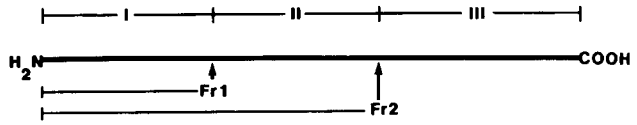

FIGURE 1 Schematic arrangement of structural regions of N-CAM. The schematic drawing indicates the linear arrangement of three defined (11) regions of N-CAM. Region I contains the binding site for N-CAM to N-CAM (homophilic) interaction; region II has been proposed to contain the bulk of the carbohydrate, and region III contains portions associated with the cell membrane. Fr1 (region I, M_r 65,000) is spontaneously produced by incubation of N-CAM in solution and contains the amino terminus of the protein and the intermolecular binding site. Fr2 (region I plus region II) is a high molecular weight fragment (M_r 108,000 after removal of sialic acid) released from cell membranes by treatment with V-8 protease. This fragment contains >80% of the sialic acid present in the intact molecule. The Roman numerals are used merely to illustrate the sequential order of the regions.

carbohydrate sites. During prolonged incubation of the molecule at 37°C, a species of M_r 65,000, fragment 1 (Fr1), containing the binding site and amino terminus but <5% of the sialic acid, is spontaneously produced. A larger, sialic acid-rich fragment, fragment 2 (Fr2), can be released from brain vesicles by limited proteolysis with *Staphylococcus aureus* V-8 protease. This fragment contains the NH_2 terminal binding region and >80% of the sialic acid of the intact molecule; upon neuraminidase treatment, it is reduced to a component of M_r 108,000 on SDS PAGE. The remainder of the N-CAM molecule is assumed to include the portion of the molecule involved in its association with the membrane because neither Fr1 nor Fr2 can be reconstituted into artificial lipid membranes (11).

Recent studies on the binding of N-CAM in reconstituted lipid membrane vesicles (12) show that its binding behavior is greatly influenced by its sialic acid content. Complete desialylation increases the rate of N-CAM binding by fourfold. Molecules with intermediate amounts of sialic acid, generated by partial neuraminidase digestion, have intermediate binding rates. Molecules lacking sialic acid bind at rates equivalent to those of the adult forms, and Fr1, when immobilized on Covaspheres, can bind to cells. Although both findings indicate that sialic acid is not required for binding, the data suggest that the sialic acid component has a striking modulatory effect upon intermolecular binding. Any changes in the sialic acid content of N-CAM that occur during E–A conversion may therefore be expected to lead to differential adhesivity within developing tissues (1, 2).

In view of the functional significance of these modulatory events, and to understand the mechanism of modulation, it is particularly important to confirm that E–A conversion involves only the loss of sialic acid and not of sugar attachment sites. To resolve this issue, we have isolated a cyanogen bromide (CNBr) fragment from embryonic and adult N-CAM that accounts for the bulk of the central region of the molecule represented by the difference between Fr1 and Fr2 (Fig. 1). The adult sialopeptide was found to have less sialic acid than the embryonic peptide, but both had three N-linked oligosaccharides. This suggests that conversion does not occur by the removal or absence of the sites themselves, and favors the hypothesis that E–A conversion occurs either by the removal of sialic acid from the E form, or its failure to be attached to one or more of these sites.

MATERIALS AND METHODS

N-CAM was prepared as described (6, 8). Reduced and alkylated (13) protein was treated with CNBr by adding 8–10 mg N-CAM to 2 ml of water; 90% formic acid was then added to a final concentration of 70%. Under these conditions, the protein was soluble after sonication. Solid CNBr (450 mg) was added to the protein solution. After 45 min at room temperature, 100 ml of water was added and the reaction mixture was lyophylized repeatedly from water until the odor of formic acid was no longer detectable. This material was dissolved in 0.1 M NH_4HCO_3, and centrifuged at 15,000 *g* for 10 min, and the supernatant was loaded onto a column of Sephacryl S-300 (1.6 × 100 cm) in 0.1 M NH_4HCO_3.

Specific anti-N-CAM polyclonal and monoclonal antibodies were prepared and screened as described (3, 4, 6, 14). N-CAM and N-CAM CNBr fragments were separated by SDS PAGE and stained with "Stains-all" (Eastman Kodak Co., Rochester, NY) (15), Coomassie Blue, or silver (16); alternatively, they were electrophoretically transferred to nitrocellulose paper, and reacted with rabbit polyclonal or mouse monoclonal antibodies followed by ^{125}I-protein A as described (17). For peptide mapping, N-CAM and fragments from gels were treated with *Staphylococcus aureus* V-8 protease (0.25 μg/lane) and the products were resolved by SDS PAGE (17–19). Gels were calibrated with the following molecular weight standards purchased from Sigma Chemical Co. (St. Louis, MO) or Bethesda Research Laboratories (Gaithersburg, MD): myosin (M_r 205,000), β-galactosidase (M_r 116,000), phosphorylase B (M_r 97,400), bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), α-chymotrypsinogen (M_r 25,700), trypsinogen (M_r 24,000), soybean trypsin inhibitor (M_r 20,100), β-lactoglobulin (M_r 18,400), bovine α-lactalbumin (M_r 14,200), and cytochrome *c* (M_r 12,300).

Digestion of N-CAM and N-CAM CNBr fragments (~1 mg/ml) with neuraminidase (1 U/ml) from *Vibrio cholera* (Calbiochem-Behring Corp., San Diego, CA) was carried out in 0.1 M sodium acetate, 0.2 mM EDTA, 2 mM $CaCl_2$ for 18 h at 37°C. Alternatively, for peptide mapping experiments, N-CAM was treated with neuraminidase on an immunoaffinity support as described (11). Digestion of N-CAM and N-CAM CNBr fragments with endo-β-*N*-acetylglucosaminidase F (Endo F) was done according to the method of Elder and Alexander (20). The substrate (5–20 μg) was dissolved in 10 μl 2.2 mM Tris-HCl, pH 7.4, 11% Trasylol, 2.2% SDS, and 0.11 M dithiothreitol, and boiled for 3 min. The solution was cooled and 5 μl of 9% Nonidet P-40, 4 mg/ml phenylmethylsulfonyl fluoride solution was added. To this mixture 30 μl of enzyme solution was added. The enzyme solution contained 100 U/ml Endo F, 0.17 M sodium phosphate buffer, pH 6.1, and 83 mM EDTA. The mixtures were incubated at 37°C for 20 h. There are several indications that proteolysis does not influence the results of glycosidase digestions. The changes in molecular weight agree with those expected for removal of sugar moieties. The products of endoglycosidase F or neuraminidase treatment of the CNBr sialopeptide are the same for incubation times ranging from 16 to 48 h. As noted above, protease inhibitors are included in the endoglycosidase F incubation mixture. Storage of the CNBr sialopeptide under a variety of conditions indicate that it is a very stable peptide. Moreover, endoglycosidase F treatment of the liver cell adhesion molecule (21), which is extremely sensitive to proteolysis yielding a distinctive fragment, shows no evidence for proteolysis. Control incubations were performed in parallel and treated as above substituting water for the enzyme. Enzyme preparations were tested using ovalbumin as a control substrate.

Sialic acid determinations were performed by reaction with thiobarbituric acid (22) after acid hydrolysis in 0.1 N H_2SO_4 for 4 h. Amino acid analyses were performed on a Beckman 121 M amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA) using *p*-flurophenylalanine as an internal standard (6, 8); hydrolyses were carried out in 6 N HCl at 110°C for 18 h in tubes sealed

RESULTS

CNBr Cleavage of Embryonic N-CAM

CNBr fragments were generated from reduced and alkylated embryonic N-CAM and were fractionated on Sephacryl S300 (Fig. 2, upper panel). Aliquots of fractions were removed for sialic acid analysis by the thiobarbituric acid assay (22) both before and after acid hydrolysis. After hydrolysis, three regions of sialic acid-containing material were seen: high molecular weight material associated with undigested or partially digested N-CAM; material of intermediate molecular weight; and low molecular weight material that in several experiments did not appear to coelute with protein. The low molecular

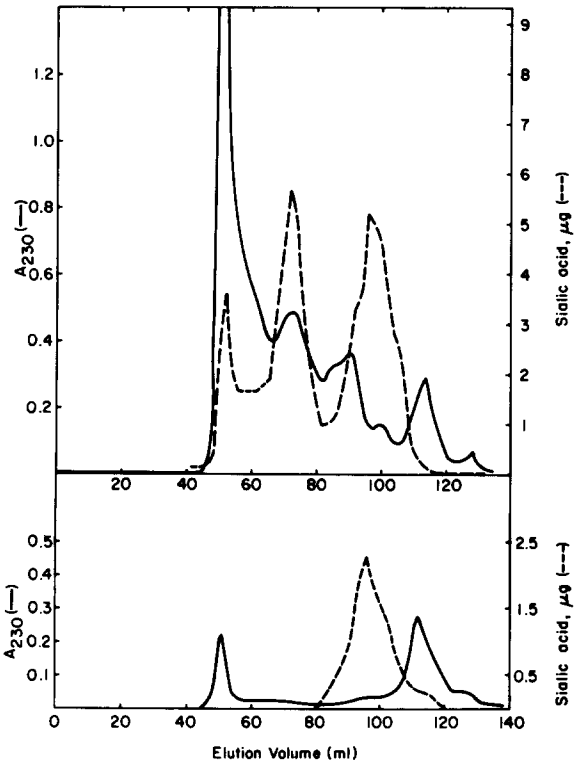

FIGURE 2 Fractionation of CNBr-treated and acid-treated N-CAM. Affinity-purified reduced and alkylated embryonic N-CAM was treated with CNBr in 70% formic acid (*upper panel*, 10 mg of N-CAM) or with 70% formic acid alone (*lower panel*, 8.5 mg of N-CAM), as described in Materials and Methods. The soluble material, in 3 ml of 0.1 M NH_4HCO_3, was chromatographed on a column (1.5 × 100 cm) of Sephacryl S-300 in the same buffer. The solid line indicates absorbance at 230 nm (left ordinate); the broken line represents total sialic acid (right ordinate) in fractions analyzed by the thiobarbituric acid assay (22) after acid hydrolysis.

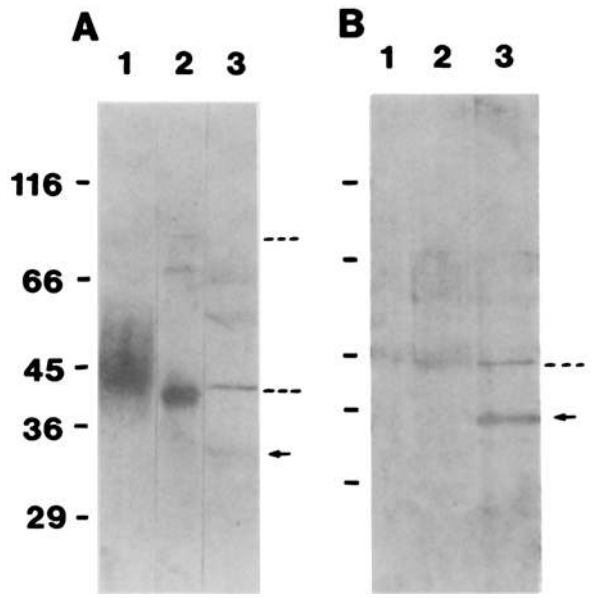

FIGURE 3 CNBr sialopeptide from embryonic and adult N-CAM. Purified embryonic CNBr sialopeptide (*A*, lanes *1–3*) and adult CNBr sialopeptide (*B*, lanes *1–3*) were treated with neuraminidase (lane *2*) or Endo F (lane *3*), as described in Materials and Methods, and separated by SDS PAGE on a 10% gel. The arrow in lane *3* points to the deglycosylated CNBr sialopeptide; the dotted lines represent components that are present in the enzyme preparation. The bands near the M_r 66,000 standard are assumed to be aggregates of the deglycosylated peptide. The purified embryonic material in lanes *1–3* was treated for 48 h with 70% formic acid for maximal release of the acid labile sialic acid component before enzyme digestions. Both gels are stained by the silver method of Morrissey (16); identical standards indicated by the horizontal bars were used for each.

weight material was not free sialic acid because acid hydrolysis was required to obtain reactivity in the assay (22), which detects only free sialic acid.

Treament of embryonic N-CAM with 70% formic acid in the absence of CNBr yielded a similar low molecular weight, sialic acid–containing fraction with no associated peptide (Fig. 2, lower panel). (Only a small amount of protein-associated absorbance was detected in the effluent because most of the N-CAM became insoluble after acid treatment.) The low molecular weight sialic acid material appeared to be polymeric sialic acid that had been attached through an acid-labile linkage inasmuch as it was retained on an 18% SDS polyacrylamide gel as a polydisperse region staining blue with "Stains-all," a carbocyanine cationic dye that forms a blue complex with anionic material including sialoproteins (23, 24), phosphoproteins (15), and acidic calcium-binding proteins (25).

The sialic acid-containing fractions of intermediate molecular weight (Fig. 2, upper panel) were pooled and rerun on the same column. This fractionation yielded polydisperse material on PAGE in the range of M_r 42,000–60,000 that was free of other detectable peptides (Fig. 3*A*, lane *1*). Upon neuraminidase treatment, the broad zone was reduced to a single M_r 42,000 band (Fig. 3*A*, lane *2*) indicating that the heterogeneity is due to the presence of a large amount of sialic acid. Furthermore, a component of M_r 35,000 was generated upon extensive Endo F treatment without prior treatment with neuraminidase (Fig. 3*A*, lane *3*), which suggested that the carbohydrate was N-linked and the sialic acid is attached to N-linked oligosaccharides. In both neuraminidase- and Endo F-treated material, components near M_r 66,000–68,000 are observed. Because these components are not present in untreated material and can be recognized by a monoclonal antibody specific for the peptide region of the fragment (see Figure 5), they are assumed to be aggregates of the deglycosylated peptide.

When CNBr fragments were prepared from affinity-purified adult N-CAM and separated on a Sephacryl S300 column, a sialic acid–containing component eluted at a slightly lower molecular weight than the embryonic sialopeptide, and no low molecular weight sialic acid–containing material was detected. The acid-labile sialic acid material thus appears to be a distinctive feature of the embryonic form of the molecule. After separation on SDS PAGE, the sialic acid-rich fraction from adult N-CAM contained a M_r 45,000 peptide (Fig. 3*B*, lane *1*) rather than the polydisperse material found in the embryonic N-CAM CNBr digest. This behavior on gels (polydisperse vs. banded) is comparable with that seen when E and A forms of the whole molecule are compared. In the same manner as the embryonic peptide, however, the adult M_r 45,000 peptide was reduced to a component of M_r 42,000 by neuraminidase treatment and to an M_r 35,000 component by Endo F treatment (Fig. 3*B*, lanes *2* and *3*). The adult N-CAM molecule thus has a sialic acid–containing region similar or identical to that in embryonic N-CAM.

The region represented by the embryonic and adult CNBr

peptides was identified as a major sialic acid-containing glycopeptide of N-CAM (11) by several criteria. The material isolated from embryonic N-CAM stained blue with "Stains-all" (Fig. 4*A*), comparable with intact embryonic N-CAM. Moreover, the sialopeptide reacted on immunoblots with monoclonal antibody 15G8 (Fig. 4*B*), the reactivity of which requires the presence of the high concentration of sialic acid associated only with the embryonic form of N-CAM (9, 10). A similar peptide was generated from Fr2, the fragment released from membranes by V-8 protease and known to contain >80% of the N-CAM sialic acid (Hemperly, J. J., and B. A. Cunningham, unpublished observation; also see below). The CNBr sialopeptide isolated from adult N-CAM contained less sialic acid, but upon neuraminidase or Endo F treatment was reduced to components of similar molecular weight as the embryonic peptide. These results suggest that the M_r 42,000 CNBr peptide is located in the central region of both the embryonic and adult molecule, representing most or possibly all of the difference between Fr1 and Fr2 (11).

Characterization of the CNBr Fragment

To determine whether the embryonic CNBr sialopeptide contained the majority of the sialic acid in N-CAM, the amino acid and sialic acid contents (Table I) of the CNBr sialopeptide were determined. As a control, N-CAM was also treated under CNBr conditions (mock CNBr), and separated from the acid-released sialic acid-containing material by gel filtration on Sephadex G-50; analyses of this material were used to evaluate the loss of sialic acid caused by the acid treatment alone (Fig. 2, lower panel). Mock treated N-CAM contained an average of 70% of the sialic acid present in the untreated molecule, indicating that as much as 30% of N-CAM sialic acid was spontaneously released under the acidic CNBr conditions. Because this material is polymeric sialic acid, is derived only from the embryonic form of the molecule, and accounts for up to 30% of the N-CAM sialic acid, we assume that it is released from the major sialic acid containing region of the molecule. In support of this notion, treatment of the purified embryonic CNBr sialopeptide with formic acid released more low molecular weight material, indicating that this material can be generated from the CNBr sialopeptide.

The ratio of sialic acid to amino acid in the CNBr sialopeptide accounted for >90% of the sialic acid remaining in mock CNBr-treated N-CAM. We conclude that the CNBr sialopeptide represents a region that contains the major sites of sialic acid attachment in the N-CAM molecule. Moreoer, the observation that the conditions used for CNBr treatment did not destroy the antigenic determinant recognized by the 15G8 antibody (Fig. 4*B*) suggest that at least part of the sialic acid moiety characteristic of embryonic N-CAM is retained in this fragment.

Location of the CNBr Sialopeptide within the Polypeptide Chain of N-CAM

The foregoing results suggested that the CNBr sialopeptide contained the majority of the N-CAM sialic acid and, therefore, that at least a portion of it was contained within Fr2 (M_r 108,000, see Fig. 1). It was important to demonstrate directly, however, whether the CNBr sialopeptide could account for the majority of the difference between Fr1 and Fr2. To show that the overlap between Fr1 and the CNBr sialopeptide was minimal and that the sialopeptide was almost completely included within Fr2, analysis of CNBr fragments with a monoclonal antibody specific for a peptide determinant of the sialic acid–rich region as well as peptide mapping procedures were used.

A number of monoclonal antibodies have been generated against various forms of N-CAM. An N-CAM monoclonal antibody, anti-(N-CAM) No. 2, was previously prepared and identified (6, 26) that reacted with both M_r 140,000 and M_r 170,000 polypeptide chains of N-CAM (26 and Fig. 5). This antibody reacts with Fr2 (Fig. 5*B*), but not with Fr1 (compare

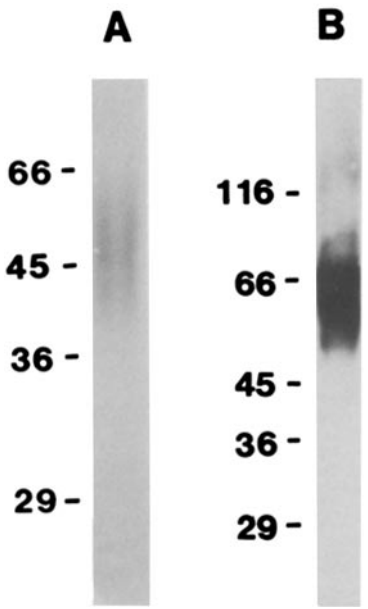

FIGURE 4 Embryonic CNBr sialopeptide. (*A*) The purified embryonic CNBr sialopeptide was resolved on a 15% polyacrylamide gel and stained with "Stains-all" (15). (*B*) The embryonic CNBr sialopeptide was resolved on a 10% gel and immunoblotted with anti-N-CAM monoclonal antibody 15G8. This material represents a separate preparation from lane *A* and from material shown in Fig. 3, accounting for the slight differences in its M_r.

TABLE I

Amino Acid and Sialic Acid Analysis of Embryonic N-CAM and N-CAM CNBr Sialopeptide

Preparation of N-CAM	M_r, exclusive of sialic acid*	Sialic acid/100 mol amino acid	Percentage of sialic acid compared with N-CAM‡
		mol	%
Untreated N-CAM§	140,000	13.2	—
N-CAM, formic acid-treated (unfractionated)	140,000	13.2	100
N-CAM, formic acid-treated (protein separated from released sialic acid)	140,000	9.6	73
CNBr sialopeptide	42,000	33.2	75

Sialic acid to amino acid ratios were calculated for N-CAM treated with formic acid to account for destruction of sialic acid by the acid treatment, for N-CAM treated with formic acid and then separated from released sialic acid material by chromatography on Sephadex G-50 to account for release of polymers of sialic acid by the acid treatment, and for the purified CNBr sialopeptide.

* Molecular weights estimated from SDS PAGE.

‡ Percentage of sialic acid retained was calculated relative to molecular weight, i.e., if the CNBr sialopeptide contained all of the N-CAM sialic acid, it, theoretically, would have 3.3 times (140,000/42,000) the amount of sialic acid per 100 mol of amino acid. As shown above, this expectation was met.

§ From reference 6.

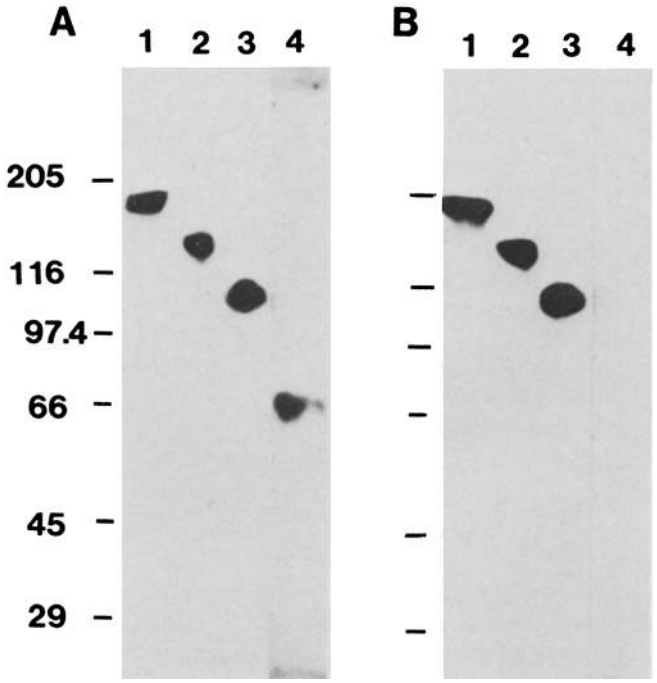

FIGURE 5 Reactivity of N-CAM and N-CAM fragments with anti-(N-CAM) No. 2. N-CAM peptides of M_r 170,000 (lane *1*) and M_r 140,000 (lane *2*) were generated by neuraminidase treatment of embryonic N-CAM on an affinity support, neuraminidase-treated Fr2 (lane *3*), and Fr1 (lane *4*) were prepared as described (11). These fragments were purified by cutting them from polyacrylamide gels, and were rerun on a 7.5% gel similar to the Cleveland procedure (18). A nitrocellulose blot (17) of these proteins was reacted with (*A*) rabbit polyclonal anti-N-CAM or (*B*) anti-(N-CAM) No. 2.

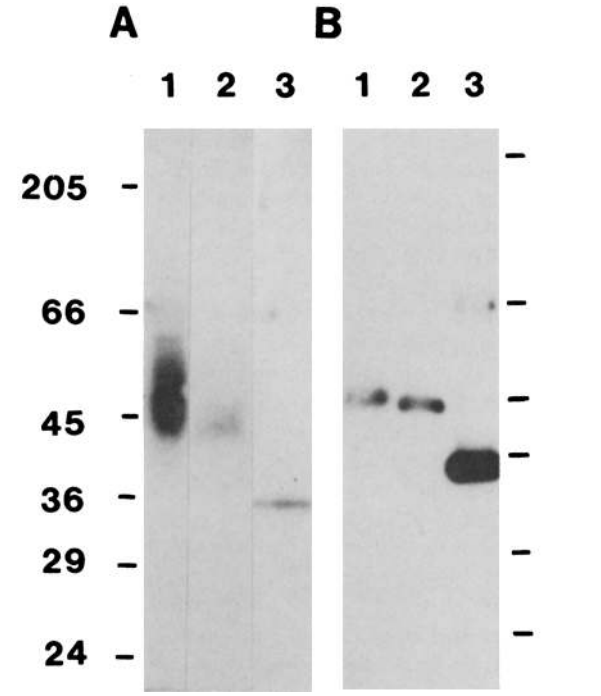

FIGURE 6 Monoclonal anti-(N-CAM) No. 2 immunoblot of CNBr sialopeptides from embryonic and adult N-CAM. The CNBr sialopeptides from embryonic (*A*) and adult (*B*) N-CAM described in Fig. 3 were immunoblotted with anti-(N-CAM) No. 2. (Lane *1*) Untreated sialopeptides; (lane *2*) neuraminidase-treated sialopeptides; (lane *3*) Endo F-treated sialopeptides. Migrations of identical standards in *B* are denoted on the right.

with Fig. 5*A*, which is stained with rabbit polyclonal anti-N-CAM). The recognition site of this antibody therefore appears to be within the sialic acid-containing region of the molecule (i.e., the region represented by the difference between Fr2 and Fr1).

Having shown that anti-(N-CAM) No. 2 reacted with the middle region of N-CAM polypeptide, we next tested its reactivity with the CNBr fragment. Immunoblot analysis of the CNBr sialopeptide demonstrated that anti-(N-CAM) No. 2 reacted with the sialic acid-containing CNBr fragment from embryonic and adult N-CAM both before and after treatment with neuraminidase or with Endo F (Fig. 6). These observations suggest that the antibody recognized a peptide determinant because Endo F removes both high mannose and complex types of N-linked sugars (20); O-linked sugars have not been demonstrated in N-CAM. Furthermore, for an equivalent amount of protein, Endo F-treated material reacted much more strongly with anti-(N-CAM) No. 2 (see Fig. 8), supporting the notion that the antigenic determinant is a peptide.

These results indicated that Fr2 and the CNBr sialopeptide had a common peptide antigenic determinant. To determine whether the CNBr sialopeptide was included within Fr2, CNBr fragments were generated from Fr2 after digestion with neuraminidase and analyzed by the immunoblot method using anti-(N-CAM) No. 2. This treatment of Fr2 yielded an anti-(N-CAM) No. 2 reactive component (Fig. 7*A*, lane *1*) that co-migrated with the purified neuraminidase-treated CNBr sialopeptide (Fig. 7*A*, lane *2*). Several higher molecular weight components were observed, and because this digest was not subjected to fractionation before the immunoblot

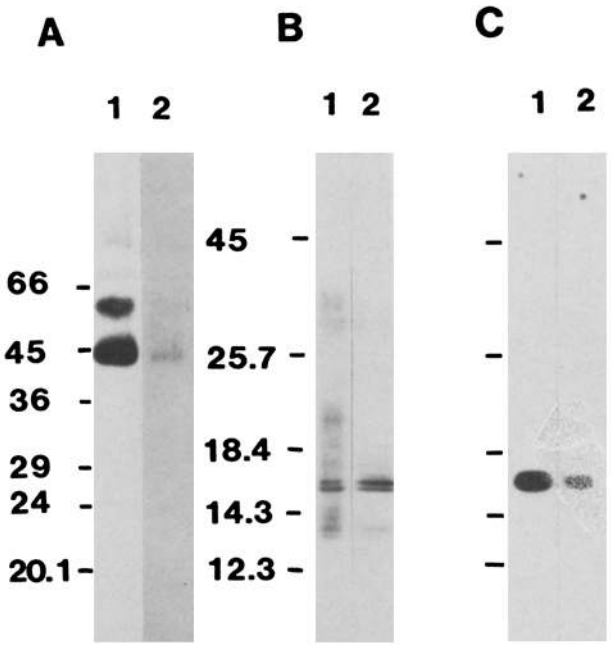

FIGURE 7 Comparison of CNBr and V-8 cleavage products of N-CAM fragments. (*A*) anti-(N-CAM) No. 2 immunoblot of unfractionated CNBr digest of neuraminidase treated Fr2 (lane *1*) and the purified neuraminidase treated CNBr sialopeptide from embryonic N-CAM (lane *2*) on a 12% polyacrylamide gel. Silver stain (*B*) and anti-(N-CAM) No. 2 immunoblot (*C*) of V-8 protease digests of neuraminidase-treated Fr2 (lanes *1*) and the neuraminidase-treated embryonic CNBr sialopeptide (lanes *2*) performed by the method of Cleveland et al. (18) using 0.25 μg of *S. aureus* V-8 protease.

analysis, were presumed to be partial CNBr products of Fr2. Similar partial cleavages have been seen in unfractionated CNBr digests of the intact molecule. These results strongly suggested that the CNBr sialopeptide is contained within Fr2.

Limited proteolytic mapping using *S. aureus* V-8 protease was also used to determine the relationship between Fr2 and the CNBr sialopeptide, and to corroborate the results obtained with monoclonal antibodies. The proteolytic map of neuraminidase-treated Fr2 and the CNBr sialopeptide was analyzed both by silver staining and by the immunoblot technique using anti-(N-CAM) No. 2 as a probe for peptides corresponding to the sialic acid–containing region. A prominent doublet of M_r 16,000 was generated from both Fr2 and the M_r 42,000 CNBr peptide (Fig. 7*B*, lanes *1* and *2*) and all fragments generated from the CNBr peptide were also found in the digest of Fr2. When the peptide maps were transferred to nitrocellulose and reacted with anti-(N-CAM) No. 2, both Fr2 and the CNBr sialopeptide yielded a major reactive band of M_r 16,000 (Fig. 7*C*), which may correspond to one or both of the components of the doublet. Furthermore, in a separate experiment, cleavage of Fr2 resulted in several anti-(N-CAM) No. 2 reactive partial cleavage products, one of which migrated with the uncleaved CNBr fragment, suggesting that the V-8 cleavage of Fr2 generates a peptide similar in size to the CNBr sialopeptide. It is important to note in this V-8 peptide analysis that Fr2 was originally generated by V-8 protease cleavage of N-CAM from membranes, and that it contained the amino terminus of intact N-CAM. Thus, fragments produced from Fr2 that do not appear in Fr1 should be derived from the carboxyl terminal region of Fr2 (see Fig. 1). The combined results indicate that the sialic acid-rich CNBr fragment is contained almost solely within Fr2, and that it is closely aligned with the difference peptide between Fr1 and Fr2. A more precise alignment must await extensive amino acid sequence determination.

Number of Oligosaccharide Attachment Sites in the CNBr Sialopeptide

After treatment with neuraminidase to reduce its polydispersity, the CNBr sialopeptide was separated from the enzyme by gel filtraton. Equal aliquots of this fragment were then exposed to various concentrations of Endo F, and the products were assessed by the immunoblot technique with anti-(N-CAM) No. 2 in order to differentiate the product from components of the enzyme, all of which have similar molecular weights. As previously found for other glycoproteins containing N-glycosidic linkages (20, 21), a spectrum of intermediate-sized species resulted from the sequential removal of the sugar chains; the highest and lowest are assumed to be, respectively, the undigested molecule and the completely digested peptide free of N-linked sugars. The total number of species generated should therefore be one greater than the number of N-linked carbohydrate attachment sites. The CNBr sialopeptide yielded a total of four components (Fig. 8*A*), suggesting that within this region there are three asparagine-linked sugar chains. Sialic acid may be a constituent of one, two, or all three of the chains.

A similar analysis was performed on adult material to determine whether the adult molecule contained the same number of attachment sites, i.e., whether E–A conversion left these sites intact. After treatment with neuraminidase and Endo F, as described above for the purified embryonic material, analysis by immunoblots also gave four components (Fig. 8*B*) of sizes similar to those obtained from embryonic material, indicating that adult N-CAM also contains three carbohydrate attachment sites within the comparable fragment.

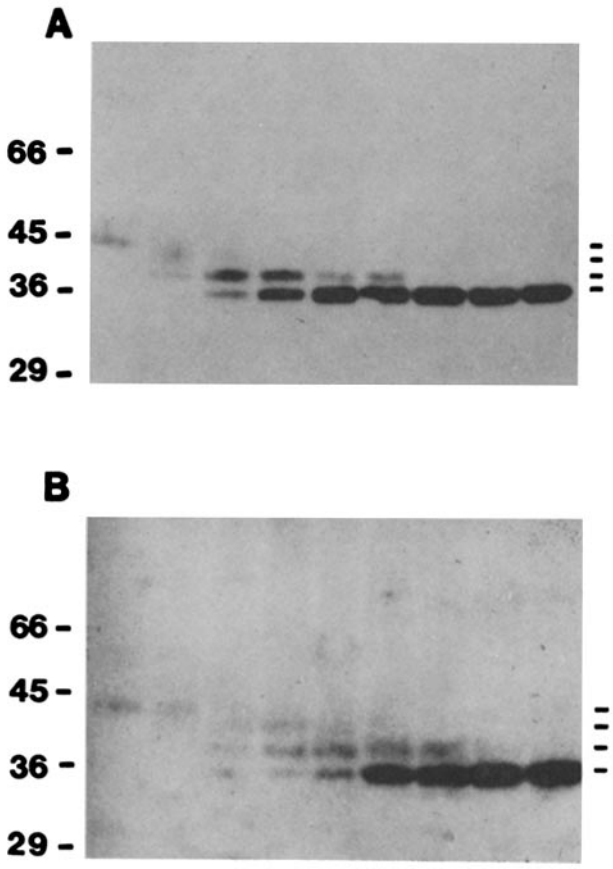

FIGURE 8 Monoclonal anti-(N-CAM) No. 2 immunoblot of partial endoglycosidase F digests of embryonic and adult CNBr sialopeptides. Equal aliquots of the neuraminidase-treated CNBr sialopeptides from embryonic (*A*) and adult (*B*) N-CAM were subjected to treatment with endo F and separated by SDS PAGE on 10% gels. The final lane contains the highest enzyme concentration (as described in Material and Methods) with serial twofold dilutions in lanes to the left. The first lane in each panel represents the control incubation, lacking enzyme. Each lane contains an equivalent amount of substrate; antibody reactivity appears to increase with sugar removal, resulting in the increasing exposure in the right lanes.

DISCUSSION

Structural analysis of N-CAM provides a unique opportunity to define mechanisms of cell surface modulation (1, 2) and to study the function of covalently linked carbohydrate in a glycoprotein that itself has a well-defined function at the cell surface. The unusual sialic acid of N-CAM has a marked effect on N-CAM binding (12) and appears to be the feature of the molecule that is altered in E–A conversion (1, 2, 9, 10). The present results are summarized and incorporated into the proposed model for N-CAM, as shown in Fig. 9. Previous studies (11) localized the sialic acid of embryonic N-CAM to a middle region of the linear sequence represented by the difference between Fr1, which is spontaneously released by

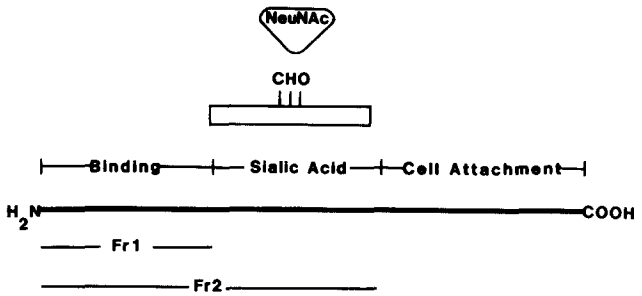

FIGURE 9 Localization of the CNBr sialopeptide within the linear model of N-CAM. The CNBr sialopeptide (block at top) from both embryonic and adult forms can be localized to the central region in a linear model of N-CAM. This fragment contains the majority of the sialic acid in N-CAM; the fragment isolated from embryonic N-CAM retains the 15G8 antigenic determinant and "Stains-all" reactivity, both of which require the polymeric sialic acid component, *N*-acetyl-neuraminic acid (*NeuNAc*). The region contains three sites of attachment for N-linked carbohydrate (*CHO*), at least two of which probably terminate in polymeric sialic acid. The peptide in this region is characterized by its reactivity with anti-(N-CAM) No. 2. Based on its molecular weight, sialic acid content, and monoclonal antibody reactivity, the sialopeptide appears to account for much of the difference peptide between Fr2 and Fr1 in embryonic and adult N-CAM, but its precise alignment remains to be determined.

incubation of solutions of N-CAM, and Fr2, which is released from membranes with V-8 protease. The major CNBr sialopeptide identified in the present studies corresponded to a large segment of this middle region.

The embryonic sialopeptide appeared as a polydisperse material ranging from M_r 42,000 to M_r 60,000 on SDS PAGE and was converted to a component of M_r 42,000 upon treatment with neuraminidase. Quantitative comparisons of sialic acid and amino acid content of this fragment with those of mock CNBr-treated N-CAM confirmed that it contained the majority of N-CAM sialic acid. CNBr fragmentation of Fr2 yielded a component that co-migrated with the purified N-CAM CNBr sialopeptide and also reacted with anti-(N-CAM) No. 2. In addition, the CNBr sialopeptide yielded a V-8 protease peptide map which overlapped the map of Fr2. These results indicated that the CNBr sialopeptide is almost completely contained within Fr2, and that it has little or no overlap with Fr1.

The CNBr sialopeptide reacted with two monoclonal antibodies specific for determinants on distinctive regions of N-CAM. Monoclonal antibody 15G8 characteristically reacts only with embryonic N-CAM, and the reactive antigenic determinant appears to depend upon the presence of polymeric sialic acid. In contrast, anti-(N-CAM) No. 2 antibodies recognized a peptide determinant in the region where the sugar moieties are attached. This antibody also detects both the M_r 170,000 and 140,000 polypeptides of N-CAM (26), suggesting that they are similar in this portion of their structure. Earlier studies (11) indicated that the two polypeptides had the same amino terminal sequence and gave similar peptide maps, but revealed no details about their possible similarities in the carbohydrate attachment region.

Characterization of the CNBr sialopeptide with Endo F indicated that there are three potential attachment sites for sialic acid in N-CAM. Attempts to perform such analyses on intact N-CAM or Fr2 were inconclusive because the decrease in molecular weight due to removal of the carbohydrate by Endo F from such a large molecule was too small to resolve bands of intermediate molecular weight; furthermore, there was ambiguity due to the existence of M_r 140,000 and 170,000 polypeptides of N-CAM (8, 11). It is important to note that, to detect the sequential digestion products of the CNBr sialopeptide, it was first necessary to remove the sialic acid from the fragment; this analysis, therefore, indicates the maximum number of chains that could terminate in sialic acid. Whether one, two, or all three sites contain sialic acid will require isolation of smaller glycopeptides and extensive carbohydrate analysis.

The CNBr sialopeptide is to date the smallest fragment isolated that contains the bulk of the sialic acid. Preliminary studies of proteolytic digests (e.g., trypsin) yield material that stains with "Stains-all" and appears on both gel filtration and SDS PAGE as a broad zone of M_r >20,000. This material separates into over overlapping zones on PAGE but no peptides are detectable with Coomassie Blue when the sialic acid is removed. Endoglycosidase F treatment of intact N-CAM releases similar high molecular weight material that stains blue with "Stains-all" and migrates on SDS PAGE in two similar zones. These results suggest that major sialic acid components may be attached to at least two of the three sites.

In view of the physiological and functional significance of E–A conversion, it was particularly important to determine whether a comparable CNBr fragment could be obtained from the A form of N-CAM and whether it had the same number of N-linked oligosaccharides. A CNBr sialopeptide from adult N-CAM was identified and shown to be analogous to the embryonic sialopeptide in size, and in its reactivity with anti-(N-CAM) No. 2. Most significantly, partial cleavage with Endo F followed by analysis on immunoblots indicated that the adult sialopeptide also contained three attachment sites for N-linked carbohydrate. This finding suggests that the adult and embryonic peptides differ only in their content and arrangement of terminal sialic acids, not in the gross location or number of N-linked oligosaccharide chains on the polypeptide. Although the precise mechanism of E–A conversion is unknown, a reasonable hypothesis is that it involves alterations in enzymes acting on N-CAM, which control the addition or removal of sialic acid (1, 2, 9, 10). The comparison of E and A forms in terms of their CNBr sialopeptides and carbohydrate attachment sites carried out here confirms that alterations in the sialic acid rather than in the sites themselves are involved in E–A conversion, and clearly focuses attention upon sialic acid-specific enzymes. Nonetheless, it should be pointed out that the presence of small amounts of sialic acid in short attachment sites other than the sites susceptible to the enzymes used in the present study cannot be excluded. Exploration of this possibility will have to await detailed studies of amino acid sequences.

It has been proposed (1, 2) that alterations in the sialic acid-rich domain modulate the binding of N-CAM either by a charge perturbation or by steric effects. The suggestion that polysialic acid occurs at possibly two or three sites near the binding domain increases the opportunities for different amounts of sialic acid to affect the binding efficacy, especially if all of the sialic acid-containing intermediates can differentially affect N-CAM binding. This model would provide a basis for diversity in binding behavior and could explain such observations as the differences in the conversion rates among different brain regions (10). Inasmuch as the presence or absence of a particular carbohydrate moiety can also affect protein structure, this charge perturbation model might also

account for the differential reactivity with monoclonal antibodies seen in different mouse brain areas (9, 10). Multiple attachment sites provide an additional means by which local cell surface modulation (1, 2) of N-CAM might perturb its function resulting in differential adhesion. Furthermore, the major carbohydrate on a given N-CAM molecule could affect its lateral interaction with another N-CAM or with other proteins within the plane of the membrane. Any or all of these interactions affect local cell surface modulation either by changing local concentrations of N-CAM (which would significantly affect binding efficacy [12]), or by changing the local environment of cell surface N-CAM as it relates to other protein or lipid domains. A detailed analysis of glycopeptides in conjunction with a study of conformation, electron microscopic appearance (27), and equilibrium binding behavior should shed light on these important questions relating carbohydrate structure to protein function.

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