A Heparin-binding Domain from N-CAM Is Involved in Neural Cell–Substratum Adhesion

Gregory J. Cole and Luis Glaser

Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. Cell–substratum adhesion in the embryonic chicken nervous system has been shown to be mediated in part by a 170,000-mol-wt polypeptide that is a component of adherons. Attachment of retinal cells to the 170,000-mol-wt protein is inhibited by the C_1H_3 monoclonal antibody and by heparan sulfate (Cole, G. J., D. Schubert, and L. Glaser, 1985, *J. Cell Biol.*, 100:1192–1199). In the present study we have demonstrated that the 170,000-mol-wt C_1H_3 polypeptide is immunologically identical to the neural cell adhesion molecule N-CAM, and that the 170,000-mol-wt component of N-CAM is preferentially secreted by cells as a component of adherons. We have identified a monoclonal antibody, designated B_1A_3, that inhibits heparin binding to N-CAM and cell-to-substratum adhesion. A 25,000-mol-wt heparin (heparan sulfate)–binding domain of N-CAM has been identified by limited proteolysis, and this fragment promotes cell attachment when bound to glass surfaces. The fragment also partially inhibits cell binding to adherons when bound to retinal cells, and the B_1A_3 monoclonal antibody inhibits retinal cell attachment to substrata composed of intact N-CAM or the heparin-binding domain. These data are the first evidence that N-CAM is a multifunctional protein that contains both cell- and heparin (heparan sulfate)–binding domains.

ESTABLISHMENT of neural connections during embryogenesis depends upon a variety of cell recognition processes, which include cell–cell and cell–matrix interactions. Cell–cell adhesion has been particularly well studied in the developing nervous system, and several macromolecules that play an integral role in these interactions have been identified (10, 12–14, 17, 23, 33). The best characterized cell adhesion molecule is N-CAM (7, 27), which mediates cell adhesion processes via a homophilic binding mechanism (28). Although these studies have provided insight into the mechanisms governing interactions between neural cells, a paucity of information exists regarding how neural cells interact with their substratum during neural development.

Cell–substratum adhesion involving fibroblast-like cells has been well characterized, with fibronectin being the molecular component primarily responsible for this process (15, 37). The interaction of fibroblasts with fibronectin in the extracellular matrix has been shown to depend upon heparan sulfate proteoglycan (25), and the binding of heparan sulfate to fibronectin was proposed to induce a conformational change in the protein (16). It has recently been demonstrated by circular dichroism that heparin does induce a conformational change in fibronectin (21). After this conformational change fibronectin interacts with the cell surface with a higher affinity (16), as measured by biological cell-binding activity. These data, therefore, suggest that the interaction between heparan sulfate and fibronectin may modulate the biological activity of fibronectin. A role for fibronectin in neuronal cell interactions has also been suggested, as neural crest cell migration is a fibronectin-dependent process (2). In addition, neurite outgrowth in vitro has been shown to involve the heparin-binding domain of fibronectin (24). However, the precise role of fibronectin, or other extracellular matrix molecules in neural cell–substratum interactions, still remains unclear since these matrix molecules are not widely distributed in the developing central nervous system.

Recent studies by Schubert and his co-workers (29, 31) demonstrated that embryonic chick neural retina cells release macromolecular components, termed adherons, into their culture medium. Adherons promote cell–substratum[1] attachment when adsorbed onto tissue culture plastic (31), and this cell attachment can be inhibited by heparin and heparan sulfate (29, 31). The latter is presumed to be the physiologically important ligand since heparan sulfate occurs on the surface of retinal cells (29), and an antiserum produced against a cell surface heparan sulfate proteoglycan has been shown to inhibit cell–adheron binding (29). However, heparin can be used as a functional analogue of heparan sulfate since it is closely related in structure to heparan sulfate.

We have previously reported the isolation of a monoclonal antibody (MAb),[2] designated C_1H_3, which recognizes a 170,000-mol-wt polypeptide that is a component of adherons

[1] In these studies, cell–substratum adhesion will refer to cell attachment to an appropriate substratum. The substrata we will refer to are adherons, which are produced by retinal cells in vitro, or identified proteins which have been covalently coupled to a glass surface.

[2] *Abbreviations used in this paper*: MAb, monoclonal antibody. N-CAM, neural cell adhesion molecule.

(3, 4). This MAb inhibits cell–substratum attachment (4, 5), and the purified antigen, when linked to an inert surface such as glass, promotes cell attachment (5). We demonstrated that heparan sulfate inhibits the binding of retinal cells to 170,000-mol-wt protein and that heparan sulfate binds to the 170,000-mol-wt protein and induces a conformational change in the molecule (6). Recent studies in several laboratories have suggested that the interaction of neural cells with the extracellular matrix glycoprotein laminin is required for neurite outgrowth (19, 34), and that the heparin-binding domain of laminin is capable of mediating this effect (8). Together, these observations raise the possibility that heparan sulfate–protein interactions are important for cell–substratum adhesion in the developing nervous system. We have therefore used MAbs to identify the molecular domain in the 170,000-mol-wt C_1H_3 polypeptide which is responsible for heparan sulfate binding. In the present study we show that after cleavage of the 170,000-mol-wt protein with subtilisin protease, a 25,000-mol-wt fragment is retained on a heparin–agarose column. This fragment, when covalently coupled to glass, promotes the attachment of retinal cells. The fragment also acts as an inhibitor of cell attachment to adherons when bound to retinal cells before the adhesion assay.

We had previously reported that the 170,000-mol-wt C_1H_3 protein was unrelated to N-CAM since N-CAM was not detected in adherons (5). This conclusion was based on experiments in which an anti-N-CAM MAb (224-1A6-A1) did not bind to intact adherons or immunoprecipitate labeled protein from solubilized adherons (5). Two recent observations prompted our reexamination of this problem. J. Covault and J. Sanes (personal communication) demonstrated by immunoblotting that the C_1H_3 MAb binds immunopurified N-CAM. In addition, a recent description of changes in N-CAM during retinal development (9) parallel our previous description of the C_1H_3 antigens; i.e., the low molecular weight form (140,000-mol-wt) appears in development before the high molecular weight form (170,000-mol-wt). Previous studies regarding N-CAM had implied that the high molecular weight form appeared first in development. Our present studies indicate that the anti-N-CAM MAb recognizes significantly less protein in conditioned medium from retinal cultures than does the C_1H_3 MAb, and this reduced sensitivity may account for our previous failure to detect immunoprecipitable protein in adherons with this anti-N-CAM MAb. Nevertheless, our observations reported below indicate that the C_1H_3 epitope is present on the majority of retinal N-CAM molecules as defined by an anti-N-CAM antibody, and also suggest that heparan sulfate–N-CAM interactions are likely to be important during neuronal development.

Materials and Methods

Production of Antibodies

The preparation and characterization of the C_1H_3 MAb has been described previously (3) and was obtained by immunizing rats with embryonic day 9 retinal cells. The B_1A_3 MAb was produced by immunizing BALB/c mice with immunopurified 170,000-mol-wt protein, and screening hybridomas using a dot-blot assay (5). Anti-N-CAM MAb was a generous gift of Dr. David Gottlieb (Washington University, St. Louis) (36) and was obtained by immunizing BALB/c mice with embryonic day 9 retinal cells. Anti-heparan sulfate proteoglycan antiserum was a generous gift of Dr. David Schubert (The Salk Institute, La Jolla, CA).

Preparation of Adherons

We prepared adherons from retinal cell cultures as described previously (5). Embryonic day 11 chick retinas were mechanically dissociated with a fire-polished pipette and incubated for 18 h at 37°C in serum-free Dulbecco's modified Eagle's medium containing transferrin, insulin, progesterone, and putrescine (1). Conditioned medium was then processed as described (5) to yield adherons. We have also demonstrated that adherons are produced in serum-containing medium, and thus may represent normally secreted retinal cell components.

Cell–Substratum Adhesion Assays

To assay adhesion of neural retina cells, day 11 retinas were mechanically dissociated and metabolically labeled for 2 h with 5 μCi/ml of [^{35}S]methionine (translation grade, New England Nuclear, Boston, MA). Labeled cells were then washed twice with Earle's balanced salt solution (EBSS) containing 0.2% bovine serum albumin (BSA), and 0.2-ml aliquots were incubated with adheron-coated dishes or 0.1-ml aliquots were incubated with glass scintillation vials that had been coated with 20 μg/ml of 170,000-mol-wt protein or 3 μg/ml of the heparin-binding domain. Glass vials were coated with protein according to published procedures (5). Assays using glass vials contained 125 μg/ml of chondroitin sulfate in the medium, which was used to decrease nonspecific cell attachment (6). After a 1 h incubation of cells with dishes, non-attached cells were removed by swirling the dishes and vacuum aspiration. Bound cells were solubilized in Triton X-100, and isotope content was measured.

The effect of antibodies on cell–substratum adhesion was examined as previously described (5, 6). The effect of the heparin-binding domain of the 170,000-mol-wt protein on cell attachment was determined by incubating labeled retinal cells for 1 h at 4°C with 10 μg/ml of the fragment. The cells were then washed twice by centrifugation and added to vials coated with the 170,000-mol-wt protein or to adheron-coated dishes.

Preparation of Heparin-binding Domain

A heparin-binding domain of the 170,000-mol-wt C_1H_3 polypeptide was identified by digesting 300 μg of partially purified protein (isolated as described in references 5 and 6) with a 1:50 enzyme-to-substrate ratio of subtilisin protease (Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C. The reaction was terminated by the addition of phenylmethylsulfonyl fluoride (to 0.3 mM) and 1,000 U of aprotinin. The sample was then incubated with heparin–agarose for 2 h at room temperature, in 15 mM NaCl, 10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7.4. The heparin–agarose was then packed into a column and eluted with 3 column volumes of incubation buffer. Bound protein was eluted with 3 column volumes of 1 M NaCl. The fractions were then desalted and concentrated in Amicon microconcentrators (Amicon Corp., Danvers, MA), and analyzed by polyacrylamide gel electrophoresis and immunoblotting.

Immunoprecipitation and Immunoblotting Analysis of Antigens

The analysis of immunopurified N-CAM, 170,000-mol-wt C_1H_3 protein, or heparin-binding fractions by immunoblotting was as described previously (3, 6). Briefly, proteins were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with MAbs. Antibody binding was visualized by reaction with Vectastain ABC reagents (Vector Laboratories, Inc., Burlingame, CA).

Immunoprecipitation of metabolically labeled retinal cell proteins was according to published methods (3). Briefly, day 7 retinas were mechanically dissociated and labeled with 100 μCi/ml of [^{35}S]methionine for 1–2 h. Cells were then homogenized in RIPA buffer (3) and incubated with MAbs and *S. aureus* cells coated with goat anti-rat IgG (Cappel Laboratories, Cochranville, PA). Conditioned medium was also immunoprecipitated with MAbs, and this involved adding an equal volume of RIPA buffer, adjusting the medium to 0.2% SDS, and heating to 60°C for 10 min. This protocol results in the solubilization of adheron complexes in the medium, and thus cleaner immunoprecipitations of the 170,000-mol-wt or N-CAM proteins. Immunoprecipitated proteins were analyzed by electrophoresis on 6% polyacrylamide gels, and radioactive proteins were visualized by fluorography.

Results

Comparison of N-CAM and the 170,000-mol-wt C_1H_3 Protein

Cell–cell adhesion in the developing nervous system has been

intensively studied, with N-CAM representing the best characterized cell adhesion molecule (7, 27). Like N-CAM, the 170,000-mol-wt C_1H_3 protein mediates cell interactions via a homophilic binding mechanism (5, 6, 28), although it is apparent that the binding of heparan sulfate to the 170,000-mol-wt protein is also necessary for its function (6). The C_1H_3 polypeptide also is similar in molecular weight to N-CAM, exhibits a developmental regulation pattern identical to that of N-CAM in chick retina, and has been shown to bind to immunopurified N-CAM (Covault, J., and J. Sanes, personal communication). It therefore appeared possible to us that the C_1H_3 protein might constitute a subset of N-CAM molecules (35), and this possibility was investigated further using immunoprecipitation and immunoblotting techniques.

An initial approach used to demonstrate immunological identity between N-CAM and the C_1H_3 antigen was immunoblotting of the immunopurified proteins. As shown in Fig. 1, the C_1H_3 MAb binds to both immunopurified 170,000-mol-wt protein and N-CAM, and an MAb against N-CAM binds the 170,000-mol-wt protein. Although the staining patterns are similar, they are not identical. It therefore appears that the two polypeptides are immunologically similar, although it is possible that the 170,000-mol-wt protein represents a unique subpopulation of N-CAM molecules (35). To test this possibility, embryonic day 7 retinal cells were pulse-labeled for 2 h with [^{35}S]methionine and immunoprecipitated with the C_1H_3 and anti-N-CAM MAbs. In Fig. 2 it can be seen that the C_1H_3 MAb immunoprecipitates both 170,000- and 140,000-mol-wt proteins from a cell extract, but only the 170,000-mol-wt protein is immunoprecipitated from conditioned medium. The anti-N-CAM MAb also reacts with both molecular weight polypeptides in cell extracts and immunoprecipitates a 170,000-mol-wt protein from conditioned medium. Our data would appear to suggest that the two proteins may be immunologically identical, since if labeled retinal cell

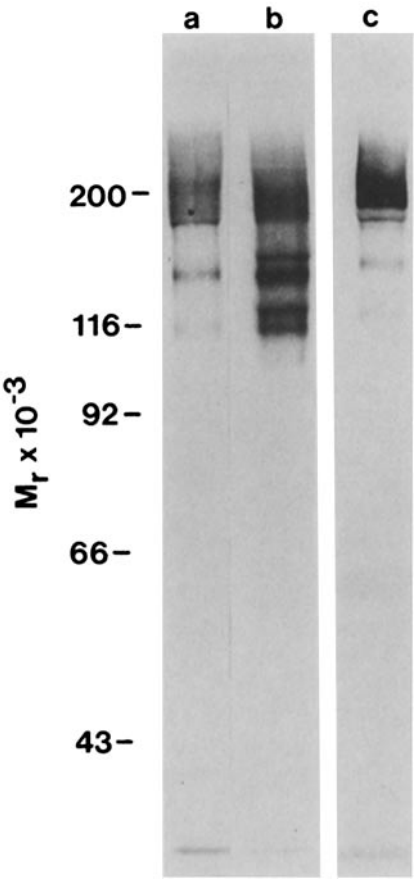

Figure 1. Immunoblotting of immunopurified 170,000-mol-wt C_1H_3 protein and N-CAM. 5-μg aliquots of N-CAM immunopurified with the C_1H_3 MAb (*a* and *c*) or anti-N-CAM MAb (*b*) were separated on a 7% polyacrylamide gel, transferred to nitrocellulose, and reacted with C_1H_3 MAb (*a* and *b*) or anti-N-CAM MAb (*c*). Antibody binding was visualized as described under Materials and Methods. The N-CAM protein blotted with anti-N-CAM MAb in lane *c* was electrophoresed on a different gel than the protein in lanes *a* and *b*.

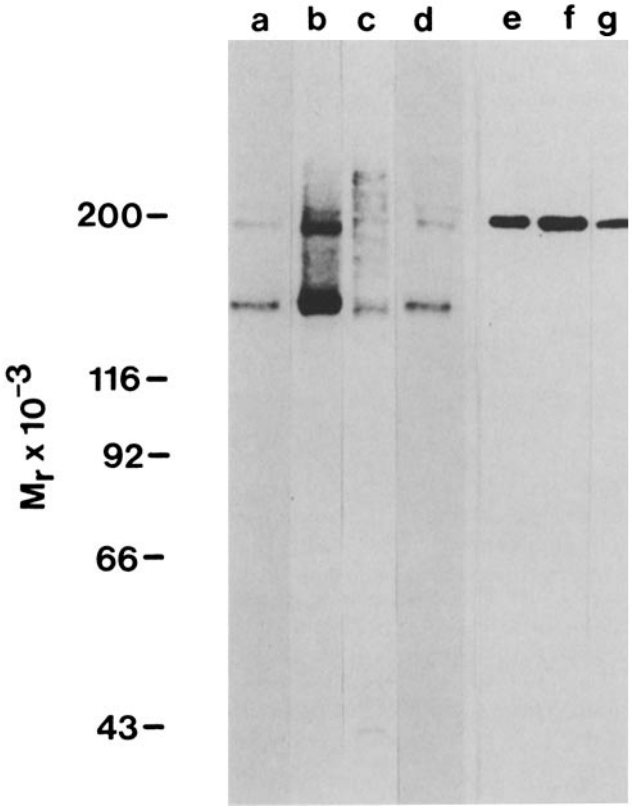

Figure 2. Immunoprecipitation of metabolically labeled retinal cell proteins. Embryonic day 7 retinal cells were labeled with [^{35}S]methionine in Spinner culture for 2 h as described under Materials and Methods. Labeled cells were then centrifuged and washed twice. Cells were then homogenized in RIPA buffer and incubated overnight at 4°C with monoclonal antibodies. Immunoprecipitated proteins were isolated by incubating with *Staphylococcus aureus* cells coated with goat anti-rat IgG. Labeled proteins were also immunoprecipitated from conditioned medium that was diluted 1:1 with RIPA buffer, adjusted to 0.2% SDS, and heated to 60°C for 10 min to solubilize adheron complexes. Immunoprecipitated proteins were analyzed by electrophoresis on 6% polyacrylamide gels and were visualized by fluorography. Lanes: *a*, cell extract immunoprecipitated with C_1H_3 MAb; *b*, cell extract immunoprecipitated with anti-N-CAM MAb; *c*, incubation with anti-N-CAM after immunoprecipitation with C_1H_3 MAb; *d*, immunoprecipitation of cell extract with B_1A_3 MAb. Lanes *e–g* show immunoprecipitation of medium with B_1A_3 (*e*), C_1H_3 (*f*), and anti-N-CAM MAbs (*g*). It should be noted that equivalent amounts of radioactivity were not loaded in each lane, and the difference in reactivity between the MAbs is slight. The starting material for lane *c* was similar to lane *b*, and thus the majority of radioactivity is removed by the C_1H_3 MAb before immunoprecipitation with anti-N-CAM MAb.

proteins are immunoprecipitated with C_1H_3 MAb, followed by immunoprecipitation with anti-N-CAM MAb, most of the N-CAM molecules are removed by the C_1H_3 MAb (Fig. 2).

We have previously shown that the 170,000-mol-wt C_1H_3 protein binds heparin, and thus this is the first evidence that N-CAM is a multifunctional protein, containing both cell- and heparin-binding domains. These data also show that early embryonic chick retinal cells synthesize the 170,000-mol-wt form of N-CAM, although a recent study by Friedlander et al. (9) demonstrated that by immunoblotting only the 140,000-mol-wt form of the protein is detected in day 7 retinal cells. Friedlander et al. (9) also demonstrated that early retina cultures synthesize primarily the 140,000-mol-wt N-CAM molecules, although they used long-term labeling protocols in the studies. We have used short pulse labeling in our studies and also have shown that most of the 170,000-mol-wt N-CAM is secreted by the cells. These data are thus in agreement with our previous findings that the synthesis of the 170,000-mol-wt protein can be induced prematurely in vitro (3). In addition, these studies indicate that the 170,000-mol-wt N-CAM component is preferentially released into the culture medium, which may suggest that this N-CAM component is associated with the extracellular matrix.

Identification of the Heparin-binding Domain of N-CAM

As stated above, we have previously shown that the 170,000-mol-wt C_1H_3 protein (designated hereafter as N-CAM) specifically binds [^{3}H]heparin, indicating that it contains a heparin-binding domain (6). Heparan sulfate also inhibits the attachment of retinal cells to glass surfaces coated with N-CAM, and an antiserum directed against a retinal cell surface heparan sulfate proteoglycan has a similar effect (6). These data therefore suggest that the heparin-binding domain of the N-CAM protein is functionally important for retinal cell attachment to the extracellular matrix. In the present study we were interested in identifying the molecular domain in the N-CAM protein responsible for heparin binding, and determining whether MAbs that recognize this domain could inhibit cell–substratum adhesion. Although heparan sulfate can be considered the physiologically important ligand, heparin is closely related in structure to heparan sulfate and can be used as a functional analogue of heparan sulfate. We have therefore used heparin in these studies, although in the nervous system heparan sulfate is the relevant molecule.

N-CAM protein was partially purified from detergent extracts of embryonic day 14 chick brain using C_1H_3 MAb coupled to Sepharose 4B (5), and in the present experiments consists of the 170,000-mol-wt protein and several lower molecular weight proteins (Fig. 3*a*). However, only the 170,000-mol-wt protein reacts with any of our MAbs that bind to the molecule on an immunoblot (data not shown). This N-CAM protein is retained on a heparin–agarose column, although only 20% of the protein binds to the column (data not shown). These data confirm our previous observations that the molecule possesses a heparin-binding domain. To identify the molecular domain responsible for heparin-binding, proteolytic digests of the N-CAM protein were incubated with heparin–agarose. We have previously demonstrated that digestion of the protein with subtilisin protease yielded a variety of fragments that reacted with the C_1H_3 MAb (6), and we thus chose to use subtilisin protease in these

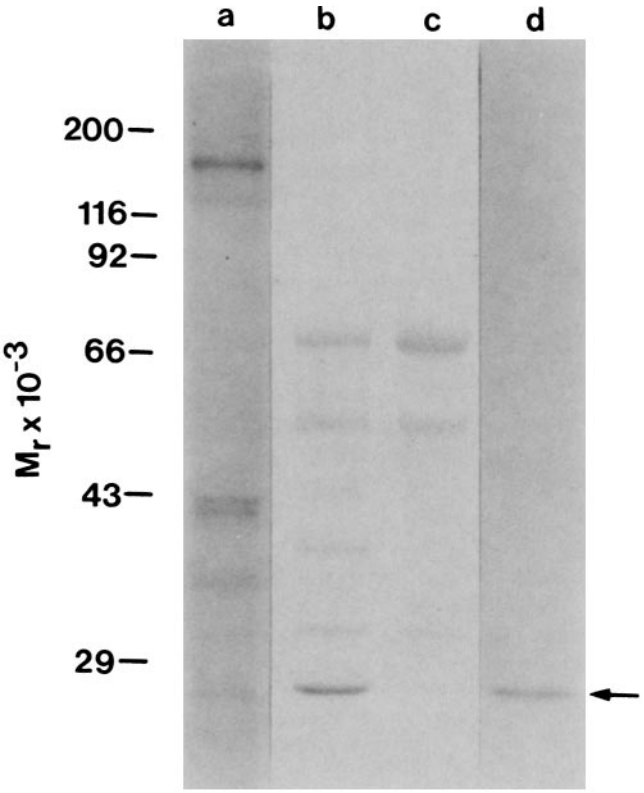

Figure 3. Identification of heparin-binding domain from N-CAM. 300 μg of immunopurified N-CAM was digested with subtilisin protease (1:50 enzyme to substrate) as described under Materials and Methods and incubated with 0.5 ml of heparin–agarose. Unbound and bound fractions were collected as described, and aliquots were electrophoresed on a 9% polyacrylamide gel, followed by staining with Coomassie Blue. (*a*) 20 μg of undigested N-CAM; (*b*) digested N-CAM before heparin–agarose chromatography; (*c*) unbound fraction; and (*d*) bound fraction. Only a 25,000-mol-wt fragment (denoted by arrow) is retained on heparin–agarose.

experiments. When N-CAM protein is incubated with subtilisin protease for 45 min, several bands ranging in molecular weight from 70,000 to 25,000 are detected by Coomassie Blue staining (Fig. 3*b*). After heparin–agarose chromatography only the 25,000-mol-wt fragment is retained on the column (Fig. 3*d*). This fragment therefore appears to represent the heparin-binding domain of N-CAM. It should also be noted that almost all of the 25,000-mol-wt fragment obtained binds to heparin–agarose, in contrast to our experiment with intact N-CAM protein, which binds heparin–agarose poorly. These results may be explained by the possibility that the heparin-binding domain is not exposed on all N-CAM molecules in solution, particularly since N-CAM is known to aggregate in solution. This may account for only 20% of the intact protein binding heparin–agarose, whereas most of the isolated domain is bound by the heparin–agarose column.

To confirm that the 25,000-mol-wt heparin-binding fragment was derived from the N-CAM protein, and to determine the precise role of this structural domain in the function of the protein, we analyzed this fragment using immunoblotting. As shown in Fig. 4*b*, the C_1H_3 MAb does not react with this fragment, which suggests that the C_1H_3 MAb inhibits cell–substratum adhesion by interacting with another functional domain. However, the C_1H_3 MAb also does not bind to any

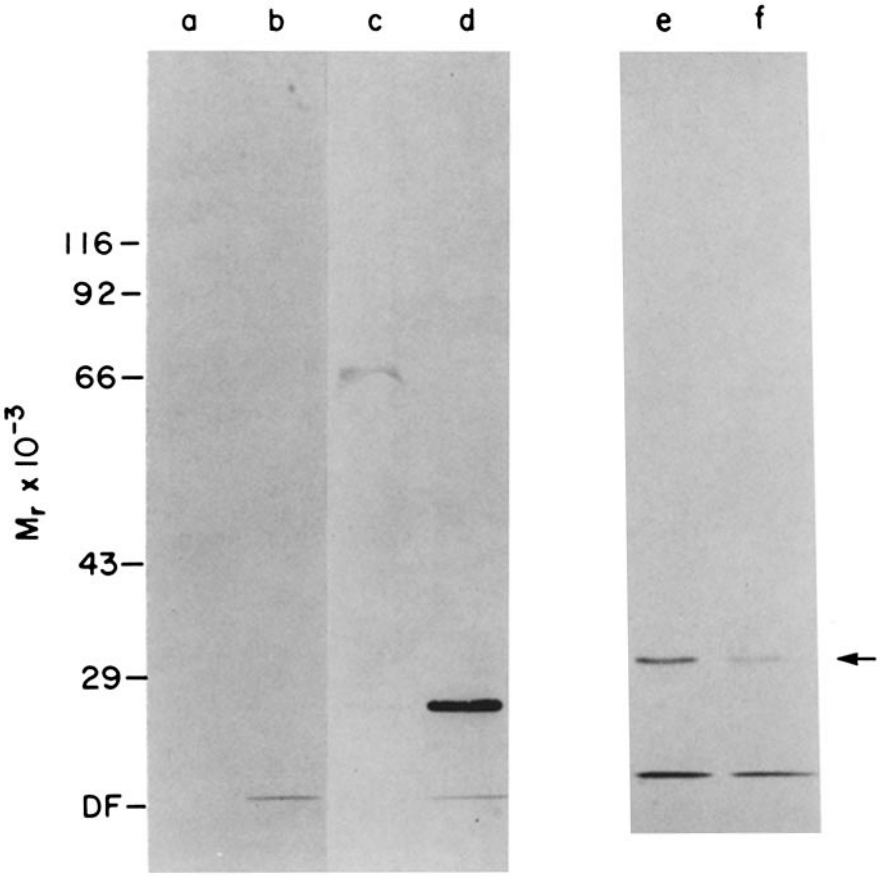

Figure 4. Immunoblotting of heparin-binding domain with anti-N-CAM MAbs. Aliquots of unbound and bound fractions obtained by heparin–agarose chromatography, as described in Fig. 3, were separated on a 9% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose blot was then incubated with C_1H_3 MAb (*a* and *b*) or B_1A_3 MAb (*c* and *d*) as described under Materials and Methods. Proteolytic fragments not retained on heparin–agarose are shown in lanes *a* and *c*; the 25,000-mol-wt fragment is present in lanes *b* and *d*. Note that only the B_1A_3 MAb reacts with the heparin-binding domain. Lanes *e* and *f* show the results of immunoblotting with B_1A_3 MAb of the heparin-binding domain isolated from N-CAM purified with the C_1H_3 MAb (50 μg starting material, lane *e*) or an anti-N-CAM MAb (15 μg starting material, lane *f*). The arrow denotes the 25,000-mol-wt fragment. These results confirm that N-CAM and the 170,000-mol-wt C_1H_3 protein are immunologically related. Molecular weight markers are indicated; *DF*, dye front of the gel.

components that are not retained by the heparin–agarose column (Fig. 4*a*). These data therefore imply that the C_1H_3 epitope does not survive the conditions used to generate the proteolytic fragments. This proposal is supported by a time-course digestion, which shows that the C_1H_3 MAb reacts with several fragments after a 10-min digestion, but under the conditions used for heparin–agarose chromatography the MAb does not recognize any proteolytic fragments (Fig. 5, *a–c*).

We have generated additional MAbs that recognize N-CAM, one of which, designated B_1A_3, reacts with an epitope distinct from the C_1H_3 antigenic determinant. As shown in Fig. 2, the B_1A_3 MAb immunoprecipitates proteins with molecular weights identical to that of N-CAM. As shown in Fig. 5, *d–f*, the B_1A_3 MAb reacts with a 65,000-mol-wt fragment that is obtained by proteolysis of N-CAM, and this fragment is converted to a 25,000-mol-wt fragment with prolonged proteolysis by subtilisin. This MAb is therefore a good candidate for reacting with the heparin-binding domain and, as demonstrated in Fig. 4*d*, the B_1A_3 MAb binds to the 25,000-mol-wt heparin-binding fragment. It can also be seen in Fig. 4, *e* and *f* that the B_1A_3 MAb reacts with a 25,000-mol-wt heparin-binding fragment obtained by digesting immunopurified N-CAM (obtained from an anti-N-CAM column as described in Fig. 1) with subtilisin protease. This experiment provides additional support that N-CAM and the C_1H_3 protein are immunologically identical. However, the yield of 25,000-mol-wt fragment from N-CAM appears to be lower than from protein immunopurified with the C_1H_3 MAb. This may therefore raise the possibility that only a subset of N-CAM contains a heparin-binding domain, with the anti-N-CAM MAb immunopurifying less of this subset than the C_1H_3 MAb.

Role of Heparin-binding Domain in Cell–Substratum Adhesion

To examine the role of the heparin-binding domain in cell–substratum adhesion, we were first interested in ascertaining whether the B_1A_3 MAb could inhibit heparin binding by the intact protein. This would therefore indicate that the MAb recognizes the region of the molecule necessary for heparin binding. To test this possibility, we used a [^{3}H]heparin-binding assay that was originally used to demonstrate that N-CAM possessed a heparin-binding domain (6). In initial studies, the B_1A_3 MAb preparation also contained a heparin-binding component, which probably resulted from other protein components in the ascites fluid. We therefore passed B_1A_3 ascites fluid over heparin–agarose, with the isolated MAb retaining activity (data not shown). When this MAb is incubated with N-CAM protein, and the N-CAM protein is then incubated with [^{3}H]heparin, binding of heparin to the protein is inhibited (Table I). We have previously shown that the C_1H_3 MAb does not inhibit heparin binding (6), which indicates that the B_1A_3 MAb specifically inhibits [^{3}H]heparin binding to N-CAM.

Since the B_1A_3 MAb inhibits heparin binding to N-CAM, this MAb can be used as a probe to determine the role of this structural domain in mediating cell–substratum adhesion. Several approaches were used to ascertain if the heparin-binding domain is required for cell–substratum adhesion. The

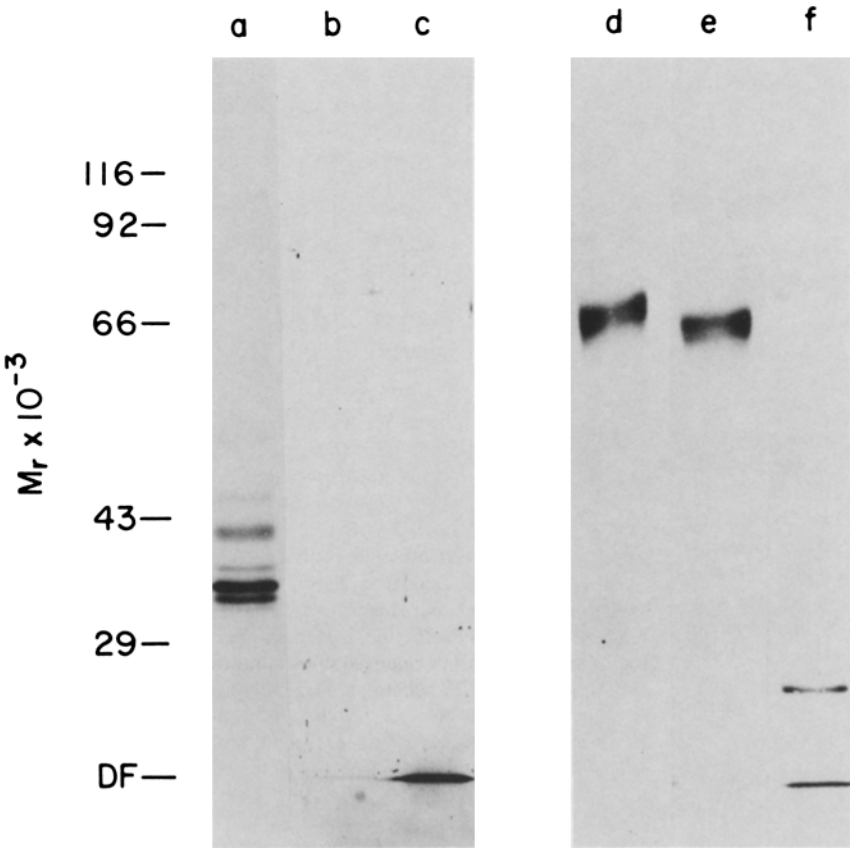

Figure 5. Kinetics of proteolytic digestion of N-CAM by subtilisin protease. 10-μg aliquots of immunopurified N-CAM were incubated 10 min (*a* and *d*) or 20 min (*b* and *e*) with 1:100 enzyme to substrate of protease, or 45 min with 1:50 enzyme to substrate of protease (*c* and *f*). The digested N-CAM protein was then electrophoresed on a 9% polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblotting with C_1H_3 MAb (*a–c*) or B_1A_3 MAb (*d–f*).

Table I. Inhibition of [^{3}H]Heparin Binding to N-CAM by the B_1A_3 Monoclonal Antibody

Treatment	Percentage of cpm bound
No protein	0.4 ± 0.0
B_1A_3 MAb	2.2 ± 0.3
N-CAM	11.6 ± 1.0
N-CAM + B_1A_3 MAb	5.7 ± 0.7

Mean of two experiments ± SD conducted in duplicate. Input radioactivity represented 20,000 cpm of [^{3}H]heparin excluded from a Sephadex G-100 column. 100 μg/ml of N-CAM protein was incubated 30 min with 200 μg/ml of B_1A_3 ascites fluid excluded from a heparin–agarose column. [^{3}H]Heparin was then added and incubated for 30 min, followed by binding to nitrocellulose (6). The samples were washed three times with PBS and counted.

first approach was to immobilize intact N-CAM protein or the heparin-binding domain on glass surfaces. The advantage of this assay system is that only one protein component can be analyzed, in contrast to adherons, which are complex in molecular composition (31). We have previously shown that retinal cells will attach to N-CAM protein covalently coupled to glass (5, 6). In the present experiment we were interested in determining if retinal cells would bind to the heparin-binding domain, which would imply that the interaction between cell surface heparan sulfate proteoglycan and N-CAM in the matrix can promote cell attachment. As shown in Fig. 6, retinal cells attach to N-CAM covalently coupled to the glass surface, and this attachment is inhibited when retinal cells are incubated with the 25,000-mol-wt fragment or C_1H_3 Fab fragments. It can also be seen that the B_1A_3 MAb does not inhibit cell binding when incubated with the substratum, and also does not prevent cell attachment when incubated with retinal cells before the adhesion assay. However, when B_1A_3 Fab fragments are present in the medium during the adhesion assay, cell attachment is inhibited. These data imply that the B_1A_3 MAb binds to the cell surface and substratum with low affinity, and must be present during the assay to competitively inhibit cell attachment. These data also confirm our earlier observations that the interaction between cell surface heparan sulfate proteoglycan and N-CAM in the matrix is required for neural cell–substratum adhesion.

When derivatized vials are coated with the 25,000-mol-wt fragment (in molar ratios comparable to the intact protein), cell attachment is also observed (Fig. 7). The percentage of cells that bind to the heparin-binding domain is similar to intact N-CAM, which implies that the 25,000-mol-wt fragment and N-CAM bind to the glass surface with equal efficiency. This cell binding is partially inhibited when B_1A_3 MAb is incubated with vials coated with the 25,000-mol-wt fragment, and addition of heparin or an antiserum raised against a retinal cell surface heparan sulfate proteoglycan (29) prevents cell–substratum adhesion to the 25,000-mol-wt fragment. The C_1H_3 MAb also does not inhibit cell attachment under these assay conditions when the MAb is incubated with retinal cells (data not shown), which suggests that the C_1H_3 MAb inhibits cell–substratum adhesion by binding to a second functional domain, presumed to be the cell-binding domain. These data thus suggest that the isolated 25,000-mol-wt fragment has sufficiently high affinity for cell surface heparan sulfate to yield stable cell-to-substratum adhesion. These data also imply that the B_1A_3 MAb binds with higher affinity to the heparin-binding domain than to the intact

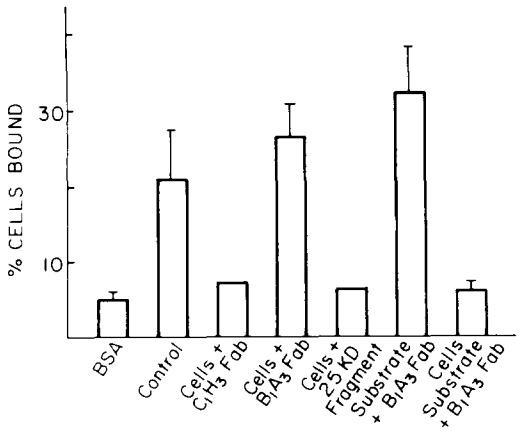

Figure 6. Role of heparin-binding domain in attachment of retinal cells to an N-CAM substratum. Glass scintillation vials were derivatized with 3-aminopropyltriethoxy silane (22, 23) and coated with 20 μg/ml of immunopurified N-CAM. Nonspecific cell attachment was determined using vials coated with albumin, and additional binding sites on N-CAM coated vials were blocked with Earle's balanced salt solution containing 0.2% albumin. Nonspecific cell binding was also reduced by including 125 μg/ml of chondroitin sulfate in the assay medium (6). Metabolically labeled day 12 retinal cells were then added to the vials for 1 h at 37°C. The effect of anti-N-CAM MAbs or the 25,000-mol-wt heparin-binding fragment on cell attachment was examined by incubating labeled retinal cells with 0.5 mg/ml of C_1H_3 or B_1A_3 Fab fragments or 10 μg/ml of the heparin-binding fragment for 1 h at 4°C. The cells were then washed twice and added to the coated vials. The effect of the B_1A_3 MAb on cell attachment was also assessed by incubating N-CAM–coated vials with 0.5 mg/ml of B_1A_3 Fab for 1 h at room temperature. The vials were then washed and used in the adhesion assay. Alternatively, N-CAM–coated vials were incubated with 0.3 mg/ml of B_1A_3 Fab fragments, and this antibody remained in the medium during the cell adhesion assay, which was conducted using retinal cells incubated with B_1A_3 Fab fragments as previously described. Cell attachment was quantitated by dissolving bound cells in Triton X-100 and measuring isotope content.

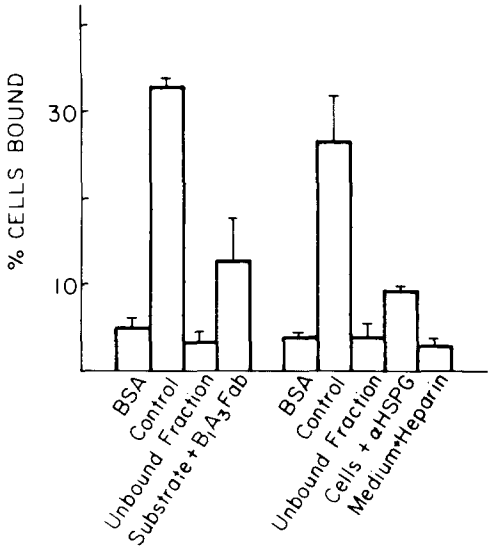

Figure 7. Retinal cell attachment to the purified heparin-binding domain of N-CAM. Glass scintillation vials were derivatized as described in Fig. 7 and coated with 3 μg of the 25,000-mol-wt heparin-binding fragment. Nonspecific cell attachment was blocked by incubating vials with Earle's balanced salt solution–0.2% albumin and by including chondroitin sulfate (125 μg/ml) in the medium. Cell attachment to the vials was measured using metabolically labeled day 12 retinal cells as described under Materials and Methods. The effect of anti–heparan sulfate proteoglycan antiserum (*αHSPG*; 29) on cell adhesion was examined by incubating retinal cells with a 1:100 dilution of the antiserum for 1 h at 4°C. The effect of the B_1A_3 MAb on cell attachment was examined by incubating the coated vials with 0.5 mg/ml of B_1A_3 Fab fragments for 1 h at room temperature. The vials were then washed twice and used in the adhesion assay. The effect of heparin on cell binding was assessed by including 50 μg/ml of heparin in the assay medium. Cell attachment was quantitated as described in Fig. 6. The data are shown as two different sets of experiments, conducted in duplicate at least three times. The unbound fraction is protein obtained by digesting N-CAM with subtilisin protease, and which is then not retained on a heparin–agarose column.

protein, since after incubation of this antibody with the substratum, followed by washing, the B_1A_3 MAb can still inhibit cell attachment (Fig. 7). This effect was not observed with substrata was comprised of intact N-CAM (Fig. 6). These data also indicate that multiple mechanisms are involved in the promotion of neural cell–substratum adhesion, with the multifunctional N-CAM protein playing an integral role in this process.

Since we have shown previously that retinal cells attach to adherons in vitro, we were interested in demonstrating that the heparin-binding domain of N-CAM is required for this process. To investigate this possibility, retinal cell adherons were adsorbed onto plastic petri dishes. As shown in Fig. 8, retinal cells attach to dishes coated with adheron protein, but not to uncoated plastic. Attachment of retinal cells to adheron-coated dishes is inhibited ~50–70% by the C_1H_3 MAb (3, 4), and likewise cell binding is inhibited 30–40% by the B_1A_3 MAb (Fig. 8). We have previously demonstrated that binding of heparan sulfate to N-CAM induces a conformational change in the protein (6), and have postulated that this conformational change is required for homophilic interactions involving N-CAM. We therefore postulate that the B_1A_3 antibody prevents cell-to-substratum attachment by preventing this conformational change. It can also be seen that the B_1A_3 MAb once again does not significantly inhibit cell attachment when bound to adherons, and then washed from the substratum, yet the 25,000-mol-wt heparin-binding fragment, when incubated with retinal cells, impairs cell attachment to adherons (Fig. 8). These data imply that the heparin-binding domain of matrix N-CAM is necessary for retinal cell interactions with the extracellular matrix. However, as shown previously, the B_1A_3 MAb must be present in the assay medium in order to inhibit cell attachment to intact N-CAM.

Discussion

In the present study we have investigated the role of a heparin-binding domain from the 170,000-mol-wt C_1H_3 polypeptide in promoting neural cell–substratum adhesion. Previous studies in our laboratory have demonstrated that the C_1H_3 MAb inhibits cell attachment to the substratum when incubated with either cells or the substratum, which indicates that neural cell–substratum adhesion occurs via a homophilic binding mechanism (4–6). The interaction between heparan sulfate and the 170,000-mol-wt protein is also required for cell attachment since an antiserum to a cell surface heparan sulfate proteoglycan disrupts cell binding (6). We therefore proposed

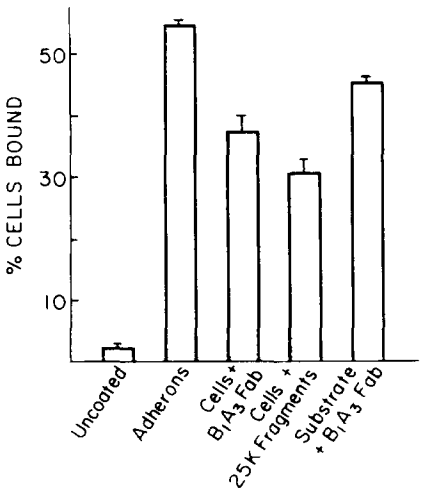

Figure 8. Role of heparin-binding domain in cell binding to adherons. 35-mm plastic petri dishes were coated with 25 μg of retina adheron protein, and nonspecific binding sites were blocked with Earle's balanced salt solution containing 0.2% albumin. Mechanically dissociated day 12 retinal cells were then labeled with [^{35}S]methionine and incubated with the dishes for 1 h at 37°C. The role of the heparin-binding domain of N-CAM in promoting cell attachment to adherons was examined by incubating aliquots of retinal cells with 0.5 mg/ml of B_1A_3 Fab fragments or 10 μg/ml of the heparin-binding domain for 1 h at 4°C. The cells were then washed twice and added to adheron-coated dishes. Adheron-coated dishes were also incubated with B_1A_3 Fab fragments (0.5 mg/ml) for 1 h at room temperature, washed twice, and incubated with labeled retinal cells. After incubation, dishes were swirled gently to dislodge weakly adherent cells, the medium was aspirated, and bound cells were dissolved in Triton X-100 in order to measure isotope content.

that the binding of heparan sulfate to 170,000-mol-wt protein in the extracellular matrix induces a conformational change in the protein, and this conformational change modulates the binding affinity of the protein (6). In this regard neural cell–substratum adhesion is similar to cell attachment involving fibronectin, since fibroblasts attach to their matrix via protein–protein and protein–glycosaminoglycan interactions (15, 16, 25, 37). Insight into the mechanism of fibronectin-mediated cell attachment has also been obtained by the isolation of specific structural and functional domains of the molecule, which are present in the intact molecule as protease-resistant fragments (22, 26, 38). Therefore, we were interested in determining whether a heparin-binding domain from the 170,000-mol-wt protein could be generated, which would permit us to characterize its role in mediating neural cell–substratum adhesion.

One important conclusion that can be made from the present study is that the 170,000-mol-wt C_1H_3 protein is immunologically identical to N-CAM, as shown by immunoblotting and immunoprecipitation analysis. Although the C_1H_3 MAb can adsorb most N-CAM molecules (recognized by an anti-N-CAM MAb) from solution, there appear to be subtle differences in the molecules these MAbs recognize when the MAbs are used to immunopurify their respective antigens. For example, the predominant polypeptide immunopurified from brain tissue with the C_1H_3 MAb is the 170,000-mol-wt form of N-CAM, with smaller amounts of the 140,000- and 120,000-mol-wt proteins present. However, the anti-N-CAM MAb immunopurifies approximately similar amounts of the three N-CAM components. Since both MAbs bind all three N-CAM components by immunoblotting procedures, these results may arise due to differences in affinities for individual N-CAM components. For example, the C_1H_3 MAb may display a higher affinity towards the higher molecular weight N-CAM component, but is capable of binding all three components. Thus, this MAb would immunopurify greater amounts of the higher molecular weight component.

The immunological identity between the C_1H_3 protein and N-CAM is of interest, since it demonstrates a novel function for the N-CAM molecule. N-CAM has been previously shown to mediate neuron–neuron (33) and neuron–muscle (11) adhesion, and recently has been shown to be present on glial cells (18, 20) and to participate in neuron–glia cell adhesion (18). Our data indicate that N-CAM contains a heparin-binding domain that is required for cell attachment to retinal cell extracellular matrix material. This is therefore the first evidence suggesting that N-CAM is a multifunctional protein. These data also imply that the heparin-binding domain may modulate the homophilic binding between N-CAM molecules. In addition, previous studies have demonstrated that retinal ganglion cell outgrowth occurs along glial endfeet in the optic tract and is mediated by N-CAM (32). In light of the evidence that neurite outgrowth can be promoted by heparin-binding domains of extracellular matrix molecules (8, 24), it is promising to speculate that glial endfeet contain a subset of N-CAM molecules that possess a heparin-binding domain. Recent studies in several laboratories suggest that distinct subsets of N-CAM are present on different classes of cells in the nervous system (18, 20, 35). Immunocytochemical staining of developing nervous tissue with the B_1A_3 MAb could provide information regarding whether this MAb recognizes a subset of N-CAM.

The role of N-CAM in promoting neural cell–substratum adhesion was first demonstrated using the C_1H_3 MAb, with the MAb inhibiting the binding of retinal cells to adherons (4). Adherons are complexes of proteins and glycosaminoglycans that are secreted by neural cells in culture, and thus resemble extracellular matrix material in molecular composition (31). As shown in previous studies in our laboratory, the protein now recognized as N-CAM is a component of adherons (4, 5), and the binding of the C_1H_3 MAb to adherons partially inhibits cell attachment. Cell–adheron binding is also inhibited by heparin or heparan sulfate (29, 31), and the binding of retinal cells to a substratum of N-CAM is inhibited by heparan sulfate (6). These data implied that N-CAM contained a heparin-binding domain, which has been confirmed in the present study. The heparin-binding domain, a 25,000-mol-wt fragment, has been purified and partially inhibits cell attachment to adherons, but abolishes cell binding to an N-CAM substratum. These data suggest that not all retinal cells binding to adherons in vitro are binding to N-CAM, which is consistent with our observation that the C_1H_3 MAb does not completely inhibit cell–adheron binding. Schubert and his co-workers have identified another adheron component, called purpurin, which is also involved in cell–adheron binding (30). Antibodies against this molecule inhibit adhesion only 40–50% (30), and the antigen is only present on a subpopulation of adherons. These data suggest that cell attachment to adherons occurs by multiple mechanisms, with several molecules being capable of binding subpopulations of neural cells. It remains to be determined whether the inhibi-

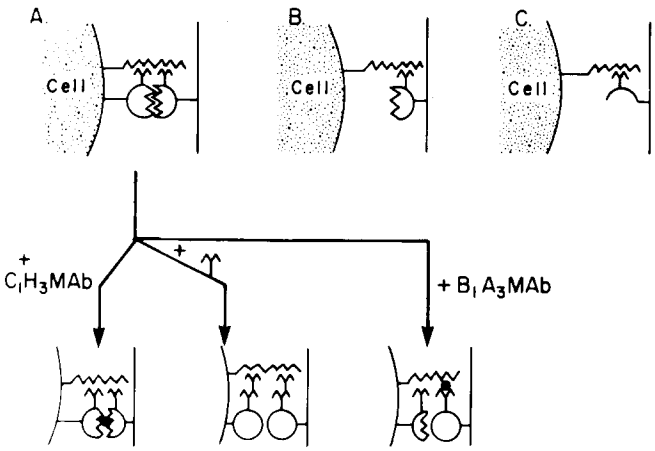

Figure 9. Schematic diagram depicting a possible model for neural cell–substratum adhesion, based on current experimental observations. In *A*, the model shows that N-CAM undergoes a conformational change after binding of heparan sulfate (6), and homophilic binding between N-CAM molecules results in cell attachment to the matrix. Cell attachment does not occur under conditions shown in B (i.e., C_1H_3 MAb has been bound to cell surface N-CAM, which is not diagrammed) since the interaction between cell surface heparan sulfate and N-CAM is weak. Cell attachment does occur in C, which implies that the isolated heparin-binding domain has a higher affinity for heparan sulfate. Possible mechanisms for the inhibition of cell–substratum adhesion by the C_1H_3 and B_1A_3 MAbs (represented by solid circles) or the heparin-binding domain of N-CAM are also depicted. It is proposed that the C_1H_3 MAb recognizes an epitope that is necessary for homophilic binding between N-CAM molecules. In contrast, the B_1A_3 MAb or heparin-binding domain prevent heparan sulfate binding to N-CAM, preventing the conformational change in the N-CAM protein that allows homophilic binding and cell attachment.

tory effect of the C_1H_3 MAb and anti–purpurin antibody will be additive, resulting in complete disruption of cell attachment.

Since it is apparent that adherons are complex structures, it is more feasible to assess the role of a particular molecule in cell–substratum adhesion by coupling that molecule to an inert surface. When N-CAM is coupled to glass surfaces the C_1H_3 MAb and the 25,000-mol-wt heparin-binding domain inhibit cell attachment when incubated with retinal cells. The B_1A_3 MAb also inhibits cell attachment when included in the assay medium and incubated with the retinal cells. When a substratum is prepared using the 25,000-mol-wt fragment, cells attach to the substratum, and this binding is inhibited by the B_1A_3 MAb, heparin, or the anti–heparan sulfate proteoglycan antiserum. The 25,000-mol-wt heparin-binding fragment of N-CAM therefore represents a distinct functional domain that can promote neural cell attachment. A scheme that summarizes our observations to date is shown in Fig. 9. We envisage that intact N-CAM protein containing the heparan-binding domain promotes cell attachment to the matrix by first binding cell surface heparan sulfate proteoglycan. This binding of heparan sulfate to N-CAM results in a conformational change in the protein, which then allows homophilic binding between N-CAM molecules to occur. It is this binding mechanism that results in stable cell attachment. Binding of cell surface heparan sulfate to N-CAM in the extracellular matrix is too weak to generate a stable cell-to-matrix attachment, even though cells can bind to the 25,000-mol-wt heparan domain, which binds this proteoglycan with higher affinity. These data are consistent with our previous observations that the C_1H_3 MAb inhibits cell–substratum adhesion but not heparin binding, and that heparan sulfate or antibodies that block heparan sulfate-N-CAM binding inhibit cell–substratum adhesion.

In conclusion, in the present study we have shown that the 170,000-mol-wt C_1H_3 protein is immunologically identical to N-CAM. We have also isolated the B_1A_3 MAb which recognizes the heparin-binding domain of N-CAM, and this MAb inhibits the binding of heparin to N-CAM. We have identified a 25,000-mol-wt heparin-binding domain derived from N-CAM, and this domain promotes cell attachment when coupled to glass surfaces, as well as inhibiting cell–adheron binding when bound to retinal cells. Thus, N-CAM represents a multifunctional protein that contains both cell- and heparin-binding domains.

This work was supported by Grant GM 18405 from the National Institutes of Health. G. J. Cole is the recipient of a National Institutes of Health Postdoctoral Fellowship (EY-0566). The authors are also grateful for the generous gifts of anti-N-CAM MAb (Dr. David Gottlieb, Washington University) and anti–heparan sulfate proteoglycan antiserum (Dr. David Schubert, The Salk Institute).

Received for publication 14 October 1985, and in revised form 7 November 1985.

References

1. Bottenstein, J. E., and G. Sato. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA.* 76:514–518.

2. Boucaut, J.-C., T. Darribere, T. J. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J. Cell Biol.* 99:1822–1830.

3. Cole, G. J., and L. Glaser. 1984. Identification of novel neural- and neural retina-specific antigens with a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 81:2260–2264.

4. Cole, G. J., and L. Glaser. 1984. Inhibition of embryonic neural retina cell-substratum adhesion with a monoclonal antibody. *J. Biol. Chem.* 259:4031–4034.

5. Cole, G. J., and L. Glaser. 1984. Cell-substratum adhesion in embryonic chick central nervous system is mediated by a 170,000-mol-wt neural-specific polypeptide. *J. Cell Biol.* 99:1605–1612.

6. Cole, G. J., D. Schubert, and L. Glaser. 1985. Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. *J. Cell Biol.* 100:1192–1199.

7. Edelman, G. M. 1983. Cell adhesion molecules. *Science (Wash. DC).* 219:450–457.

8. Edgar, D., R. Timpl, and H. Thoenen. 1984. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1463–1468.

9. Friedlander, D. R., R. Brackenbury, and G. M. Edelman. 1985. Conversion of embryonic form to adult forms of N-CAM in vitro: results from *de novo* synthesis of adult forms. *J. Cell Biol.* 101:412–419.

10. Grumet, M., S. Hoffman, and G. M. Edelman. 1984. Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron-neuron and neuron-glia adhesion. *Proc. Natl. Acad. Sci. USA.* 81:267–271.

11. Grumet, M., U. Rutishauser, and G. M. Edelman. 1982. Neural cell adhesion molecule is on embryonic muscle cells and mediates adhesion to nerve cells in vitro. *Nature (Lond.).* 295:693–695.

12. Grunwald, G. B., R. S. Pratt, and J. Lilien. 1982. Enzymation dissection of embryonic cell adhesive mechanisms. *J. Cell Sci.* 55:69–83.

13. Hausman, R. E., and A. A. Moscona. 1976. Isolation of retina-specific cell-aggregating factor from membranes of embryonic neural retina tissue. *Proc. Natl. Acad. Sci. USA.* 73:3594–3598.

14. Hirn, M., M. S. Ghandour, H. Deagostini-Bazin, and C. Goridis. 1983. Molecular heterogeneity and structural evolution during cerebellar ontogeny detected by monoclonal antibody of the mouse cell surface antigen BSP-2. *Brain Res.* 265:87–100.

15. Hynes, R. O., and K. M. Yamada. 1982. Fibronectin: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369–377.

16. Johansson, S., and M. Höök. 1984. Substrate adhesion of rat hepatocytes: on the mechanism of attachment to fibronectin. *J. Cell Biol.* 98:810–817.

17. Jorgensen, O. S., A. Delouvee, J. P. Thiery, and G. M. Edelman. 1980. The nervous system specific protein D2 is involved in adhesion among neurites from cultured rat ganglia. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 111:39–42.

18. Keilhauer, G., A. Faissner, and M. Schachner. 1985. Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies. *Nature (Lond.).* 316:728–730.

19. Lander, A. D., D. K. Fujii, and L. F. Reichardt. 1985. Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned medium. *Proc. Natl. Acad. Sci. USA.* 82:2183–2187.

20. Noble, M., M. Albrechtsen, C. Moller, J. Lyles, E. Bock, C. Goridis, M. Watanabe, and U. Rutishauser. 1985. Glial cells express N-CAM/D2-CAM-like polypeptides *in vitro*. *Nature (Lond.).* 316:725–728.

21. Osterlund, E., I. Eronen, K. Osterlund, and M. Vuento. 1985. Secondary structure of human plasma fibronectin: conformational change induced by calf alveolar heparan sulfates. *Biochemistry.* 24:2661–2667.

22. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell.* 26:259–267.

23. Rathjen, G., and M. Schachner. 1984. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1–10.

24. Rogers, S. L., J. B. McCarthy, S. L. Palm, L. T. Furcht, and P. C. Letourneau. 1985. Neuron-specific interactions with two neurite-promoting fragments of fibronectin. *J. Neurosci.* 5:369–378.

25. Rollins, B. J., M. K. Cathcart, and L. A. Culp. 1982. Fibronectin-proteoglycan binding as the molecular basis for fibroblast adhesion to extracellular matrices. *In* The Glycoconjugates. Volume III. M. Horowitz, editor. Academic Press, Inc., New York. 282–329.

26. Rouslahti, E., E. G. Hayman, E. Engvall, W. C. Cothran, and W. T. Butler. 1981. Alignment of biologically active domains in the fibronectin molecule. *J. Biol. Chem.* 256:7277–7281.

27. Rutishauser, U. 1984. Developmental biology of a neural cell adhesion molecule. *Nature (Lond.).* 310:549–554.

28. Rutishauser, U., S. Hoffman, and G. M. Edelman. 1982. Binding properties of a cell adhesion molecule from neural tissue. *Proc. Natl. Acad. Sci. USA.* 79:685–689.

29. Schubert, D., and M. LaCorbiere. 1985. Isolation of a cell surface receptor for chick neural retina adherons. *J. Cell Biol.* 100:56–63.

30. Schubert, D., and M. LaCorbiere. 1985. Isolation of an adhesion mediating protein from chick neural retina adherons. *J. Cell Biol.* 101:1071–1077.

31. Schubert, D., M. LaCorbiere, F. G. Klier, and C. Birdwell. 1983. A role for adherons in retinal cell adhesion. *J. Cell Biol.* 96:990–998.

32. Silver, J., and U. Rutishauser. 1984. Guidance of optic axons *in vivo* by a preformed adhesive pathway on neuroepithelial endfeet. *Dev. Biol.* 106:485–499.

33. Thiery, J.-P., R. Brackenbury, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neural cells of chick retina. II. Purification and characterization of cell adhesion molecule from neural retina. *J. Biol. Chem.* 252:6841–6845.

34. Unsicker, K., S. D. Skaper, G. E. Davis, M. Manthorpe, and S. Varon. 1985. Comparison of the effects of laminin and the polyornithine-binding neurite promoting factor from RN22 Schwannoma cells on neurite regeneration from cultured newborn and adult rat dorsal root ganglion neurons. *Dev. Brain Res.* 17:304–308.

35. Williams, R. K., C. Goridis, and R. Akeson. 1985. Individual neural cell types express immunologically distinct N-CAM forms. *J. Cell Biol.* 101:36–42.

36. Witte, D., and D. I. Gottlieb. 1983. Time of appearance and tissue distribution of a cell surface antigen in early chick development. *Dev. Brain Res.* 9:63–67.

37. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761–799.

38. Yamada, K. M., D. W. Kennedy, K. Kimata, and R. M. Pratt. 1980. Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. *J. Biol. Chem.* 255:6055–6063.