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

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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 C1H3 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 C1H3 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 B1A3, 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 B1A3 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 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), designated C1H3, 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.
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$\text{H}_3$ 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$\text{H}_3$ 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-I-A6-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$\text{H}_3$ 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$\text{H}_3$ 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$\text{H}_3$ 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$\text{H}_3$ 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$\text{H}_3$ MAb has been described previously (3) and was obtained by immunizing rats with embryonic day 9 retinal cells. The B10.A 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).

Results

Comparison of N-CAM and the 170,000-mol-wt C$\text{H}_3$ 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 CH₃ 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 CH₃ 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 CH₃ 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 CH₃ antigen was immunoblotting of the immunopurified proteins. As shown in Fig. 1, the CH₃ 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 [³⁵S]methionine in Spinner culture 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 CH₃ MAb; b, cell extract immunoprecipitated with anti-N-CAM MAb; c, incubation with anti-N-CAM after immunoprecipitation with CH₃ MAb; d, immunoprecipitation of cell extract with B₄A₃ MAb. Lanes e-g show immunoprecipitation of medium with B₄A₃ (e), CH₃ (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 CH₃ MAb before immunoprecipitation with anti-N-CAM MAb.

**Figure 1.** Immunoblotting of immunopurified 170,000-mol-wt CH₃ protein and N-CAM. 5-μg aliquots of N-CAM immunopurified with the CH₃ MAb (a and c) or anti-N-CAM MAb (b) were separated on a 7% polyacrylamide gel, transferred to nitrocellulose, and reacted with CH₃ 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 [³⁵S]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 CH₃ MAb; b, cell extract immunoprecipitated with anti-N-CAM MAb; c, incubation with anti-N-CAM after immunoprecipitation with CH₃ MAb; d, immunoprecipitation of cell extract with B₄A₃ MAb. Lanes e-g show immunoprecipitation of medium with B₄A₃ (e), CH₃ (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 CH₃ MAb before immunoprecipitation with anti-N-CAM MAb.
proteins are immunoprecipitated with C1H3 MAb, followed by immunoprecipitation with anti-N-CAM MAb, most of the N-CAM molecules are removed by the C1H3 MAb (Fig. 2).

We have previously shown that the 170,000-mol-wt C1H3 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 C1H3 protein (designated hereafter as N-CAM) specifically binds [3H]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 C1H3 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. 3a). 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 C1H3 MAb (6), and we thus chose to use subtilisin protease in these 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. 3b). After heparin–agarose chromatography only the 25,000-mol-wt fragment is retained on the column (Fig. 3d). 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. 4b, the C1H3 MAb does not react with this fragment, which suggests that the C1H3 MAb inhibits cell-substratum adhesion by interacting with another functional domain. However, the C1H3 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 CIH3 MAb (a and b) or BIA3 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 BIA3 MAb reacts with the heparin-binding domain. Lanes e and f show the results of immunoblotting with BIA3 MAb of the heparin-binding domain isolated from N-CAM purified with the CIH3 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 CIH3 protein are immunologically related.

components that are not retained by the heparin-agarose column (Fig. 4a). These data therefore imply that the CIH3 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 CIH3 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 BIA3, reacts with an epitope distinct from the CIH3 antigenic determinant. As shown in Fig. 2, the BIA3 MAb immunoprecipitates proteins with molecular weights identical to that of N-CAM. As shown in Fig. 5, d–f, the BIA3 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 BIA3 MAb binds to the 25,000-mol-wt heparin-binding fragment. It can also be seen in Fig. 4, e and f that the BIA3 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 CIH3 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 CIH3 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 CIH3 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 BIA3 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 [3H]heparin-binding assay that was originally used to demonstrate that N-CAM possessed a heparin-binding domain (6). In initial studies, the BIA3 MAb preparation also contained a heparin-binding component, which probably resulted from other protein components in the ascites fluid. We therefore passed BIA3 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 [3H]heparin, binding of heparin to the protein is inhibited (Table I). We have previously shown that the CIH3 MAb does not inhibit heparin binding (6), which indicates that the BIA3 MAb specifically inhibits [3H]heparin binding to N-CAM.

Since the BIA3 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 CIH3 MAb (a-c) or B1A3 MAb (d-f).

Table I. Inhibition of [3H]Heparin Binding to N-CAM by the B1A3 Monoclonal Antibody

| Treatment                  | Percentage of cpm bound |
|----------------------------|--------------------------|
| No protein                 | 0.4 ± 0.0                |
| B1A3 MAb                   | 2.2 ± 0.3                |
| N-CAM                      | 11.6 ± 1.0               |
| N-CAM + B1A3 MAb           | 5.7 ± 0.7                |

Mean of two experiments ± SD conducted in duplicate. Input radioactivity represented 20,000 cpm of [3H]heparin excluded from a Sephadex G-100 column. 100 μg/ml of N-CAM protein was incubated 30 min with 200 μg/ml of B1A3 ascites fluid excluded from a heparin-agarose column. [3H]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 CIH3 Fab fragments. It can also be seen that the B1A3 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 B1A3 Fab fragments are present in the medium during the adhesion assay, cell attachment is inhibited. These data imply that the B1A3 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 B1A3 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 CIH3 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 CIH3 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 B1A3 MAb binds with higher affinity to the heparin-binding domain than to the intact...
protein, since after incubation of this antibody with the substrate, followed by washing, the B1A3 MAb can still inhibit cell attachment (Fig. 7). This effect was not observed with substrata 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 C10H3 MAb (3, 4), and likewise cell binding is inhibited 30–40% by the B1A3 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 B1A3 antibody prevents cell-to-substratum attachment by preventing this conformational change. It can also be seen that the B1A3 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 the substratum (Fig. 8). These data imply that the heparin-binding domain of matrix N-CAM is necessary for retinal cell adhesion when incubated with retinal cells, implying that both heparin and N-CAM proteins are involved in this process.

Discussion

In the present study we have investigated the role of a heparin-binding domain from the 170,000-mol-wt C10H3 polypeptide in promoting neural cell-substratum adhesion. Previous studies in our laboratory have demonstrated that the C10H3 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...
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-H3 protein is immunologically identical to N-CAM, as shown by immunoblotting and immunoprecipitation analysis. Although the C-H3 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-H3 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-H3 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-H3 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 it 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-A3 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-H3 MAb, with the MAb inhibiting the binding of retinal cells to adherons (4). Adherens 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 adherens (4, 5), and the binding of the C-H3 MAb to adherens partially inhibits cell attachment. Cell–adherens 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 adherens, but abolishes cell binding to an N-CAM substratum. These data suggest that not all retinal cells binding to adherens in vitro are binding to N-CAM, which is consistent with our observation that the C-H3 MAb does not completely inhibit cell–adherens binding. Schubert and his co-workers have identified another adherens component, called purpurin, which is also involved in cell–adherens binding (30). Antibodies against this molecule inhibit adhesion only 40–50% (30), and the antigen is only present on a subpopulation of adherens. These data suggest that cell attachment to adherens occurs by multiple mechanisms, with several molecules being capable of binding subpopulations of neural cells. It remains to be determined whether the inhibi-
binding between N-CAM molecules to occur. It is this binding of heparan sulfate to N-CAM results in a conformational change in the protein, which then allows homophilic binding by first binding cell surface heparan sulfate proteoglycan. This 25,000-mol-wt heparin-binding domain promotes cell attachment to the matrix and N-CAM, and this domain promotes cell attachment when coupled to glass surfaces, as well as inhibiting cell-adenon binding when bound to retinal cells. Thus, N-CAM represents a multifunctional protein that contains both cell- and heparin-binding domains.

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., CH3 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 CH3 and B A3 MAb (represented by solid circles) or the heparin-binding domain of N-CAM are also depicted. It is proposed that the CH3 MAb recognizes an epitope that is necessary for homophilic binding between N-CAM molecules. In contrast, the B A3 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.

The inhibitory effect of the CH3 MAb and antipurpurin 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 CH3 MAb and the 25,000-mol-wt heparin-binding domain inhibit cell attachment when incubated with retinal cells. The B A3 MAb also inhibits cell attachment when included in the assay medium and incubated with the retinal cells. When a substrate is prepared using the 25,000-mol-wt fragment, cells attach to the substrate, and this binding is inhibited by the B A3 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 CH3 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 CH3 protein is immunologically identical to N-CAM. We have also isolated the B A3 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-adenon binding when bound to retinal cells. Thus, N-CAM represents a multifunctional protein that contains both cell- and heparin-binding domains.

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