A Role for Polysialic Acid in Neural Cell Adhesion Molecule Heterophilic Binding to Proteoglycans*

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The neural cell adhesion molecule (NCAM) is known to participate in both homophilic and heterophilic binding, the latter including mechanisms that involve interaction with proteoglycans. The polysialic acid (PSA) moiety of NCAM can serve as a negative regulator of homophilic binding, but indirect evidence has suggested that PSA can also be involved in heterophilic binding. We have examined this potential positive role for PSA in terms of the adhesion of PSA-expressing mouse F11 cells and chick embryonic brain cells to substrates composed of the purified heparan sulfate proteoglycans agrin and 6C4. This adhesion was specifically inhibited by polyclonal anti-NCAM Fab antibodies, monoclonal anti-PSA antibodies, PSA itself, and enzymatic removal of either PSA or heparan sulfate side chains. By contrast, the adhesion was not affected by chondroitinase, and cell binding to laminin was not inhibited by any of these treatments. A specific NCAM-heparan sulfate interaction in this adhesion was further indicated by its inhibition with monoclonal anti-NCAM Fab antibodies that recognize the known heparin-bind-
ing domain of NCAM and with the HBD-2 peptide derived from this region, but not with antibodies directed against other regions of the protein including the homophilic binding region. Together, the results suggest that PSA can act in vitro either as a receptor in NCAM heterophilic adhesion or as a promoter of binding between heparan sulfate proteoglycans and the NCAM heparin-binding domain.

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§ The abbreviations used are: NCAM, neural cell adhesion molecule; HSPG, heparan sulfate proteoglycan; PSA, polysialic acid; GAG, glycosaminoglycan; PBS, phosphate-buffered saline; endo N, endo-eruminidase N; HBD, heparan-binding domain of NCAM; CEB cells, chick embryonic day 11 brain cells; BSA, bovine serum albumin.

The most familiar and best characterized binding activity of the neural cell adhesion molecule (NCAM) is a homophilic receptor (1–4) primarily involving isostructural interaction between NCAM Ig domain 3 on apposing cells (5–8). However, there is also ample evidence for two distinct heterophilic functions, one involving NCAM Ig domain 2 binding to certain heparan sulfate proteoglycans (HSPGs) (9–16) and the other thought to involve binding of NCAM to the polypeptide core of the chondroitin sulfate proteoglycans neurocan and phosphancan (17–20). Although the biological significance of NCAM heterophilic binding remains to be established, two HSPGs with interesting developmental expression patterns, namely agrin and the 6C4 antigen (21–23), have been shown to serve as heterophilic receptors for NCAM (15, 16, 24). Furthermore, the lethality of soluble NCAM produced in transgenic mice is most consistent with its interaction with a non-NCAM receptor (25).

The possibility of multiple NCAM binding activities presents a challenge to the interpretation of NCAM perturbation and gene mutation studies, particularly in terms of molecular mechanism. One aspect of this ambiguity concerns the mode of action of the polysialic acid (PSA) moiety attached to NCAM. PSA has been proposed to function as a negative regulator of cell-cell interactions, possibly through steric effects stemming from its large size and abundance (26). Extensive evidence has been obtained both in vitro and in vivo to support this role and to establish its importance in promoting plasticity in cell interactions during development, notably in specific patterns of axon outgrowth and cell migration (27). However, there has also been an indirect suggestion that there might be an additional role for PSA in heterophilic binding. Specifically, a monoclonal anti-NCAM antibody was shown to block heterophilic binding of cells to an NCAM substrate less well when that substrate had a higher content of PSA, an effect that could be explained by a positive role of PSA in the adhesion process (28).

In this study, we have used the in vitro adhesion assay utilized by Storms et al. (15, 16) to obtain direct evidence for a positive role for PSA in cell adhesion mediated by heterophilic binding of NCAM to two purified HSPG substrates, agrin and 6C4. Mouse F11 cells bearing moderate levels of PSA and chick embryonic brain cells bearing high levels of PSA were used in these assays. A combination of specific antibody, enzyme, and competitive inhibitors (NCAM peptides and PSA fragments) was used to establish that this influence of PSA involves the known heparin-binding domain of NCAM and is distinct from the homophilic binding domain.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal anti-chick NCAM antibodies 5e, 79b, PP, 9a, and 105 were produced by methods previously described (29). These antibodies recognize different structural and functional elements in the NCAM polypeptide (see “Results”). Polyclonal anti-mouse NCAM and anti-chick NCAM antibodies were produced from rabbit antiserum using standard methods. The PSA-recognizing IgM monoclonal antibody 5A5 was produced by methods previously described (30). Fab antibody fragments of anti-NCAM antibodies were produced by digestion with pepsin. Ascites fluids containing monoclonal antibody 6D2 directed against chick agrin from embryonic day 10 retinal basal laminae (21) and monoclonal antibody 6C4 directed against an as yet unnamed chick HSPG from the same tissue (6C4 antigen) (22) were generous gifts of Dr. Willi Halfter (University of Pittsburgh, Pittsburgh, PA). Monoclonal antibody 6D2 (anti-agrin) was the generous gift of Dr. Gregory Cole (University of Ohio, Columbus, OH).

Enzymes and Reagents—The glycosaminoglycan (GAG)-cleaving enzymes heparinase I (EC 4.2.2.7), heparinase III (heparin-sulfate lyase, EC 4.2.2.8), and chondroitinase ABC (chondroitinase ABC lyase, EC 4.2.2.4) were obtained from Sigma. GAG-cleaving enzymes were diluted to a final concentration of 10 milliunits/ml in PBS for GAG digests. All
FIG. 1. F11 and chick brain cells adhere to agrin and 6C4 HSPGs, but not to laminin, by an NCAM-mediated mechanism. Mouse F11 (A) and CEB (B) cell interactions with substrate-immobilized agrin, 6C4 antigen HSPG, brain NCAM, laminin (Ln), and BSA substrates were measured using the spot cell adhesion assay. Probe cells were untreated (black bars) for 4 h prior to the spot adhesion assay. Mouse F11 (A) and CEB (B) cell interactions with agrin, 6C4 antigen, NCAM, laminin (Ln), and BSA substrates were measured using the spot cell adhesion assay.

GAG-cleaving experiments were carried out at 37 °C for 4 h. In some cases, GAG-cleaving experiments were carried out in the presence of a protease inhibitor mixture previously described (16) comprising 1 mM a-toluene-sulfonyl fluoride, 2 μg/ml leupeptin, 1 mM EDTA, and 0.1 mg/ml BSA. The results obtained with and without the protease inhibitor were identical, suggesting that they did not reflect protease contamination. Endoeneraminidase N (endo N) was produced in phage- 

FIG. 2. Removal of PSA from NCAM inhibits heterophilic adhesion of F11 and chick embryonic brain cells to agrin and 6C4 HSPGs while enhancing homophilic adhesion to NCAM. Probe cells were untreated (black bars), treated with endo N (gray bars), or treated with endo N and polyclonal anti-NCAM Fab antibodies (Ab; white bars) for 4 h prior to the spot adhesion assay. Mouse F11 (A) and CEB (B) cell interactions with agrin, 6C4 antigen, NCAM, laminin (Ln), and BSA substrates were measured using the spot cell adhesion assay.

The sequence of the heparan-binding domain of NCAM (HBD-2) (12) was synthesized by Research Genetics (Huntsville, AL). The sequence of HBD-2 is IWKHKGRDVILKKDVR-

Tissue Culture—F11 cells are a PSA-positive hybrid of mouse E17 primary sensory neurons and rat neuroblastoma cells formed by fusing the two cell types (32). F11 cells were maintained in culture in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Chicken embryonic brain cells with highly polysialylated NCAM (hereafter referred to as CEB cells) were prepared on the day of the experiment by dissecting whole brains from day 11 chick embryos. The brains were passed through a 300-μm nylon mesh and incubated in suspension at room temperature in PBS (pH 7.4) with 1 mM EDTA for 5 min. This cell suspension was centrifuged; the pellet was resuspended in PBS; and the cell suspension was passed through a 50-μm nylon mesh. Pasteur pipettes with flame-rounded tips were used to break up the remaining clumps of cells until a single cell suspension was achieved. These cells were then plated in experimental medium (Dulbecco's modified Eagle's medium + 0.1% BSA) and maintained in a tissue culture incubator until needed (~4 h).

Spot Cell Adhesion Assay—The spot cell adhesion assay used has been described previously (14, 15). This assay is a modification of the methods of Lagenaur and Lemmon (33). Briefly, 0.25-ml aliquots of nitrocellulose solution (5-cm² nitrocellulose in 6 ml of methanol) were applied to 3-cm tissue culture plates and allowed to air dry for 30 min. Then, 2-μl spots of substrate solutions (0.1–0.5 μg/spot) were applied to the nitrocellulose and incubated for 1 h. Each plate had 10 or fewer spots, and the spots were labeled on the reverse side of the plates for identification. The nitrocellulose was blocked for 1 h with BSA solution (10 mg/ml in PBS). After blocking, the plates were washed twice with PBS, and any substrate treatments were carried out.

On the day of the assay, nearly confluent tissue culture plates of F11 cells or of freshly prepared CEB cells that were to be treated with endo N were treated for 4 h. Cells were then prepared by incubating the cells

A

B

Substrate

Agrin 6C4 NCAM Ln BSA Agrin 6C4 NCAM Ln BSA

Cells / Field

(mean ± SEM)

Cells / Field

(mean ± SEM)
in 0.1 mM EDTA for 5 min and gently removing the cells with a Pasteur pipette from tissue culture dishes. Cells were washed twice by resuspension in PBS. Cells were then centrifuged in a tabletop centrifuge for 10 min, resuspended in Dulbecco's modified Eagle's medium, counted, and adjusted to a final concentration of 2 × 10⁶ cells/ml of experimental medium. Cells were allowed to recover on ice for 30–60 min. Cells that were to be treated with antibodies were treated with the appropriate antibodies during this period at a final concentration of 0.1–0.5 mg of antibody/ml of medium. The amount of antibody added was well in excess of the amount needed to saturate specific antigenic sites on the cell surface. Cells were added to appropriate experimental dishes (2 ml/dish) and incubated in a tissue culture incubator for 1 h. Experimental dishes were washed twice with Dulbecco's modified Eagle's medium, and the cells remaining in contact with each substrate spot were counted using an inverted phase-contrast microscope at magnification × 200. Three 1-mm² fields of view were counted for each spot using a grid reticule in the subjective lens of the microscope. Each experiment was repeated at least three times.

In these studies, it was generally observed that variation within an experimental series using a single cell preparation was quite low, whereas the overall level of binding could vary more significantly from day to day. In our experience, this type of variation results from differences in the cell preparations. Therefore, to compare results from different days, each experiment included a full set of substrates and controls so as to assure a consistent relative picture of the binding.

**RESULTS**

NCAM-mediated Adhesion of PSA-positive F11 and CEB Cells to HSPGs—Spot cell adhesion assays were used to measure interaction of F11 and CEB cells, bearing moderate and high levels of PSA, respectively, with two substrate-immobilized HSPGs, agrin and the 6C4 antigen, as well as with control substrates of NCAM, laminin, and BSA (Fig. 1). The F11 cells adhered rapidly to the two HSPGs and laminin, moderately well to NCAM, and poorly to BSA. The CEB cells gave similar results, with moderate binding levels for NCAM and the two HSPGs. It should be noted, however, that the ability of these different substrates to adhere to nitrocellulose varies and is particularly low for proteoglycans. Thus, the absolute levels of cell binding should not be compared for the different substrates, but rather the relative changes in binding produced by specific perturbations, as featured in this study.

To investigate whether these interactions were mediated by NCAM on the cells, the cells were treated with the appropriate anti-NCAM Fab antibody for 1 h and then washed prior to the spot cell adhesion assay. Under these conditions, adhesion to the NCAM substrate as well as to both HSPG substrates was strongly inhibited by anti-NCAM Fab antibody treatment, whereas adhesion to laminin was unaffected.

PSA Is Required for Adhesion of F11 and CEB Cells to HSPGs—The above assay for heterophilic binding of PSA-positive cells to defined HSPG substrates was then carried out with and without pretreatment of the cells with endo N, an enzyme known to have a strict specificity for PSA (2, 34) (Fig. 2). If PSA was functioning in the assay as a negative regulator of adhesion, then it would have been expected that endo N treatment would increase binding. This was in fact the result obtained for adhesion to the NCAM substrate, an interaction that appears to be dominated by homophilic adhesion in that it is strongly inhibited by treating the cells alone with anti-NCAM Fab antibody. By contrast, adhesion to the HSPGs was strongly inhibited by the endo N treatment, in fact to the same extent obtained with anti-NCAM Fab antibody (Fig. 2). This finding indicates a positive role for PSA in heterophilic binding that is clearly distinct from its role as a negative regulator of cell interactions.

A second test of the direct role of PSA in the binding of F11 cells to HSPGs was to use fragments of PSA (obtained as colominic acid derived from bacterial cell coats), as a competitive inhibitor. As shown in Fig. 3, increasing concentrations of...
PSA fragments caused a progressive decrease in the binding to either agrin or heparin, but did not alter attachment to NCAM, fibronectin, or BSA.

Adhesion to HSPGs Is Inhibited by Anti-PSA Antibodies—A second difference in the function of PSA in adhesion to HSPGs was revealed by the effect of anti-PSA antibodies. When functioning as a negative regulator of cell interactions, PSA has been proposed to operate as a steric inhibitor of cell and/or molecular contacts, and thus, it is not surprising that the addition of anti-PSA antibodies does not affect PSA function in this context. By contrast, in binding events to which PSA contributes positively, as shown here for cell-HSPG interactions, the attachment of an antibody might be expected to inhibit this function. This was found to be the case for adhesion of both cell types to the HSPG substrates, but not to laminin (Fig. 4).

Molecular Characterization of PSA-dependent Heterophilic Adhesion—As described in the Introduction, there is substantial literature on heterophilic adhesion of NCAM to proteoglycans, involving at least two distinct mechanisms. To establish the relationship of this study to those works, a series of experiments were carried out to characterize the nature of the PSA-dependent interaction of NCAM with the agrin and 6C4 proteoglycans.

To confirm the role of heparan sulfate side chains in the adhesion of F11 and CEB cells to agrin and 6C4, the substrates were treated with either a mixture of heparinases I and III or chondroitinase ABC for 4 h at 37 °C prior to the adhesion assay. Adhesion of both cell types to agrin and the 6C4 antigen was nearly eliminated by the treatment with the heparinase I/III mixture, but was unaffected by chondroitinase treatment (Fig. 5). It should be noted, however, that this protocol does not establish that the chondroitinase is sufficiently active under the exact conditions of the study, and thus, a role for chondroitin sulfate side chains cannot be dismissed entirely by a negative result. To control for possible effects of protease contamination in the enzyme preparations, cell treatments with heparinase or chondroitinase were also carried out in the presence of a protease inhibitor mixture and gave similar results (data not shown). It should also be noted that adhesion to laminin and BSA substrates was unaffected by either enzyme treatment (Fig. 5).

The second Ig domain of NCAM has been shown to mediate NCAM-heparin interaction, and a synthetic peptide spanning a 19-residue sequence of this region has been shown to inhibit NCAM-heparin binding (12). When this peptide (HBD-2) was tested in the present assay, adhesion to HSPG substrates (agrin and the heparin substrate used in the original studies) was also carried out in the presence of a protease inhibitor mixture and gave similar results (data not shown). It should also be noted that adhesion to laminin and BSA substrates was unaffected by either enzyme treatment (Fig. 5).

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Finally, the heterophilic binding of chick NCAM to HSPGs is known to be inhibited by Fab antibodies recognizing the heparin-binding region, but not by a variety of other antibodies directed against different regions of the NCAM polypeptide (10, 29, 35). Accordingly, the monoclonal Fab antibodies 5e and 79b, which recognize the heparin-binding region (35), were found to inhibit adhesion of CEB cells to both agrin and 6C4, whereas Fab antibodies PP (which inhibits homophilic binding), 9a (which enhances homophilic binding), and 105 (which has no affect on binding) (29) did not alter heterophilic adhesion to HSPGs. None of these antibodies affected adhesion to laminin.

**DISCUSSION**

The primary goals of this study were to evaluate a direct role for PSA in promotion of NCAM-mediated heterophilic cell adhesion and to characterize the molecular components and specificity of that adhesion. It has been found that PSA is indeed required for the adhesion of cells to defined HSPG substrates. Furthermore, the molecular components of this PSA-dependent binding conform precisely to the heparin-binding activity of NCAM previously described by others (9–13).

Thus, to the findings of earlier studies showing that heparan sulfate and the HBD of NCAM are necessary for NCAM-HSPG interaction (11, 12) is now added a role for intact polysialic side chains on NCAM. Thus, either PSA removal with endo N or the binding of anti-PSA antibodies was found to be functionally equivalent to enzymatic removal of heparan sulfate or antibody blockage of the HBD region of NCAM. The correspondence, in terms of monoclonal antibody inhibition patterns, of the present adhesion activity with the NCAM heparin-binding domain is also compelling, i.e., both antibodies directed against the heparin-binding domain of Ig domain 2 were inhibitory, whereas all others, including those directed against the homophilic binding site, were not.

The addition of a second activity for PSA that, at the level of adhesion, acts in the opposite manner as the previously described negative regulatory activity represents an important consideration in the mechanistic interpretation of PSA function in biological systems. However, in preliminary studies on the possible role of this type of adhesion in the PSA-dependent bundling of neuronal processes (30) and the migration of neuronal precursors in vitro (36), we did not find any detectable effects with either the HBD-2 peptide or heparinases. Thus, a biological role for heterophilic NCAM-HSPG interactions remains to be demonstrated. On the other hand, given the specificity of this adhesion with respect to proteoglycan type, the presence of a distinct HSPG-binding site on NCAM, and the abundance of HSPGs in the in vivo environment of PSA/

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3 S. D. Storms and U. Rutishauser, unpublished observations.
NCAM-expressing cells, it seems unlikely that this phenomenon is simply an in vitro artifact. However, whether this binding by itself mediates adhesion under physiological conditions, as opposed to being a component of a more complex form of interaction, remains unknown.

Finally, although the results obtained indicate that PSA is required for NCAM-dependent binding of cells to an HSPG substrate via a specific HSPG-binding site located at least in part in NCAM Ig domain 2, it remains unclear whether PSA actually binds to HSPGs or indirectly modulates an NCAM-HSPG interaction. Three possible mechanisms to explain the present results are illustrated schematically in Fig. 8. The simplest is that stable attachment of the cells to the substrate requires an affinity of the HSPG for PSA itself as well as for the HSPG-binding region on NCAM Ig domain 2, either as distinct additive sites or as complementary components of a single site (dual binding model). In addition to explaining the inhibition of attachment by endo N, soluble PSA fragments, the HBD-2 peptide, and heparinases, this model would be easiest to reconcile with the inhibition by antibodies that bind to PSA. However, it remains to be established whether either the HSPG core proteins or glycomoieties have an affinity for the PSA polymer.

A second potential mechanism would be that the presence of PSA induces a conformational change in the NCAM polypeptide to produce or enhance the HSPG-binding site (conformation model). However, there is no direct evidence for such an effect, and preliminary studies on the binding of heparin to NCAM-coated Sepharose did not reveal a difference in binding when high or low PSA-containing NCAM was used. Another indirect model is that clustering of NCAM in the plane of the membrane, which has been proposed on the basis of kinetic arguments in cell membrane adhesion studies (4), might be inhibited by PSA. If such clustering served to mask HSPG-binding sites, attachment of the membranes to an HSPG substrate could be compromised (clustering model). Although we have not observed an effect of PSA on the spontaneous or antibody-induced clustering of NCAM to form patches visible by light microscopy, it is quite possible that this effect would involve aggregates of a small number of molecules.

In sum, the present findings provide a quite precise description of a molecular interaction involving two very abundant components of the extracellular environment of many tissues, thus validating earlier suggestions (28) that the role of PSA in NCAM binding properties might be more complex than previously reported. At the same time, they raise a far greater number of questions as to the exact basis for that interaction and the biological realm in which it operates. Clearly, a resolution of these issues is going to require a far more complete understanding of the mechanisms of action of NCAM and the full range of NCAM-mediated biology and thus are likely to remain an issue in the field for quite some time.

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