Binding Properties of Detergent-solubilized NCAM

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Abstract. An assay has been designed for the identification of NCAM-binding proteins present in an NP-40 detergent extract of brain membranes. This method, which is capable of analyzing both heterophilic and homophilic interactions, uses species-specific antibodies against NCAM in combination with radioiodination, so that after unlabeled chicken and iodinated frog brain membrane proteins were allowed to interact, the chicken NCAM could be specifically isolated by immunoaffinity adsorption. The radiolabeled frog proteins coisolated with chicken NCAM were then characterized by one- and two-dimensional gel electrophoresis in combination with immunoblotting. The only detectable NCAM-binding proteins were identified as the 140- and 180-kD forms of NCAM. The presence and absence of polysialic acid on NCAM did not change the amount or nature of the frog proteins immunopurified under these conditions. As an alternative for detecting heterophilic ligands, a simplified immunoprecipitation method was employed using either iodine or sulfate radiolabels. Again under these conditions only NCAM was detected. These results are consistent with the hypothesis that the major binding protein for NCAM is NCAM itself, and suggest that differences in polysialic acid content do not directly alter the properties of NCAM's homophilic binding site.

THE neural cell adhesion molecule (NCAM)[1] is a transmembranous glycoprotein present on many cell types, including neurons, glia, and muscle (for review, see Rutishauser and Jessell, 1988). It has been proposed that NCAM forms a homophilic bond during cell–cell adhesion, that is, NCAM on one cell binds to NCAM on an apposing cell (Rutishauser et al., 1982). Several lines of evidence were used initially to propose this mechanism: (*a*) NCAM-mediated adhesion between two cells is strongly inhibited by treating only one of the cells with Fab fragments that block NCAM binding function, (*b*) purified NCAM binds to cells that express NCAM; (*c*) purified NCAM specifically binds to an NCAM-Sepharose column; and (*d*) lipid vesicles containing only NCAM bind both to cells that express NCAM, and to each other (Rutishauser et al., 1982). More recently, it has been possible to specifically confer low levels of NCAM-mediated adhesion by transfection of L cells with NCAM cDNA (Edelman et al., 1987). In addition, EM indicates that the purified molecule can self-associate in detergent solution through contact between extracellular domains thought to be important for cell adhesion (Hall and Rutishauser, 1987; Frelinger et al., 1986). This combination of evidence suggests a direct role for NCAM in mediating cell–cell adhesion as well as the ability of the molecule to serve as a homophilic ligand.

However, in each of these studies NCAM alone was monitored, and therefore the important distinction remained as to whether NCAM is its own primary binding protein, or just one of several on the cell surface. To search more generally for NCAM-binding proteins, a procedure was devised that assays the full complement of detergent-extractable membrane components. Moreover, it takes advantage of the fact that NCAM-mediated adhesion can occur with comparable efficiency between membranes from different vertebrates (Hoffman et al., 1984; Hall and Rutishauser, 1985). Thus, by using neural membranes from two species in combination with species-specific anti-NCAM antibodies and radioiodination, it is possible to identify individual components even in a homophilic binding mechanism.

1. *Abbreviations used in this paper*: endo-N, a soluble form of endoneuraminidase produced by K1F bacteriophage; NCAM, neural cell adhesion molecule.

Materials and Methods

Preparation of Brain Membrane Vesicles

Brain membrane vesicles from E9/10 white Leghorn chickens and adult *Rana pipiens* frogs (Hazan Farms, Alburg, VT) were prepared by sucrose density centrifugation as described (Hoffman et al., 1982). Brains were homogenized at a ratio of 0.5 g:10 ml PBS plus 100 KIU aprotinin (Sigma Chemical Co.) in a Dounce homogenizer fitted with a loose pestle. Homogenates were layered on discontinuous sucrose gradients of 10% above 42% in PBS and spun at 35,000 rpm for 45 min at 4°C in a Ti60 rotor (Beckman Instruments, Inc., Palo Alto). The interface between 10 and 42% was collected and washed twice in PBS/aprotinin at 13,000 rpm for 10 min in a Sorvall GSA rotor (Sorvall, Inc., Norwalk, CT). Most vesicle pellets were resuspended in PBS/aprotinin to 10% vol/vol.

Removal of the polysialic acid from NCAM was performed on some vesicles using a purified phage endoneuraminidase (endo-N; Vimr et al., 1984) that specifically cleaves alpha-2,8 polysialic acid (gift of E. R. Vimr, University of Illinois, Urbana-Champaign, IL). For thorough digestion, 1 μl (15 units) of endo N was mixed with 100 μl of 10% membrane vesicles at

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0°C for 30 min with intermittent agitation. After endo-N treatment, vesicles were washed three times with 100 vol of PBS to remove excess enzyme.

Radiolabeling of Membrane Proteins

Membrane proteins were iodinated by lactoperoxidase treatment of membrane vesicles as described (Cook and Lilien, 1982). 200 μl of 10% vesicles were washed twice in PBS and resuspended in 2 pellet vol of ice-cold lactoperoxidase (50 U/ml in Hepes-buffered saline plus 10 mM glucose). 500 μCi $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) was mixed with 1 pellet vol of 200 μM H_2O_2 in Hepes-buffered saline/glucose at room temperature. To start iodination, the lactoperoxidase/membrane suspension was rapidly warmed to room termperature and mixed with peroxide/iodine for 90 s. Iodination was stopped with the addition of 20 vol ice-cold PBS containing 5 mM KI. Vesicles were then washed three times with cold PBS/aprotinin before use.

$^{35}SO_4$-labeling was performed by inoculation of E9 chick embryos in ovo with 600 μCi $Na_2{}^{35}SO_4$, followed by incubation at 37°C for 6 h. Brain membranes were isolated as above. These conditions maximize the labeling of proteoglycans (Carrino and Caplan, 1984), which tend to be less efficiently labeled by iodine. Proteoglycans labeled with $^{35}SO_4$ by this procedure were characterized by ion-exchange chromatography, gel filtration, and nitrous acid sensitivity. After retention on a DEAE column, <1% of the total sulfate label in the extract was eluted by 0.25 M NaCl, representing most of the glycoprotein, whereas 85% was eluted with 1 M NaCl, as is characteristic of proteoglycans. These proteoglycans were found in the void volume after G50 gel filtration, and 60% of the sulfate label in this fraction moved into the dye front on G25 after treatment with nitrous acid to degrade heparan sulfate proteoglycan. Thus the bulk of the $^{35}SO_4$ label is associated with proteoglycans, and in particular with heparan sulfate proteoglycan, and very little is associated with glycoproteins, such as NCAM.

Antibodies

mAbs directed against localized epitopes on chicken NCAM (Watanabe et al., 1986; Frelinger and Rutishauser, 1986) were 4D and 30B, which recognize intracellular epitopes, and 5E and 12B, which react with extracellular epitopes near the amino-terminal region of each of the three major NCAM polypeptides. Frog NCAM was detected using polyclonal antibody R16 (Jacobson and Rutishauser, 1986), which also cross-reacts with chicken NCAM.

Binding Assays

40 μl of a 10% vol/vol chicken vesicle suspension 120 μl of iodinated frog vesicle suspension (10% vol/vol), diluted 1:1 in PBS/DNase I (20 μg/ml; Sigma Chemical Co.) were mixed for 20 min at 37°C, and then pelleted at 13,750 *g* for 1 min. The pelleted vesicles were solubilized in 20 vol of extraction buffer (PBS plus 0.5% NP-40) for 30 min at 4°C. After extraction, insoluble material was removed by centrifugation at 13,750 *g* for 5 min at 4°C. Chicken NCAM and NCAM-associated frog molecules were then immunoadsorbed for 2 h at 4°C to mAb 5E coupled to Sepharose 4B. The amount of 5E on the Sepharose was sufficient to adsorb all the chicken NCAM: 20 μg immunoglobulin/20 μl beads was used for <1 μg total chicken NCAM, and the supernatants after adsorption contained no chicken NCAM as detected by SDS-PAGE and immunoblot reactivity (data not shown). Immunoadsorbed proteins were washed three times with 100 vol of extraction buffer, then mixed with 40 μl Laemmli buffer and heated at 100°C for 3 min before fractionation by SDS-PAGE. To control for nonspecific adsorption, beads coupled with an mAb directed against an irrelevant antigen (anti-trinitrophenol) were used. To determine the effects of adding unlabeled frog membranes at solubilization, 500 μl of a 10% suspension of unlabeled frog vesicles was added to the labeled frog vesicles just before mixing with the chicken vesicles. In this case, all chick NCAM was adsorbed by the 5E beads. In the simpler assay using iodine or sulfate-labeled chicken membranes alone, 80 μl of pelleted vesicles was solubilized as above, and immunoadsorbed with 50 μl 5E beads and prepared for analysis by SDS-PAGE.

Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE (Laemmli, 1970) using 8 × 6 cm gels of 7.5% acrylamide. Samples of the Sepharose bead/Laemmli buffer mixture, containing the immunoadsorbed and solubilized proteins were loaded directly into gel sample wells. Some samples were subjected to two-dimensional electrophoresis (O'Farrell, 1975). In this case, bead/buffer samples prepared in Laemmli buffer were mixed 1:1 with O'Farrell buffer, and 50 μl loaded onto the 1-mm i.d. IEF gels containing a mixture of ampholines in the pH 4–6 range. First dimensional tube gels were subjected to electrophoresis for at least 5,000 v-h, then incubated with Laemmli buffer and applied to a 7.5% gel for second dimensional molecular weight analysis.

After electrophoresis, proteins were transferred to nitrocellulose and detected by immunoblotting techniques (Burnette, 1981; Towbin et al., 1979). Nonspecific sites on the nitrocellulose were blocked by incubation with Blotto (Johnson et al., 1984) for 1 h at 37°C. Filters were then incubated with 10 μg/ml anti-chicken NCAM monoclonal antibody 5E or 20 μg/ml anti-frog NCAM R16 in Blotto for a minimum of two hours at RT. After washing with PBS, filters were incubated with a 1:1,000 dilution in Blotto of peroxidase coupled second antibody for 2 h at room temperature. Filters were washed again, and antigens visualized by reaction of bound peroxidase using 4-chloro, 1-napthol as described (Hawkes et al., 1982). To visualize radiolabeled proteins, immunoblots were exposed for autoradiography for ~1 wk at −70°C using Kodak X-AR 5 film (Eastman Kodak Co., Rochester, NY) and a Lightning Plus intensifying screen (Dupont Co., Wilmington, DE) (Laskey and Mills, 1977). Scanning densitometry of autoradiographs was performed using a Shimadzu CS-930 gel scanner at 550 nm (Shimadzu Scientific Instruments Inc., Columbia, MD).

Results

Assay for NCAM-Binding Proteins

The assay used in these studies is illustrated schematically in Fig. 1. Unlabeled chicken and radioiodinated frog membranes were mixed together, their membrane proteins were solubilized with a nonionic detergent, and the chicken NCAM was specifically isolated using 5E Sepharose. Iodinated frog proteins, which were isolated together with the chicken NCAM, were then fractionated by SDS-PAGE and detected by immunoblotting and autoradiography.

In this procedure, the molecular and species specificity of the 5E monoclonal is critical. The specificity of 5E for NCAM has been documented in several of our previous studies (for example, Sunshine et al., 1987); it is also useful to note that mAbs against other abundant cell adhesion molecules, such as L1/G4, do not coisolate NCAM (Rathjen et al., 1987). Absolute species specificity is a more difficult problem. Four mAbs against chicken NCAM were initially tested for their ability to isolate solubilized brain membrane proteins from chicken but not frog (Fig. 2). Two antibodies, 4D and 30B, which react with intracellular epitopes on chicken NCAM (Watanabe et al., 1986; Frelinger and Rutishauser, 1986), immunoadsorbed proteins from both frog and chick brain membranes with the electrophoretic mobilities and immunoblot profiles characteristic of NCAM. In contrast, immunoaffinity isolation with antibodies 5E and 12B, which recognize extracellular epitopes on chicken NCAM, isolated chicken NCAM, but not frog NCAM. Further, 5E and 12B did not show Western immunoblot reactivity with any frog proteins in a total frog brain homogenate (data not shown). On this basis, mAb 5E was judged suitable for the purpose of specifically isolating chick NCAM from a mixture of chick and frog proteins.

It is important to note that this method of adsorption does not select a subpopulation of NCAM molecules, as a large excess of immunoadsorbent was used and it was determined that no detectable chicken NCAM remained in solution after adsorption. Although 5E itself specifically adsorbs chicken NCAM, lower molecular weight proteins (<60 kD) were also adsorbed from iodinated brain membrane proteins by a

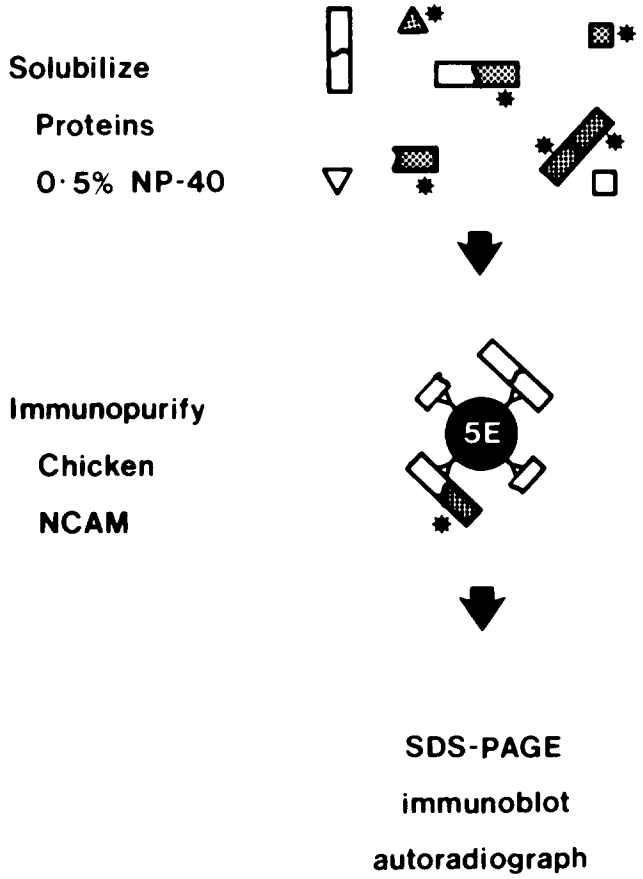

Figure 1. Assay to identify NCAM binding components. An NP-40 detergent extract containing chicken brain membrane proteins (*unshaded*) and iodinated frog brain membrane proteins (*shaded, with asterisk*) was incubated with mAb 5E-Sepharose, which specifically isolates chicken NCAM. Iodinated frog proteins that coisolate with chicken NCAM on 5E were detected by SDS-PAGE, followed by immunoblotting and autoradiography.

variety of immunoglobulin-Sepharose beads and even Sepharose beads which had been chemically activated and reacted with ethanolamine, but not coupled to protein (Fig. 3). These lower molecular weight proteins are assumed to reflect a nonspecific interaction of proteins with the Sepharose beads in this assay. In using SDS-PAGE to identify binding molecules, it is also important to recognize that small proteins, of ~30 kD or less, would not be resolved from the label in the dye front of the 7.5% SDS-PAGE gels used in the assay.

Characterization of NCAM-binding Proteins

Immunoadsorption of chicken NCAM from extracts of chick-frog vesicle mixtures resulted in the coisolation of small but readily detected quantities of high molecular weight frog protein. While the signal generated in these assays required autoradiographic exposures up to a week, the results were reproducible among the two to five independent experiments used to identify NCAM binding proteins in each study.

When increasing amounts of frog extract were incubated with the same amount of chicken extract, the immunoadsorbed label increased proportionately (Fig. 4). Thus the chicken NCAM precipitated by 5E in this assay was not saturated in its binding capacity for frog proteins. In each case, all chicken NCAM was immunoisolated by 5E-Sepharose, and was not detected in the supernatant. Nevertheless, even with an intermediate amount of frog vesicles, not all frog NCAM bound to the chicken NCAM, and remained in the supernatant (data not shown).

While the assay should allow the detection of any radioiodinated protein over 30 kD capable of binding stably to chicken NCAM in detergent solution, only iodinated frog proteins at ~140 and 180 kD were specifically isolated by 5E-Sepharose from extracts of the mixed vesicle populations (Fig. 5).

Two-dimensional IEF/SDS-PAGE analysis combined with immunoblotting for NCAM established that the iodinated proteins are NCAM (Fig. 6). The R16 immunoblot largely matches the autoradiographic profile, and in addition demonstrates that the iodinated species have pIs of ~5.6 and 5.0, corresponding to NCAM with a low and high content of polysialic acid, respectively (Hoffman et al., 1982). It is important to note that R16 reacts with both chicken and frog

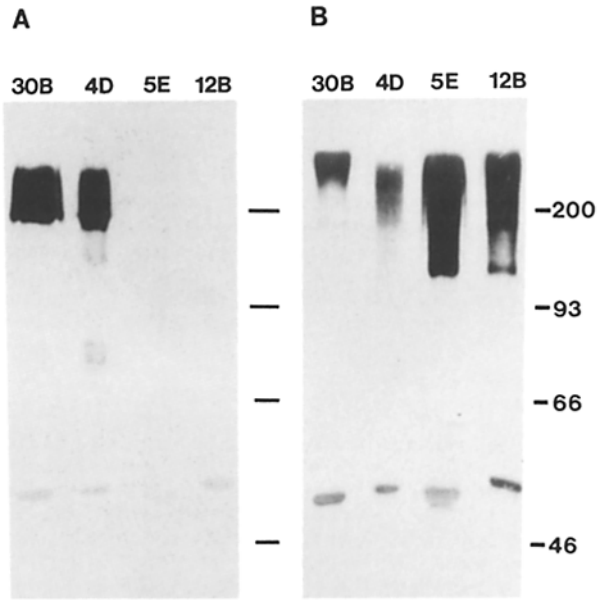

Figure 2. Species specificity of NCAM antibodies. Monoclonal antibodies covalently coupled to Sepharose 4B were used to immunoisolate proteins from detergent-solubilized brain membranes from adult frog and E9/10 chicken. Isolated proteins were separated by SDS-PAGE, and their identity established by immunoblotting. NCAM proteins from frog were visualized by reactivity with polyclonal anti-frog NCAM R16 (*A*) and chicken NCAM proteins recognized by monoclonal anti-NCAM 5E (*B*). Intracellular epitopes which react with antibodies 30B and 4D are found only on the longest NCAM-180 proteins from both frog (A; lanes *1* and *2*) and chicken (*B*; lanes *1* and *2*), and therefore appear with a characteristic high molecular weight NCAM profile. However, immunoadsorption using antibodies 5E and 12B, which react with extracellular epitopes found on all major forms of NCAM, did not isolate frog NCAM (A; lanes *3* and *4*). In parallel control experiments, these reagents immunoisolate chicken NCAM with characteristically broad molecular weight profile reflecting NCAM-140 and NCAM-180 polypeptides with variable amounts of polysialic acid (*B*; lane *3* and *4*). The lower molecular weight bands are due to second antibody reactivity with the heavy chain of immunoglobulin which had been released from the affinity support.

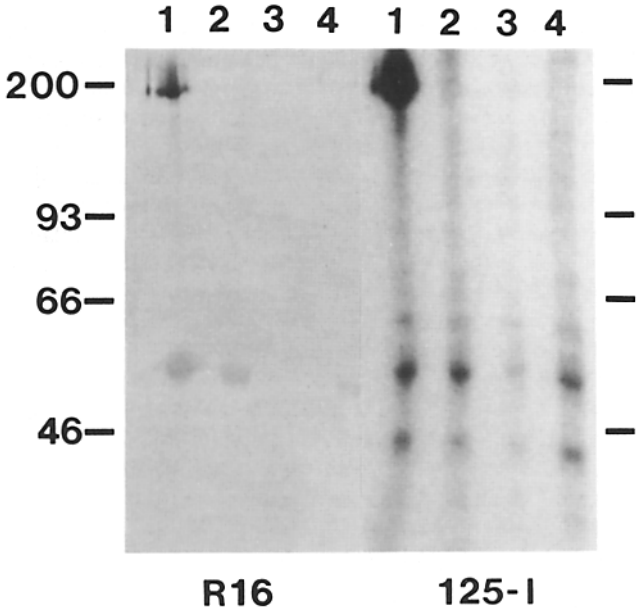

Figure 3. Low molecular weight proteins nonspecifically adsorbed to Sepharose 4B. Iodinated frog brain membranes were solubilized in extraction buffer containing NP-40 and insoluble material removed by centrifugation. Equal volumes of detergent-extracted frog proteins were incubated with the indicated derivatized Sepharose 4B for 2 h at 4°C. The beads were then washed extensively, mixed with an equal volume of Laemmli buffer, and separated by electrophoresis. After protein transfer to nitrocellulose, frog NCAM was detected using polyclonal antibody R16. The R16 profile of frog NCAM and the corresponding autoradiograph are shown for beads coupled with (*a*) mAb 4D; (*b*) mAb 5E; (*c*) no immunoglobulin; and (*d*) mAb against an irrelevant antigen (trinitrophenol).

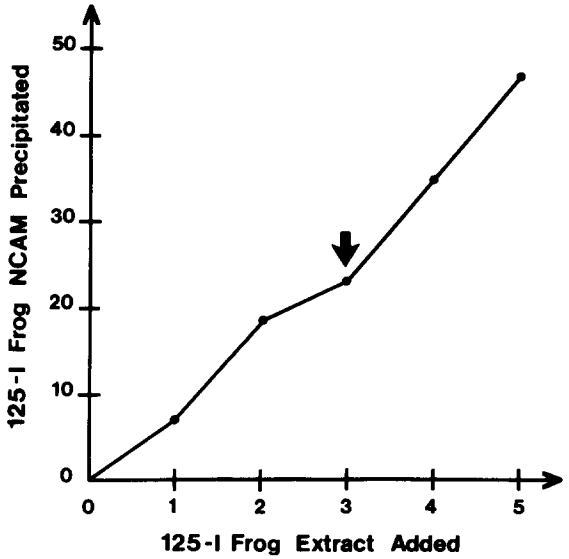

Figure 4. Immunoadsorbed chicken NCAM is not saturated with frog NCAM. Increasing amounts of iodinated frog brain vesicles were combined with 40 μl of 10% vol/vol chicken brain vesicles. In each case, the same amount of chicken NCAM was isolated by mAb 5E Sepharose, and analyzed by SDS-PAGE. The total amount of ^{125}I frog NCAM coisolated was quantified by scanning densitometry and is plotted in arbitrary units against the ratio of frog to chick membranes used. The amount of ^{125}I frog NCAM coisolated is proportional to the amount of frog membrane added. The bold arrow indicates the amount of frog vesicles used in the assay for NCAM-binding proteins.

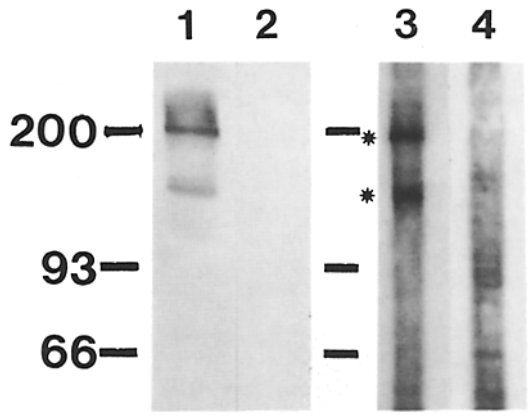

Figure 5. Specific coisolation of frog proteins during immunoadsorption of chicken NCAM from solubilized membranes. A mixture of chicken and frog brain vesicles were solubilized in NP-40 and the chicken NCAM immunoisolated using mAb 5E. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and characterized by immunoblotting using antibodies to frog NCAM (*Rl6*), or to chicken NCAM (*5E*), and by autoradiography (^{125}*I*). Frog NCAM in total frog brain homogenate has characteristic 120-, 140-, and 180-kD forms (lane *1*), whereas iodinated frog brain membranes isolated by 5E-Sepharose contain no frog NCAM (lane *2*). Iodinated frog proteins isolated by 5E-Sepharose from frog brain vesicles after incubation in the presence (lane *3*) or absence (lane *4*) of chicken brain vesicles containing NCAM. Asterisks indicate the 140- and 180-kD frog proteins specifically isolated in the presence of chicken and frog vesicles.

NCAM, so that the immunoblot profile reflects a combination of NCAMs from both species, while the autoradiogram represents only the frog protein. The adult frog NCAM in this assay is less polysialylated and appears as more distinct bands at M_r 180 and 140 kD, with smaller amounts of more heavily sialylated forms ranging up to 250 kD. Moreover, the R16 antibody reacts predominantly with the 180-kD form of frog NCAM (see Levi et al., 1987), so that the actual abundance of NCAM-140 is greater than that indicated by the immunoblot. By contrast, the two-dimensional analysis of two other cell adhesion molecules, chicken G4/L1 glycoprotein (visualized by polyclonal antibody R20) and chicken N-Cadherin (visualized by polyclonal antibody RR2, gift of Dr. Jack Lilien, University of Wisconsin, Madison, WI) produced a clearly different pattern (data not shown). The two radiolabeled components at M_r 50–60 are present in the immunoblot analysis of NCAM, and represent their nonspecific interaction with the Sepharose beads, as shown in Fig. 3.

The addition of fivefold excess unlabeled frog vesicles to the chicken-frog vesicle pellet just before solubilization caused a large decrease in the amount of iodinated NCAM coisolated with the chicken NCAM (Fig. 7). Although the chicken and frog vesicles were allowed to aggregate together before solubilization, the ability of added frog vesicles to decrease the amount of radiolabel coisolated with chicken NCAM suggests that the interactions detected primarily represent events that occurred in detergent solution.

Effects of Polysialic Acid on NCAM Binding

The presence or absence of polysialic acid on NCAM did not change the nature or amount of frog NCAM polypeptides identified. In these experiments, unlabeled chicken and iodin-

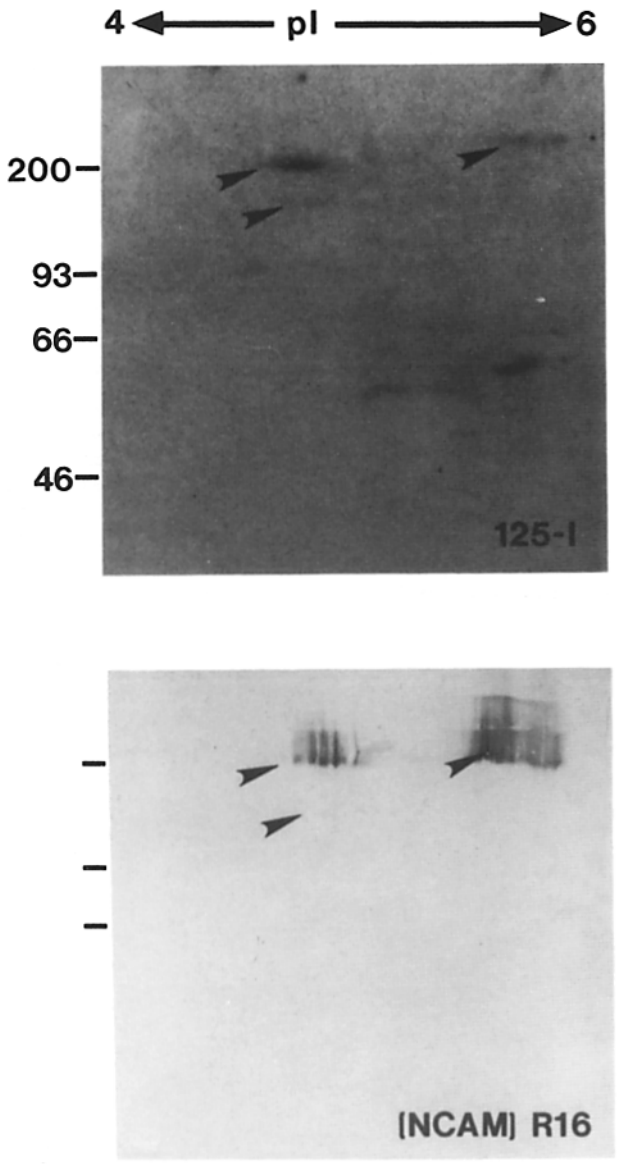

Figure 6. Comparison of coisolated iodinated proteins with NCAM using two-dimensional analysis. 5E-Sepharose immunoisolates of a solubilized mixture of chicken and frog membranes contain iodinated proteins that comigrate with NCAM detected by immunoblot with R16 (*arrows*). The broader bands and lower level of NCAM-140 reactivity seen in the R16 blots as compared with the autoradiogram reflect the specificity of this antibody (see text). The iodinated components <60 kD reflect proteins nonspecifically adsorbed to the Sepharose (see Fig. 3). IEF was carried out in the first dimension with ampholines in the range pH 4–6. The second dimension was SDS-PAGE in 7.5% acrylamide. Isoelectric points were estimated by measuring the pH of 1-cm slices from reference gels in the first dimension.

ated frog vesicles were each treated with endo-N to remove NCAM polysialic acid before mixing. Embryonic chicken brain NCAM is highly sialylated while NCAM from adult frog brain is less sialylated, but NCAM from both species had a faster mobility and more distinct appearance after endo-N removal of polysialic acid (Fig. 8 *A*, compare lanes *5E*, *1* and *2* for chicken NCAM, and *Rl6*, *1* and *2* for frog NCAM). When endo-N-treated vesicles were used in the assay, the iodinated frog proteins that coisolated with chicken NCAM had the electrophoretic mobilities of desialylated NCAM (Fig. 8 *A*, 125-I). Except for this expected change in mobility, however, the results were similar to those obtained without the use of endo-N. Quantification by scanning densitometry (Fig. 8 *B*) of the frog NCAM isolated from control and endo-N-treated vesicles indicated that the ratios of NCAM-180 to NCAM-140 were similar after endo-N treatment. Further, a comparison of the total NCAM radiolabel obtained (including endo-N sensitive polysialylated NCAM) indicated that similar amounts of the molecule were immunoaffinity isolated under both conditions, with possibly a slight decrease after endo-N treatment. Therefore, although a substantial fraction of chicken NCAM binding sites remain available (Fig. 4) and some frog NCAM remained in solution after immunoadsorption of the chicken NCAM, the removal of NCAM polysialic acid from both NCAMs did not change the nature or amount of frog NCAM bound.

Comparison of Iodine and Sulfate Labeling

To examine more directly the possibility of heterophilic NCAM interactions, simple immunoadsorptions of chicken NCAM, combined with either ^{125}I or $^{35}SO_4$ labeling, were monitored by SDS-PAGE and autoradiography. While iodination is an effective label for NCAM and most other cell surface proteins, the efficiency of labeling is dependent on tyrosine content, and some cell surface components might be underrepresented in the search for heterophilic binding. Of particular concern are sulfated proteoglycans, which have a low tyrosine content and have been implicated in NCAM-mediated binding (Cole et al., 1986*a*). To address this prob-

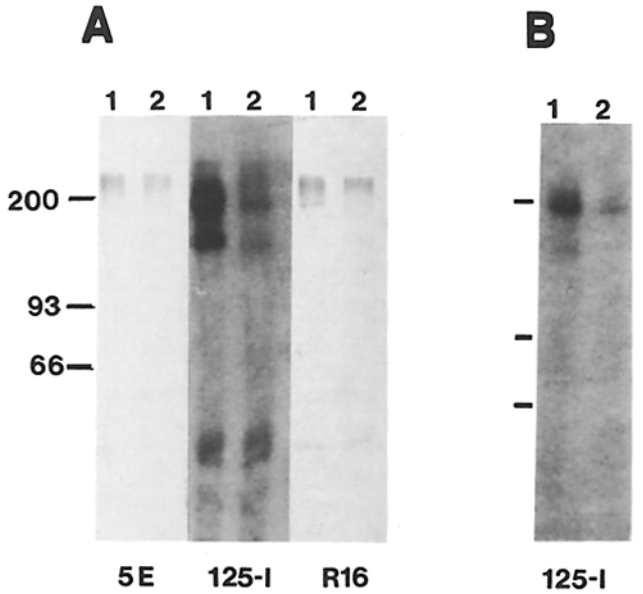

Figure 7. Effects of dilution with unlabeled frog vesicles on coisolated labeled frog proteins. The addition of unlabeled frog brain vesicles to the chicken/frog vesicle mixture before (*A*) or during solubilization (*B*) diminished the intensity of the iodinated frog NCAM co-isolated with chick NCAM. The chicken NCAM in the undiluted control (*1*) and dilution samples (*2*) is detected by immunoblotting with 5E. The frog NCAM immunoreactivity with R16 is shown in the control (*1*) and dilution (*2*) samples. The corresponding ^{125}I autoradiogram of coisolated frog proteins is shown for the control (*1*) and dilution (*2*) in each case.

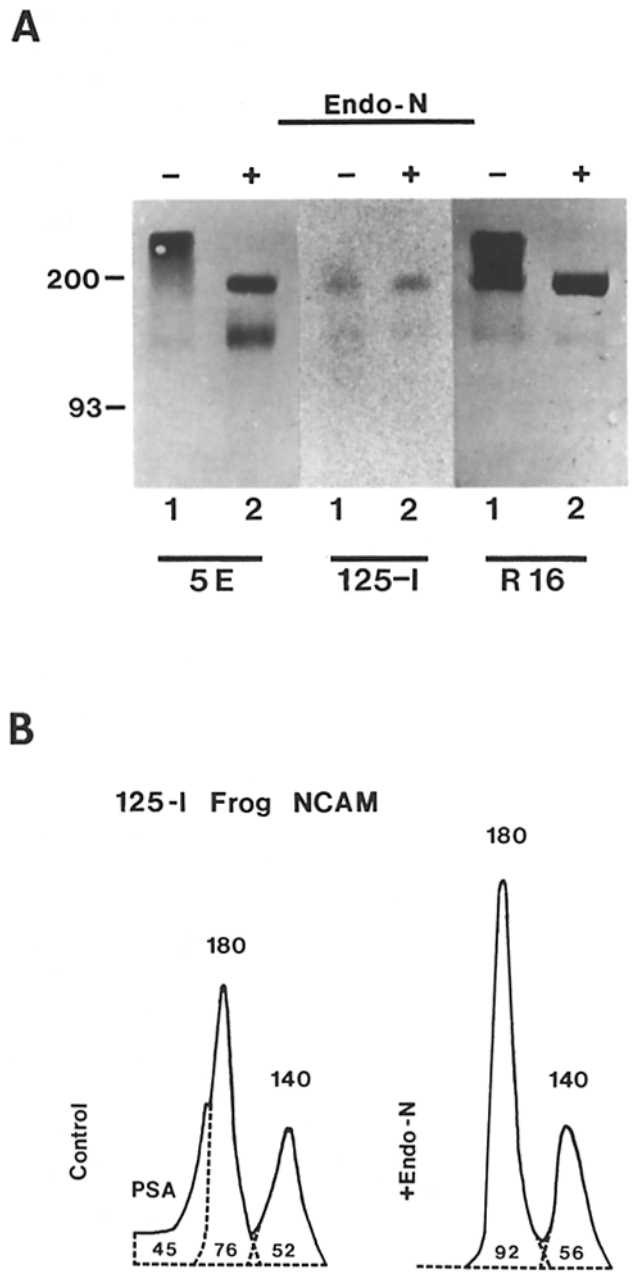

Figure 8. Enzymatic removal of NCAM polysialic acid by endo-N does not change the nature or amount of coisolated frog proteins. (*A*) The effect of NCAM polysialic acid on the molecular species coisolated from frog was evaluated by treating both chicken and frog membranes with endo-N before aggregation (+) and comparing them with untreated controls (−). (*5E*) The change in electrophoretic mobility of chicken NCAM upon endo-N treatment from a broad smear (*1*) to focused bands at 140 and 180 kD (*2*) is seen using antibody 5E. (*^{125}I*) Iodinated frog proteins immunoisolated from chicken and frog vesicle coaggregates, which had either the normal complement of NCAM polysialic acid (*1*) or had polysialic acid removed by endo-N (*2*). (*RI6*) Adult frog NCAM is relatively desialylated in the control (*1*), and the remaining NCAM polysialic acid was removed by incubation with endo-N (*2*). (*B*) The form and amount of NCAM detected in autoradiogram lanes (^{125}I) were quantified by scanning densitometry. The profiles of NCAM material detected with and without endo-N treatment are shown, and the integrated areas representing 140 kD, 180 kD, and polysialylated NCAM are indicated in arbitrary units at the base of each peak.

lem, the iodine and sulfate label were compared. In this assay, the direct immunoadsorption of labeled chicken proteins to 5E beads has the advantage of increasing the sensitivity of the assay, so that less prevalent binding interactions might be observed. Moreover, because species specificity is not important, it was possible to carry out the immunoadsorption with another mAb against a different region of the molecule (mAb 4D, which recognizes an intracellular epitope). The use of a different antibody serves as a control for the possibility that mAb 5E might itself compromise a heterophilic interaction.

When either ^{125}I- or $^{35}SO_4$-labeled chick brain vesicles were solubilized in 0.5% NP-40 and NCAM adsorbed to 5E Sepharose, the single component detected by SDS-PAGE and autoradiography colocalized with the NCAM in the sample, as detected by immunoblotting (Fig. 9, lanes *1–3*). The same result was obtained when a second mAb (4D) against an intracellular epitope was used (data not shown). To exclude the presence of a non-NCAM component that comigrated with NCAM, the immunoadsorbed $^{35}SO_4$-labeled material was treated with endo-N to remove polysialic acid from NCAM, and then reanalyzed. The endo-N treatment resulted in a characteristic shift in the mobility of NCAM (Fig. 9, lane *4*) and an identical shift in the mobility of the $^{35}SO_4$-material detected by autoradiography (Fig. 9, lane *5*). Endo-N treatment of $^{35}SO_4$-labeled extracts before immunoadsorption yielded similar results.

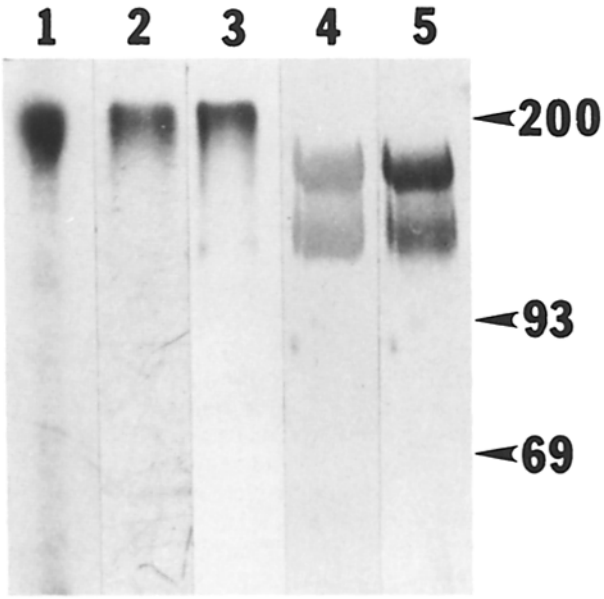

Figure 9. ^{125}I- and $^{35}SO_4$-labeled chick brain proteins isolated by direct immunoprecipitation with 5E-Sepharose. Membrane components were labeled with $^{35}SO_4$ or ^{125}I (see Materials and Methods) and mixed with PBS, pH 7.4, containing 0.5% NP-40. Soluble material was then incubated with 5E Sepharose, the beads washed in the same buffer, and the sample prepared for SDS-PAGE analysis. Some samples were treated with endo-N to remove polysialic acid, after adsorption to the beads. (*1*) ^{125}I label autoradiogram; (*2*) $^{35}SO_4$ label autoradiogram; (*3*) 5E immunoblot of $^{35}SO_4$ label sample; (*4*) 5E immunoblot of $^{35}SO_4$ label sample after endo-N treatment; (*5*) autoradiogram of $^{35}SO_4$ label after endo-N treatment. The top gel interface, at which position some proteoglycans would appear, is included within the figure but did not contain detectable radiolabel.

Discussion

Previous studies suggested that NCAM on one cell can serve as a direct or indirect receptor for NCAM on an apposing cell. However, it has remained unclear whether NCAM is the only or predominant receptor among the complete repertoire of cell surface proteins. This problem largely reflects the lack of an assay that unequivocally identifies homophilic binding among NCAMs in a detergent extract of cell surface membranes. The present approach addresses this difficulty by utilizing radiolabeling methods and brain membranes from two different species with compatible adhesion systems.

With the use of different species, the assumption is made that chicken and frog have the same NCAM-binding proteins and that there is no species specificity in their function. The rate of adhesion of frog vesicles to chick vesicles is as rapid as that between chick vesicles, and in both cases the adhesion is blocked by anti-chicken NCAM Fab, suggesting that any such differences are subtle (Hall and Rutishauser, 1985).

Together, the assays used in this study should identify any detergent-solubilized ^{125}I- or $^{35}SO_4$-labeled cell surface protein that binds stably and detectably to NCAM. Thus, the observation that NCAM was the only protein specifically detected supports the hypothesis that NCAM is a homophilic ligand. Both NCAM-180 and NCAM-140 participated in the cross-species formation of NCAM-NCAM bonds. This result is consistent with the fact that these polypeptide variants of NCAM are very similar in their extracellular NCAM binding regions (Cunningham et al., 1983). However, because both NCAM-180 and NCAM-140 were present in each membrane, our studies would not reveal whether there is any preferential binding among the different polypeptides.

Because the immunoadsorption protocol involves extensive washing with detergent-containing buffer of the 5E Sepharose after incubation with the membrane extracts, the present studies may have failed to detect molecules with a lower affinity for NCAM. Also, given the amount of background in most of the autoradiograms, which stems from the large amount of total protein required in this type of analysis, it is unlikely that molecules that might bind tightly but at low levels would have been reliably identified. Notable by its absence is heparan sulfate proteoglycan, which is proposed to associate with NCAM and participate in the cell–cell binding mechanism (Cole et al., 1986*a*), and recently has been found to coisolate with immunoaffinity-purified NCAM from extracts of chick retina and brain (Cole and Burg, 1989). Although the majority of sulfate-labeled macromolecules in our brain extract are proteoglycans and most of these are nitrous acid sensitive (see Materials and Methods), the only immunoadsorbed sulfate obtained from chick brain was in NCAM itself. Thus it would appear that any detectable level of interaction of this proteoglycan with NCAM does not survive the conditions of our assay. That NCAM-NCAM interactions can occur without detectable coisolation of sulfated proteoglycan is consistent with a less direct role of this molecule in adhesion, such as the promotion or stabilization of homophilic NCAM binding (Cole et al., 1986*a*), and the demonstration that binding among purified NCAMs is not affected by the presence of heparin (Moran and Bock, 1988). However, an alternative that cannot presently be ruled out is that one molecule of proteoglycan might serve as a bridge between large numbers of CAMs.

Although intact membranes were mixed initially, the assay used largely reflects interactions that occurred in detergent solution. The observation that NCAM can self-associate in solution does not prove that such an interaction necessarily occurs at the cell surface to mediate cell–cell adhesion. However, in our previous studies, electron microscopic visualization of immunopurified NCAM multimers dried from an NP-40 solution suggested that the predominant NCAM interaction in this detergent occurred through the domains of the molecule believed to participate in formation of cell-cell bonds (Hall and Rutishauser, 1987). In addition, the presence of nonionic detergent has been shown to be effective in minimizing nonspecific aggregation through hydrophobic transmembrane domains (Becker et al., 1989). In the present study, the most convincing evidence for specificity is the fact that while the full complement of extractable membrane proteins was allowed to interact with NCAM, only NCAM-NCAM interactions were detected.

Polysialic acid can alter the kinetics of NCAM-mediated adhesion (Hoffman et al., 1983; Rutishauser et al., 1985). However, in the present assay the presence or absence of polysialic acid on vesicles did not affect the amount or molecular form of the NCAM-binding proteins detected. This result suggests that NCAM polysialic acid does not change the avidity or specificity of NCAM-NCAM binding itself. This observation is consistent with the previous findings that neither the molecular weights of purified NCAM aggregates (Hoffman et al., 1982) nor the aggregation state observed by EM (Hall and Rutishauser, 1987) are substantially altered after removal of polysialic acid. Dramatic changes in NCAM-mediated binding upon removal of polysialic acid have only been observed when NCAM is associated with membranes or attached to a physical support (Hoffman and Edelman, 1983; Rutishauser et al., 1982; Cunningham et al., 1983). Thus the sum of observations on NCAM's binding properties with respect to polysialic acid are most consistent with the more recent proposal (Rutishauser et al., 1988) that this carbohydrate affects cell–cell interaction by a mechanism whereby physical or repulsive properties of the hydrated sugar alters overall membrane–membrane apposition.

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