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# Solid-phase binding analysis of N-CAM interactions with brain fodrin

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**Abstract**

The large cytoplasmic domain form of the neural cell adhesion molecule N-CAM has been reported to interact specifically with fodrin, a submembranous cytoskeletal protein. We tested the abilities of fodrins from bovine brain and embryonic chicken brain to bind to N-CAM that had been isolated from differentiated or undifferentiated mouse N2A neuroblastoma cells or from the brains of embryonic day 11 or day 14 chickens. Labeled fodrin samples bound with immobilized fodrin at a minimum soluble fodrin concentration of $2.5 \cdot 10^{-8}$ M, but the labeled fodrin did not bind to the immobilized N-CAM when incubated at 20-fold higher fodrin concentrations.

*Key words:* Cell adhesion; Fodrin; Spectrin; N-CAM

## 1. Introduction

The neural cell adhesion molecule N-CAM is a transmembrane glycoprotein that is expressed at the surface of a number of neural and non-neural vertebrate cell types and that can mediate adhesion between cells in vitro and in vivo [1–12]. N-CAM is expressed as a number of alternative polypeptide isoforms as the result of alternative splicing of RNA transcripts from a single gene [13–21]. Photobleaching experiments in mouse N2A neuroblastoma cells using isoform-specific monoclonal and polyclonal antibodies suggested that the 140 kDa transmembrane isoform (referred to as the sd or small cytoplasmic domain isoform) of N-CAM is freely mobile in the plane of the membrane but that the 180 kDa transmembrane isoform (referred to as the ld or large cytoplasmic domain isoform) is relatively immobile [22,23]. Immunoblot analyses revealed a switch in expression from both ld and sd isoform to the ld isoform alone upon neuronal differentiation and suggested that the ld isoform also accumulates at areas of cell-cell contact [22,23] and at newly forming synapses [24], although a later study [25] showed that the sd isoform also can accumulate at points of cell-cell contact in N2A cells. A series of immunoaffinity chromatography experiments and solid-phase radioligand binding assays provided evidence that the ld isoform of N-CAM interacts with the cytoskeletal protein fodrin [22,23], thus suggesting that an interaction with fodrin may serve as an anchoring mechanism that limits the lateral mobility of N-CAM within the plane of the membrane.

Fodrin is the brain isoform of spectrin, a component of the membrane cytoskeleton. Spectrin was originally isolated from erythrocytes [26–30], where it functions to maintain the cell's unique biconcave structure. It is a flexible rod-shaped protein found as a 1 million molecular weight tetramer consisting of two alpha and two beta subunits [31,32]. Fodrin is a closely related protein found in many nonerythroid tissues including the brain [33–36]. In the mammalian brain, the alpha subunit of fodrin has a molecular mass of 240 kDa and the beta subunit has a molecular mass of 235 kDa [37,38]. In the chicken brain the beta subunit has a molecular mass of 230 kDa [39].

Spectrin tetramers are arranged in a net-like fashion just beneath the erythrocyte cell membrane. Spectrin tetramers are joined at their ends by actin-containing

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junctional complexes and are attached to integral proteins of the plasma membrane through the junctional complexes and by interactions with adaptor proteins including ankyrin [40–43]. A similar arrangement is likely to hold for fodrin tetramers in nonerythroid cells [42]. Fodrin has been shown to interact with synaptic vesicles and can codistribute with the vesicles in the axons of neurons [44]. Fodrin molecules accompany the reorganization of the integral membrane proteins during the capping phenomenon in which antigens on the lymphocyte cell surface reorganize to the poles upon treatment with antiserum [45]. Fodrin is involved in generating specialized plasma membrane domains in polarized cells [46] and is associated with proteins of the major histocompatibility complex [47]. These results suggest that the lateral mobility of membrane proteins may be influenced by interactions with fodrin, and they make it plausible that an interaction of the ld isoform of N-CAM with fodrin may limit the lateral mobility of N-CAM within the plane of the membrane, leading to an increase in the local concentration of N-CAM at areas of cell-cell contact [23]. Furthermore, these interactions may be involved in the transduction of molecular binding interactions into adhesion between cells.

We therefore performed a number of binding experiments in order to quantitate the interaction between fodrin and the ld isoform of N-CAM. We tested both mammalian and chicken proteins in order to extend the analysis to the chicken, where much of the work on N-CAM biochemistry and developmental distribution has been done. Surprisingly, we were unable to detect a specific interaction between N-CAM and fodrin under several sets of conditions in which fodrin-fodrin binding was easily observed.

## 2. Materials and methods

*Materials.* Polyclonal rabbit antibodies and monoclonal mouse antibodies against chicken N-CAM (IgG) were gifts of B.A. Cunningham and G.M. Edelman [48]. Conditioned media from cultures of hybridoma cells expressing monoclonal antibody 224-1A6-A1 against chicken N-CAM [49] were obtained from Dr. Marianne Bronner-Fraser (University of California, Irvine). Monoclonal antibodies against mouse N-CAM (Mab310, IgG) and rabbit antibodies against mouse IgG were from Chemicon (Temecula, CA). Bovine brain spectrin (fodrin) was a gift from Dr. Velia Fowler (The Scripps Research Institute, La Jolla, CA). Bolton-Hunter reagent (Tagit) and *Vibrio cholerae* neuraminidase (2 units/ml) were purchased from Calbiochem (La Jolla, CA). Tissue culture and other reagents were from Irvine Scientific (Santa Ana, CA) or Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose and Sepharose 4B were obtained through Sigma. Fertilized chicken eggs were purchased from K&R Enterprises (Westminster, CA). Centricon filters were from Amersham (Arlington Heights, IL). PD-10 desalting columns containing Sephadex G-25 beads were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). The N2A mouse neuroblastoma cell line [50] was obtained from the American Type Culture Collection (Rockville, MD), CCL #131.

*Cell culture.* N2A cells [50] were maintained in the high glucose version of Dulbecco's modification of Eagle's medium containing 10% fetal calf serum and 100 $\mu$g/ml gentamicin on poly-D-lysine-coated plates. N2A cells were induced to differentiate in the same medium containing 0.2% fetal calf serum and 2% dimethylsulfoxide for 4 days [51]. Cells were passaged as needed by treatment with 2 mM ethylenediamine tetraacetic acid (EDTA).

*Purification of N-CAM.* Chicken or mouse N-CAM was purified from detergent extracts of embryonic brain membranes or cultured cells by affinity chromatography on immobilized anti-N-CAM monoclonal antibodies as previously described [48,52]. The amount of N-CAM recovered was estimated by measuring the absorbance at 280 nm of the recovered material and assuming that an absorbance of 1.0 in a 1 cm path length cell corresponds to a protein concentration of 1 mg/ml.

*Fodrin isolation from chicken brains.* Fodrin was isolated from 14 day chicken embryo brains according to a published procedure [53]. All procedures were performed at 4°C. Brains were collected from 240 chicken embryos at embryonic day 14 and were homogenized in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate (pH 7.4)) containing aprotinin (0.1 trypsin inhibitory units/ml) using a Sorvall Omnimixer for 90 s at the highest speed. The homogenate was centrifuged at $105\,000 \times g$ for 1 h. The pellet was washed once with homogenization buffer plus aprotinin and centrifuged at $105\,000 \times g$. Proteins were extracted with high salt buffer (10 mM Tris-HCl, 50 mM KCl, 3 mM $NaN_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol (DTT) (pH 8.2)) including aprotinin (0.1 trypsin inhibitory units/ml). Ammonium sulfate was added to a final concentration of 45% to precipitate proteins and the pH was maintained at 7 by the continuous addition of solid KOH. The precipitate was collected and dialyzed against TKE/KI buffer (10 mM Tris-HCl, 700 mM KI, 0.5 mM EDTA, 0.1 mM DTT (pH 8.2)) The dialyzate was cleared by centrifugation (60 min at $200\,000 \times g$). The supernatant was fractionated over a Sepharose 4B column. Fractions were collected and samples of the proteins were fractionated by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS PAGE) and the gel was

stained with Coomassie brilliant blue. Fractions containing the highest levels of fodrin were pooled and ammonium sulfate was added to a final concentration of 30%. The precipitate was collected by centrifugation, resuspended, dialyzed overnight and centrifuged as described above. The purified fodrin was concentrated with Centricon 10 filters (Amersham) and stored at −20°C in the presence of 0.2 mM DTT and 0.2 mM $NaN_3$.

*Fodrin iodination.* Fodrin was labeled with $^{125}I$ by the iodination method of Bolton and Hunter [54] using Bolton-Hunter reagent (Tagit) that had been iodinated with chloramine T and $Na^{125}I$ [55]. Briefly, Tagit was iodinated with 2 mCi $Na^{125}I$ solution in the presence of chloramine T and sodium metabisulfite. The reaction was quenched with nonradioactive NaI. Unincorporated $Na^{125}I$ was extracted with benzene. The aqueous phase was dried under nitrogen gas. 20 μg of fodrin at 1 μg/μl was added to the dried Tagit. The labeling reaction was allowed to proceed on ice for 1 h. The reaction was quenched with 0.2 M glycine for 5 min. Labeled fodrin was separated from unreacted $^{125}I$-labeled Tagit by chromatography over Sephadex G-25 in a prepacked Pharmacia PD-10 desalting column that was pre-equilibrated with PBS + 2 mg/ml ovalbumin + 0.1% Triton X-100. The column was developed using the same buffer. 0.5 ml fractions were collected and counted in a gamma counter. Peak fractions were identified and 0.2 mM $NaN_3$ and 0.2 mM DTT (final concentrations) were added to each. Fractions were quick frozen and stored at −20°C until used (usually the same day, always within 1 week).

*Immunoprecipitations.* Cells or cell membranes were dissolved in either a 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesufonate (Chaps) containing detergent buffer {150 mM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.0), 1 mM EDTA, 10 mM Chaps, 1 mM phenylmethylsulfonylfluoride} or a Triton X-100 containing detergent buffer (10 mM Hepes (pH 7.0), 150 mM NaCl, 1 mM EDTA, 2.5% Triton X-100, 0.1 trypsin inhibitor units/ml aprotinin). Cells were broken by 10 strokes in a Dounce homogenizer on ice followed by centrifugation at 225 000 × *g* in a Ti50 rotor using a Beckman ultracentrifuge. Antibodies (2 μl of a 5 mg/ml IgG stock) and radioimmunoassay grade bovine serum albumin (10 μl of a 30 mg/ml solution) were added directly to 500 μl of the supernatant and incubated 4 h at 4°C. When monoclonal antibodies were used to precipitate N-CAM, 2 μl of a 5 mg/ml solution of rabbit anti-mouse IgG was added to facilitate precipitation. 20 μl of a 50% (vol/vol) slurry of protein A-Sepharose was added and the mixture was incubated with shaking at 4°C overnight. The following day the Sepharose was pelleted in a microfuge and the supernatant discarded. The pellet was washed six times by cycles of resuspension in the detergent lysis buffer followed by pelleting in the microfuge.

*Solid phase radioligand binding analysis.* N-CAM was immunoprecipitated from either day 11 or 14 chick brains or mouse N2A cell by incubation of extracts with polyclonal or monoclonal antibodies directed against chicken or mouse N-CAM, followed by incubation with protein A-Sepharose. When monoclonal antibodies were used, 2 μl of a 5 mg/ml solution of rabbit anti-mouse IgG was added to facilitate precipitation. Where indicated, the pellets were incubated with 0.004 units of neuraminidase in 50 mM sodium acetate (pH 5.0), 2 mM $CaCl_2$, 2 mM EDTA for 3.5 h. The neuraminidase was inactivated by boiling the pellet in double-strength denaturing sample buffer.

For blot overlay assays, one volume of double strength denaturing sample buffer [56] was added to each pellet, boiled 3 min, then subjected to separation by SDS-PAGE. The proteins were transferred to nitrocellulose electrophoretically. The transfer was then washed three times 5 min in PBS, then incubated for 30 min at 37°C in PBS to remove SDS and reductant in an attempt to allow the proteins on the blot to refold. The transfer was incubated with 3 mg/ml bovine serum albumin in PBS or 30% normal goat serum in PBS to block nonspecific binding sites and then was incubated with 0.5–10 μg/ml $^{125}I$-labeled bovine or chicken brain fodrin in 5 ml fodrin binding buffer either at 4°C or at room temperature for a period of up to 24 h. We tested three different binding buffers that had been used in previous solid-state analyses of fodrin binding: 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 40 mg/ml bovine serum albumin, 0.2% (v/v) Triton X-100, the conditions used by Pollerberg et al. [23]; 0.1 M NaCl, 30 mM Tris-HCl (pH 8.0), 0.1 mM $MgCl_2$, 0.1 mM ATP, 0.5 mM DTT, which was used in the analysis of red cell spectrin binding to actin and protein 4.1 [57]; and 10 mM sodium phosphate (pH 7.5), 0.1 M NaCl, 0.1 mM DTT, 0.05% $NaN_3$, 0.25% gelatin, which was used in the analysis of brain fodrin binding to neurofilament subunit proteins [58]. Unbound fodrin was removed by six 1 min washes with PBS + 1% Triton X-100. The blots were exposed to Kodak XAR film with a Cronex (Dupont) intensifying screen at −80°C overnight.

For immobilized binding assays on Sepharose beads, the pellets were resuspended in 40 μl binding buffer (PBS + 2 mg/ml ovalbumin + 0.1% (v/v) Triton X-100 + 1 mM DTT + 10 mM $NaN_3$) containing 1–5 μg $^{125}I$-labeled chicken brain fodrin. The solution was incubated with agitation at room temperature or 37°C for 30 min. The protein A-Sepharose beads then were washed five times with 1 ml of PBS + 1% Triton X-100. The remaining radioactivity associated with the Sepharose pellets was determined with a gamma counter.

*Immunoblots.* Following electrophoretic separation, unlabeled immunoprecipitated material was transferred to nitrocellulose, blocked with ovalbumin (30 mg/ml in 10 mM Tris-HCl (pH 8.0), 137 mM NaCl, 0.1% Triton X-100) for 1 h before the addition of 50 μg of rabbit anti-N-CAM IgG, and allowed to incubate overnight at 4°C. The following day, the blot was rinsed five times with 10 mM Tris-HCl (pH 8.0), 137 mM NaCl, 0.1% Triton X-100 and blocked again with ovalbumin for 30 min before the addition of $^{125}$I-labeled protein A (2.5 μCi/10 ml). After 6 h the blot was rinsed 5 times with 10 mM Tris-HCl (pH 8.0), 137 mM NaCl, 0.1% Triton X-100 and exposed to Kodak XAR film with a Cronex (Dupont) intensifying screen at −80°C overnight.

## 3. Results

### *$^{125}$I-labeled mammalian fodrin did not interact with mouse or chicken N-CAM in a solid phase blot binding assay*

To examine the interaction between N-CAM and fodrin, chicken brain membrane proteins (20 mg) or between 1 and 20 μg of immunoprecipitated N-CAM from detergent extracts of embryonic chicken brain membranes or from undifferentiated or differentiated mouse N2A cell membranes were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose paper. As a control, 2 μg of purified bovine brain fodrin was included on the gel. The protein blots were incubated with radiolabeled bovine brain fodrin (0.5–2 μg/ml) for 4–24 h at room temperature or at 4°C with several different salt and detergent combinations [23,57,58]. Under all conditions tested, the radiolabeled fodrin bound to the immobilized fodrin on the nitrocellulose. The lowest concentration of fodrin that resulted in a detectable interaction with the bound fodrin was calculated to be $2.5 \cdot 10^{-8}$ M (assuming the molecular weight for fodrin to be approximately 200 000). The maximum concentration of fodrin used in these experiments ($1 \cdot 10^{-7}$ M) did not result in an interaction with N-CAM. Fig. 1 shows an autoradiogram of a representative experiment.

### *Mouse and chicken N-CAM did not interact with chicken fodrin*

In order to test the species specificity of the interaction of N-CAM with fodrin, we purified fodrin from embryonic day 14 chicken brains according to the procedure of Cheney et al. [53]. Because we could also purify large amounts of chicken N-CAM from the embryonic day 14 chicken brains by immunoaffinity chromatography, we could test both the species specificity of the interaction and the concentration dependence. The purification procedure yielded approximately 200 μg of fodrin from 600 g of chicken brain tissue. Fig. 2 shows a Coomassie stained gel of this material. The chicken fodrin was radiolabeled with $^{125}$I and a sample of the radiolabeled material was separated by SDS-PAGE and subjected to autoradiog-

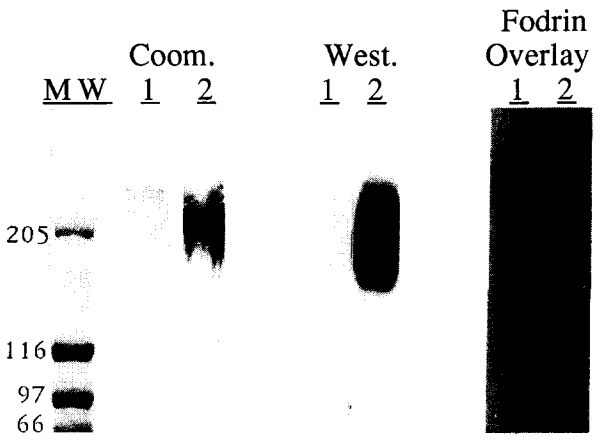


Fig. 1. Solid-phase binding of $^{125}$I-labeled bovine brain fodrin. SDS-PAGE, electrophoretic transfer and blotting procedures were carried out as described in Materials and methods. Lane MW: molecular weight standards with sizes indicated in kDa. Lanes 1: 2 μg purified bovine brain fodrin. Lanes 2: 20 μg affinity-purified embryonic day 21 chicken brain N-CAM. Coom.: Coomassie brilliant blue-stained gel. West.: immunoblot (Western blot) probed with rabbit polyclonal antibodies against chicken N-CAM. Fodrin Overlay: blot probed with 2 μg/ml $^{125}$I-labeled bovine brain fodrin.

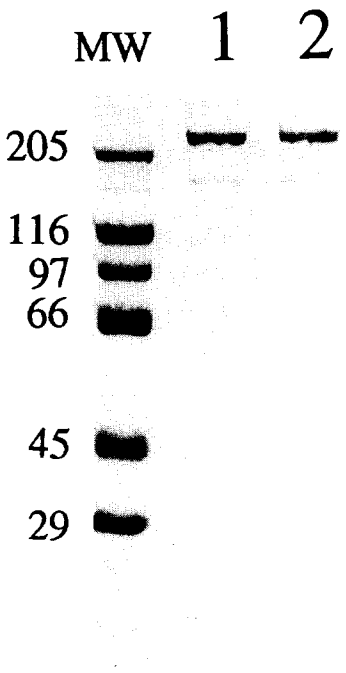


Fig. 2. Purification of chicken brain fodrin. Samples of chicken brain fodrin purified as described in Materials and methods were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue. MW: molecular weight standards with sizes indicated in kDa. Lanes 1 and 2: final concentrated fodrin preparation.

raphy. The resulting purified chicken fodrin showed the same pattern of polypeptides as did the radiolabeled bovine material, indicating a similar level of purity (data not shown).

The chicken fodrin was tested for its ability to interact with chicken or mouse N-CAM by the methods previously described. Increasing the amount the N-CAM on the blot to 20 $\mu$g and the concentration of the fodrin with which it was incubated to 10 $\mu$g/ml ($5 \cdot 10^{-6}$ M) did not result in a detectable interaction between N-CAM and fodrin. The embryonic form of N-CAM is rich in polysialic acid residues which could interfere with the interaction between N-CAM and fodrin. Therefore, we also tested the interaction between fodrin and chicken N-CAM from which the sialic acid residues had been removed by treatment with neuraminidase. Fig. 3 shows that the labeled fodrin reacted with the fodrin immobilized on the membrane but did not interact with either the polysialylated or neuraminidase treated N-CAM. Treatment with neuraminidase can result in non-specific proteolytic cleavage of the larger ld isoform of N-CAM, which causes it to comigrate with the sd isoform on a polyacrylamide gel [59]. This probably accounts for the apparent increase in the amount of the sd isoform on the autoradiograph of the immunoblot.

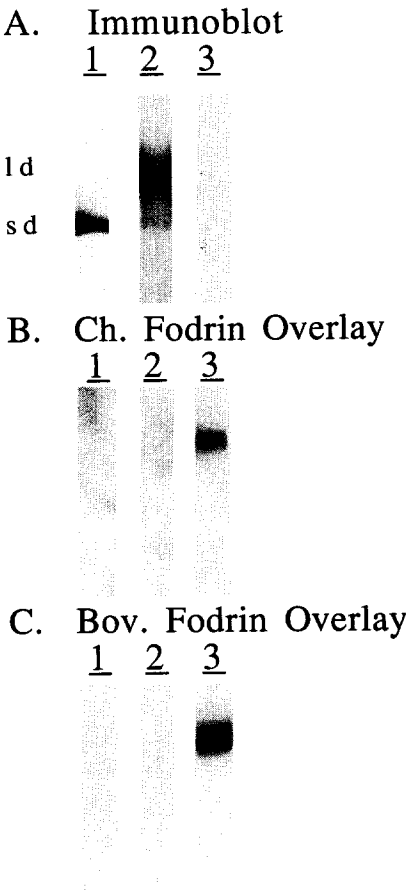


Fig. 3. Fodrin overlay analysis with chicken and bovine fodrin. Immunoprecipitation of chicken N-CAM was performed in triplicate and immunoprecipitated material was subjected to SDS-PAGE along with purified chicken fodrin. Proteins were transferred electrophoretically to nitrocellulose and were overlaid with anti-N-CAM antibodies or with fodrin as indicated. Lanes 1: neuraminidase-treated embryonic day 11 chicken brain N-CAM. Lanes 2: untreated embryonic day 11 chicken brain N-CAM. Lanes 3: 2 $\mu$g bovine brain fodrin. (Panel A) Blot probed with rabbit polyclonal anti-N-CAM antibodies. (Panel B) Blot probed with 10 $\mu$g/ml radiolabeled chicken fodrin. (Panel C) Blot probed with 10 $\mu$g/ml radiolabeled bovine brain fodrin.

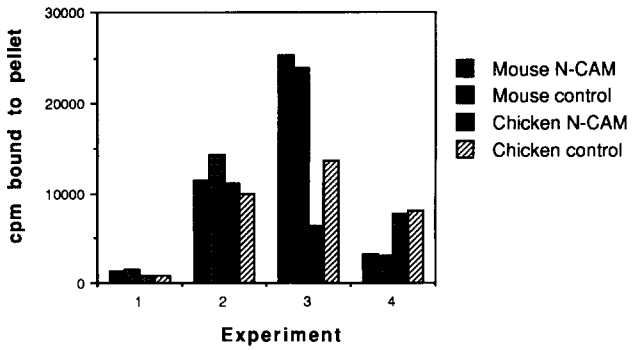


Fig. 4. Chicken brain fodrin binding to immunoprecipitated N-CAM. Four independent experiments are shown. Mouse or chicken N-CAM was immunoprecipitated from Chaps extracts of membrane fractions (experiments 1, 2 and 4) or whole cells (experiment 3). 1–5 $\mu$g of N-CAM immobilized on protein A-Sepharose beads were incubated for 30 min at 37°C (experiments 1, 3 and 4) or at room temperature (experiment 2) with 0.5–2 $\mu$g $^{125}$I-labeled chicken brain fodrin in a total volume of 40 $\mu$l. Pellets were washed five times and counted in a gamma counter. Bars labeled 'Mouse N-CAM' show the interaction of fodrin with beads resulting from precipitation of N2A cell membrane extracts with a monoclonal antibody against mouse N-CAM (Mab310). Bars labeled 'Chicken N-CAM' show the interaction of fodrin with beads resulting from precipitation from embryonic day 11 chicken brain membrane extracts with monoclonal antibody Anti N-CAM No. 1 [48] (experiment 1), with rabbit polyclonal anti-chicken N-CAM antibodies (experiments 2 and 3), or with monoclonal antibody 224-1A6-A1 against chicken N-CAM [49] (experiment 4). Bars labeled 'Mouse control' show the background interaction of fodrin with beads resulting from precipitation of N2A cell membrane extracts with rabbit antibodies directed against mouse IgG. Bars labeled 'Chicken control' show the background interaction of fodrin with beads resulting from precipitation of embryonic day 11 chicken brain membrane extracts with rabbit antibodies directed against mouse IgG.

*Fodrin failed to interact with N-CAM in solution*

Separation of N-CAM by SDS-PAGE and subsequent transfer to nitrocellulose might impair the ability of the protein to interact with fodrin. Therefore we tested the ability of N-CAM and fodrin to interact in solution. In these experiments, N-CAM was immunoprecipitated from nonionic detergent extracts of membrane preparations from N2A mouse neuroblastoma cells or from embryonic day 14 chicken brain membranes using either polyclonal anti-chicken N-CAM antiserum or monoclonal antibodies against mouse N-

CAM followed by incubation with protein A-Sepharose beads. As a control for nonspecific interactions, rabbit antibodies against mouse IgG were employed. The precipitate was then incubated in the presence of radiolabeled fodrin and washed. The amount of radiolabeled fodrin remaining associated with the immunoprecipitated material was quantified in a gamma counter. Protein A-Sepharose beads were incubated separately with labeled fodrin to determine the level of background binding. Fig. 4 shows the results of four experiments. Although the levels of nonspecific background binding varied considerably among experiments, no specific interaction between N-CAM and fodrin was detected.

## 4. Discussion

Interactions of N-CAM with cytoskeletal elements have been suggested as a possible mechanism by which N-CAM regulates its activity. One way for an adhesion molecule to strengthen an adhesive interaction is to become anchored to the cytoskeleton. The cytoskeletal element fodrin was suggested to interact with the large cytoplasmic domain isoform of N-CAM [23].

We have attempted to characterize further a specific interaction between the ld isoform of mammalian N-CAM with mammalian fodrin in a solid phase binding assay and to quantitate that interaction. Species specificity was tested by isolating both N-CAM and fodrin from avian and mammalian sources. We isolated N-CAM from either differentiated or undifferentiated N2A cells in case there was some post-translational modification associated with the differentiated neural cells that might be necessary for the interaction. We compared the highly sialylated form of chicken N-CAM to that which had been treated with neuraminidase to remove the sialic acid residues. Several different ionic conditions were tested based on the studies of others who have looked at the interactions of fodrin with integral membrane proteins. None of these variations allowed the detection of an N-CAM-fodrin interaction.

If an interaction between N-CAM and fodrin exists, it must require fodrin concentrations at least 20-fold greater than does the fodrin-fodrin interaction, which we observed at fodrin concentrations as low as $2.5 \cdot 10^{-8}$ M under the conditions tested here. The interaction of the radiolabeled fodrin with fodrin immobilized on nitrocellulose indicates that each was biologically active and that the concentrations and conditions were appropriate for an interaction to occur. Previous work [23] identified an interaction between fodrin and N-CAM at a fodrin concentration of 1 $\mu$g/ml ($5 \cdot 10^{-8}$ M), but the quantity of N-CAM immobilized on the nitrocellulose was not reported. If considerably more than 20 $\mu$g of N-CAM was employed in that study, a weaker interaction with fodrin might have been observed.

We tested the interaction between N-CAM isolated from murine N2A cells and embryonic chicken brains and fodrin from either adult bovine brain or embryonic chicken brains. Previous work [22,23,51] measured the interaction of adult bovine brain fodrin with adult murine brain N-CAM. It is possible, therefore, that there may be some chemical modification or structural feature of the adult mouse N-CAM that is required for interaction with fodrin but that was not present on the N-CAM isolated from murine tissue culture cells or from embryonic chicken brains.

Investigators studying the interactions of fodrin with other membrane associated proteins have used the solid phase binding assay to detect interactions between fodrin and synapsin I or ankyrin [60,61]. Ankyrin binds to fodrin in solution with a dissociation constant of $2.5 \cdot 10^{-8}$ M [60] and synapsin I binds to fodrin with a dissociation constant of $7 \cdot 10^{-7}$ M [62]. The interaction between fodrin and N-CAM, if it exists, presumably is considerably weaker than these interactions since it could not be detected in our experiments.

Perhaps the N-CAM cannot renature efficiently in the blot assay as we have carried it out; however, it would seem that the interaction should have been detected in the immunoprecipitation assay, in which the N-CAM is not denatured. Previous work from our laboratory also addresses this point [25]. N2A neuroblastoma cells were metabolically labeled with [$^{35}$S]methionine, solubilized in nonionic detergents, and immunoprecipitated using polyclonal and monoclonal anti-N-CAM antibodies. No specific coimmunoprecipitation of fodrin was observed on autoradiograms of gels of these immunoprecipitates. For reasons described below, at least some of the antibodies used in these experiments would not be expected to interfere with fodrin-N-CAM binding.

It is possible that the antibodies used to attach the N-CAM to beads for the solution binding assays might sterically hinder fodrin binding. While such interference cannot be ruled out for N-CAM immobilized by polyclonal antibodies, several of the binding experiments employed monoclonal antibodies that are known to bind to the extracellular portion of the N-CAM polypeptide. Monoclonal antibody Mab310 binds to mouse N-CAM on the surface of unpermeabilized N2A cells [25] and monoclonal antibody 224-1A6-A1 stains unpermeabilized normal and transfected cells expressing chicken N-CAM polypeptides (Ref. 49 and our unpublished observations), thus indicating that these antibodies bind to extracellular determinants. Monoclonal antibody anti-N-CAM No. 1 also binds to the surface of and inhibits the adhesion of chicken retinal cells [48] and binds to the surface of unpermeabilized transfected fibroblasts and neuroblastoma cells (Ref.

25 and our unpublished observations). Furthermore, peptide binding experiments indicate that this antibody binds in the region of the second and third immunoglobulin-like domain [16], that is, towards the extracellular extremity of the N-CAM molecule. These results makes it less likely that these antibodies would hinder binding of fodrin to the cytoplasmic domain of the N-CAM polypeptide and thus support the conclusion that N-CAM and fodrin do not bind in solution under these conditions.

We conclude that the N-CAM to fodrin interaction is very weak. Whether or not this interaction is biologically significant must await further experimentation.

## 5. Acknowledgments

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