Membrane Glycoproteins Involved in Neurite Fasciculation

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Abstract. Lectin affinity chromatography combined with mAb production was used to identify chick neural cell surface molecules related to L1 antigen, a mouse neural glycoprotein implicated in cell–cell adhesion (Rathjen, F. G., and M. Schachner, 1984, *EMBO* (*Eur. Mol. Biol. Organ.*) *J.*, 3:1–10).

A glycoprotein, G4 antigen, isolated by mAb G4 from adult chick brain is described which comprises a major 135-kD component, a minor doublet at 190 kD, and diffusely migrating bands at 80 and 65 kD in SDS PAGE. This molecule is structurally related to mouse L1 antigen according to NH_2-terminal amino acid sequence (50% identity) as well as the behavior of its components in two-dimensional IEF/SDS PAGE gels. A second chicken glycoprotein, F11 antigen, was isolated from adult chick brain using mAb F11. This protein has also a major 135-kD component and minor components at 170 kD and 120 kD. Both immunotransfer analysis with polyclonal antibodies to mAb G4 and to mAb F11 isolate and the behavior on IEF/SDS PAGE gels indicates that the major 135-kD component of F11 antigen is distinct from G4 antigen components. However, the 135-kD component of F11 antigen shares with G4 antigen and the neural cell adhesion molecule (NCAM) the HNK-1/L2 carbohydrate epitope.

In immunofluorescence studies, G4 and F11 antigenic sites were found to be associated mainly with the surface of process-bearing cells, particularly in fiber-rich regions of embryonic brain.

Although Fab fragments of polyclonal antibodies to mAbs G4 or F11 immunoaffinity isolate only weakly inhibit the Ca^{2+}-independent aggregation of neural cells, they strongly inhibit fasciculation of retinal axons. Together these studies extend the evidence that bundling of axons reflects the combined effects of a group of distinct cell surface glycoproteins.

The formation of specific connections in the nervous system during embryonic development is dependent on a variety of processes, including the adhesion of cells to each other and to extracellular matrices. In the last few years several neural cell surface glycoproteins have been identified which appear to be involved in adhesive interactions between neurons, glial cells, and matrices. Among these are the major Ca^{2+}-independent cell–cell adhesion molecules, of which the known ones can be classified into two groups: the very closely related neural cell adhesion molecules (NCAMs)[1] (10, 39) and the L1 group of antigens (20, 38, 40).

Mouse L1 antigen is biochemically and immunologically distinct from NCAM (13, 37). However, there is some functional similarity between the two molecules in that both anti-NCAM and anti-L1 Fab fragments inhibit the calcium-independent aggregation of cultured mouse cerebellar cells and neuroblastoma N2A cells (37, 38). Moreover, the inhibition of aggregation of N2A cells by a combination of anti-NCAM and anti-L1 is synergistic rather than additive (37). Anti-L1 Fab fragments have also been shown to modify the migration of external granular cells from mouse cerebellum in an in vitro migration system (32).

NCAM appears on both the cell body and processes and is involved in several types of interactions among neurons, glial, and muscle cells (39). In contrast, the L1-like antigens are present mainly on fasciculated axons during the development of the neocortex and spinal cord (19, 38, 41), and it has been shown that Fab fragments of polyclonal antibodies to either L1 antigen (16) or the rat nerve growth factor–induced large external (NILE) protein (40), which is immunologically related to L1 antigen (3), also inhibit the fasciculation of neurites from explants of embryonic brain tissue. Together, these results strongly suggest that the function of L1-like antigens may be focused on the formation and organization of axon bundles.

Despite these advances, the molecular basis of fiber–fiber interactions during development remains poorly defined. As in recently developed assay systems (4, 5, 23, 42), we used tissues from chicken embryos for investigations of axon interactions. Thus our studies depend on the identification of

1. *Abbreviations used in this paper*: NCAM, neural cell adhesion molecule; NgCAM, neuron–glia cell adhesion molecule; WGA, wheat germ agglutinin.

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glycoproteins in the chicken which are homologous and/or functionally related to mouse L1 antigen.

In the present report we describe (*a*) a neural glycoprotein (recognized by mAb G4) which is structurally homologous to mouse L1 antigen, and (*b*) an additional L1-like molecule (defined by mAb F11), which is related to the G4 antigen in that it is distributed in fiber-containing regions and in that polyclonal antibodies prepared against this molecule interfere with fasciculation of retinal cell axons. These results suggest that bundling of axons and the formation of fiber order involves a group of distinct cell surface glycoproteins. In an accompanying paper (8), these antibodies are further analyzed in a neurite outgrowth assay system which demonstrates that the L1-like molecules can mediate interactions between axons.

Materials and Methods

Production of mAb's

For isolation of glycoprotein fractions used for immunization, plasma membranes were prepared by the method of Hoffman et al. (25) from adult chicken brains obtained from a local slaughterhouse. 50 ml of packed membranes were solubilized in 1,000 ml solubilization buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 40 U/ml Aprotinin [Sigma Chemical Co., St. Louis, MO] pH 7.4). Nonsolubilized material was pelleted by centrifugation at 100,000 *g* for 1 h.

The supernatant was passed through lectin affinity columns containing wheat germ agglutinin (WGA) immobilized on Sepharose. Columns were washed with 50 vol of solubilization buffer without Aprotinin and PMSF, and elution was effected by three column volumes of 200 mM *N*-acetyl-D-glucosamine in 0.5% NP-40, 50 mM Tris-Cl, 150 mM NaCl, pH 7.4. The resulting glycoprotein fraction was then depleted of NCAM by passage through a column of anti-NCAM-Sepharose and used as antigen in the first step of mAb production. BALB/c mice were primed with 40-50 μg of these WGA-adhesive glycoproteins. Booster injections were given with the same amount of antigen 2 and 6 wk later.

In the second step of mAb production, solubilized plasma membrane proteins (see above) were depleted of NCAM, F11 antigen, and of WGA-binding proteins by passage over the appropriate column. The flowthrough was then passed over a lentil lectin (lens culinaris hemagglutinin) column, which was washed with 50 vol of solubilization buffer. Elution was achieved by three column volumes of 200 mM α-methyl-D-glucopyranoside in 0.5% NP-40, 50 mM Tris-Cl, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, pH 7.4. The resulting glycoprotein fraction (40-50 μg) was used to prime mice, which were then boosted twice with a 135-kD fraction (10 μg) 2 and 6 wk later. This fraction had been obtained by preparative SDS PAGE from lens culinaris hemagglutinin-binding proteins followed by electroelution (33). In both procedures, Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant for subsequent injections.

Animals with high antiserum titers received a fourth injection of the appropriate antigen without adjuvant 3 d before cell fusion. Fusion of splenocytes and the myeloma cell line P3X63-AG 8.653 (28) was carried out essentially as described by Fazekas de St. Groth and Scheidegger (15). Wells containing growing cells were screened for antigen-reactive antibodies by an ELISA. Positive wells were subcloned at least twice by limiting dilution on mouse peritoneal macrophages.

ELISAs were performed essentially as described by Engvall (12) using polystyrene plates (Nunc, Roskilde, Denmark) coated with 100-300 ng of detergent-depleted lens culinaris hemagglutin- or WGA-binding glycoproteins per well. Samples consisted of 100 μl culture supernatant. Rabbit anti-mouse IgG (H+L)-peroxidase conjugate (Miles Scientific Div., Naperville, IL) served as second antibody. *O*-Phenylenediamine (Sigma Chemical Co.) was used as chromogen (12). Detergent was removed from glycoprotein fractions by treatment with SM2 beads (Bio-Rad Laboratories, Richmond, CA) as described by Holloway (26).

Isolation of G4, F11, and L1 Antigens by Immunoaffinity Chromatography

For isolation of G4 and F11 antigens, detergent extracts of adult chicken brain plasma membranes prepared as described above were passed through immunoaffinity columns which had been prepared by coupling the respective mAb to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) or to Affi-Gel 10 (Bio-Rad Laboratories), according to the manufacturer's instructions. 3 mg of purified mAb was used per ml packed agarose. Columns were washed with 50 vol of solubilization buffer and bound antigens were eluted with three column volumes of 100 mM diethylamine, 0.5% NP-40, pH 11.5. The eluate was neutralized immediately by addition of 0.2 vol of 1 M Tris-Cl, pH 6.8. All procedures were carried out at 0-4°C. Mouse L1 antigen was isolated by a similar procedure from solubilized adult mouse brain plasma membranes using mAb to L1 antigen (38).

Polyclonal Antibodies and Fab Fragments

Polyclonal antibodies were produced by subcutaneous injection of outbred rabbits at multiple sites with 10-15 μg of immunoaffinity isolate of mAb G4 and mAb F11 (see above) at fortnightly intervals. Complete adjuvant was used for the first injection, and incomplete for subsequent injection. Blood was collected not before 7 or 8 wk. Polyclonal antibodies to chick NCAM (20 μg per injection) were prepared in the same way.

Fab fragments were produced from the IgG fraction of rabbit immune or nonimmune sera by mercuripapain digestion according to Porter (36). Affinity-purified polyclonal antibodies to mAb F11 immunoaffinity isolate were prepared by passing the IgG fraction over a column containing mAb F11 isolate (~300 μg) immobilized on CNBr-activated Sepharose.

Immunocytological Procedure

Retinal or tectal cells prepared from 7-9-d-old chick embryos were cultured in DME supplemented with 10% FCS (Gibco, Grand Island, NY) on glass slides coated with 100 μg/ml poly-L-lysine. Retinal explants from 6-d-old chick embryos were cultured in F12 medium supplemented with 10% FCS on glass slides coated with laminin (Bethesda Research Laboratories, Bethesda, MD). For immunocytological localization of antigens, live monolayer or explant cultures were incubated with mAb's or affinity-purified polyclonal antibodies at a concentration of 5-25 μg/ml in PBS (136 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4), 0.1% BSA for 30 min at room temperature. Cells were washed three times with PBS/BSA and fixed for 5 min in 3.7% formaldehyde in PBS at 0°C. Cells were washed twice with PBS/BSA, incubated with FITC-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (Miles Scientific Div.) at a 1:100 dilution, and washed again. Slides were mounted in PBS/glycerol (1:1). Essentially the same procedure was used for immunohistology of formaldehyde fixed cryostat sections (12 μm) of chick brain or retina.

Reaggregation of Chick Cells In Vitro

Ca^{2+}-independent reaggregation of dissociated 7-9-d embryonic chick retinal and tectal cells was performed essentially as described by Brackenbury et al. (7), except that particles were counted using a ZF Coulter Counter fitted with a 200-μm capillary.

Fasciculation of Neurites In Vitro

Studies on fasciculation of neurites in vitro were performed with rhodamine-labeled retinal explants prepared from 6-d-old chick embryos (4). Explants were placed on Nuclepore filters (0.1 μm pore size) coated with stripes of plasma membrane vesicles prepared from 9-d-old chick anterior tectal halves and incubated in F12 medium supplemented with 10% FCS and 0.4% Methocel (Dow Corning Corp., Midland, MI) for 50-60 h. Vesicle-coated strips were ~90 μm in width and arranged in parallel. Details of the procedure will be given elsewhere (Walter, J., and F. Bonhoeffer, manuscript in preparation). Antibodies were assayed for defasciculating activity by incubating the explants in culture medium with 0.5-1 mg/ml Fab fragments from polyclonal rabbit antibodies to mAb G4, mAb F11, and mAb C5 immunoaffinity isolate. Fab fragments (1 mg/ml) prepared from serum of unimmunized rabbits served as a control. Fasciculation behavior was not affected by the presence of nonimmune Fab fragments. Antibodies were added to the cultures at the time when retina strips were explanted.

Gel Electrophoretic Analyses

SDS PAGE was carried out on 7% acrylamide gels according to Laemmli (31). The following proteins served as molecular mass markers: myosin (200 kD), β-galactosidase (116.25 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), and ovalbumin (45 kD) (Bio-Rad Laboratories). IEF followed by SDS PAGE in the second dimension was performed essentially as de-

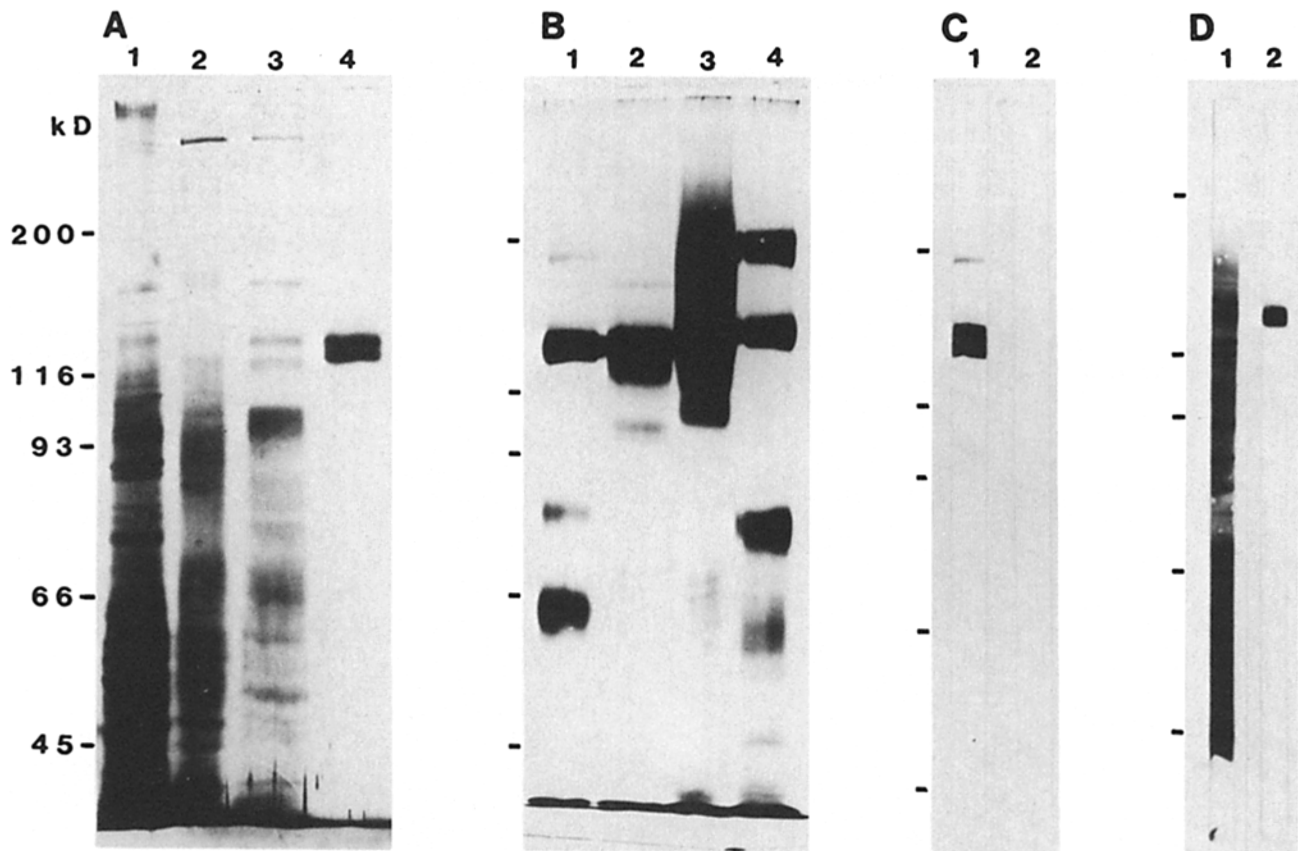


Figure 1. 7% SDS PAGE of lectin eluates (*A*), mAb immunoaffinity isolates (*B*), and immunotransfers using monoclonal antibody G4 (*C* and *D*). (*A*) Isolated chick plasma membrane proteins used to generate mAb's. (Lane *1*) Detergent-solubilized plasma membrane proteins; (lane 2) NCAM-depleted eluate of WGA used to immunize mice in the first round of mAb production; (lane *3*) eluate of lentil lectin after removal of L1-like antigens identified in the first group of hybridomas used to immunize mice in the second round of mAb production; (lane *4*) 135-kD fraction of lane *3* used to boost mice in the second round of mAb production. (*B*) Immunoaffinity isolates using mAb G4 (lane *1*) and mAb F11 (lane 2). For comparison, lanes *3* and *4* show isolates of chick NCAM and mouse L1 antigen, respectively. In *A* and *B*, isolates were from adult chicken brains except that in lane *4* of *B*. L1 was isolated from brains of adult mice. Proteins were visualized by silver staining (2). In *C*, we show antigenic determinants recognized by mAb G4 in the immunoaffinity isolates of mAb G4 and mAb F11. The immunoaffinity isolates of mAb G4 (lane *1*) and mAb F11 (lane 2) were resolved by SDS PAGE, transferred to nitrocellulose, and analyzed with mAb G4. In *D*, we show immunotransfer of chick brain plasma membranes with mAb G4. Plasma membrane proteins from adult chicken brain were fractionated by SDS PAGE, transferred to nitrocellulose, and either stained with India ink (lane *1*) or analyzed with mAb G4 (lane 2). In *C* and *D*, binding of antibodies was visualized by peroxidase-conjugated second antibodies. Positions of the indicated protein standards are marked to the left of each panel.

scribed by O'Farrell (34) using Servalyte pH 5-8 as ampholytes. IEF gels were calibrated by shaking 1-cm slices of control gels in 1 ml of degassed H_2O and measuring the pH. Proteins were visualized by silver staining according to Ansorge (2). Transfer of proteins from SDS PAGE (43) to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) and subsequent immunodetection of antigens was performed as described by Wolff et al. (44). mAb G4 was used at 10 μg/ml; the IgG fractions of polyclonal antibodies to G4 or F11 antigens were used at 25 μg/ml. mAb HNK-1 (1) was supplied as culture supernatant. Nitrocellulose strips were stained for total protein by India ink (24). Protein determinations were carried out according to Peterson (35).

NH_2-terminal Amino Acid Sequence Analysis

100–200 μg of affinity-purified G4 or L1 antigen were subjected to preparative SDS PAGE on 3-mm thick gels. The 135-kD component of G4 antigen and the 140-kD component of L1 antigen were excised from the gel, using guide strips stained by Coomassie Brilliant Blue and electroeluted as described by Hunkapiller et al. (27). Purity of the electroeluted samples was confirmed by analytical SDS PAGE. NH_2-terminal amino acid sequence analysis was performed on a gas phase sequenator constructed and operated as described in reference 18. 100–200 pmol of antigen were sequenced per run. Phenylthiohydantoin amino acids were identified by HPLC as described (17). Each sample was sequenced twice.

Tryptic Fingerprinting of ^{125}I-labeled Antigens

2–10 μg of purified L1 or G4 antigen were labeled using 200 μCi ^{125}I (New England Nuclear, Boston, MA) by the chloramine T method (22). Unbound iodine was removed by gel filtration on Sephadex G25 fine (Pharmacia Fine Chemicals) and the proteins were fractionated by SDS PAGE. Bands were localized on dried gels by autoradiography and excised. Tryptic digestion and two-dimensional separation of the resulting peptides by thin layer electrophoresis and thin layer chromatography were performed essentially as described by Elder et al. (11). Chromatograms were impregnated with 2,5-diphenyloxazole/2-methyl-naphthaline (6). Autoradiography was performed at −70°C using Kodak X-Omat AR X-ray film.

Results

Production of mAb's That Recognize L1-like Antigens

For the production of mAb's against L1-like antigens, a simple two-step cascade procedure of lectin and mAb affinity chromatography was used. In the first step, WGA affinity chromatography followed by removal of NCAM by im-

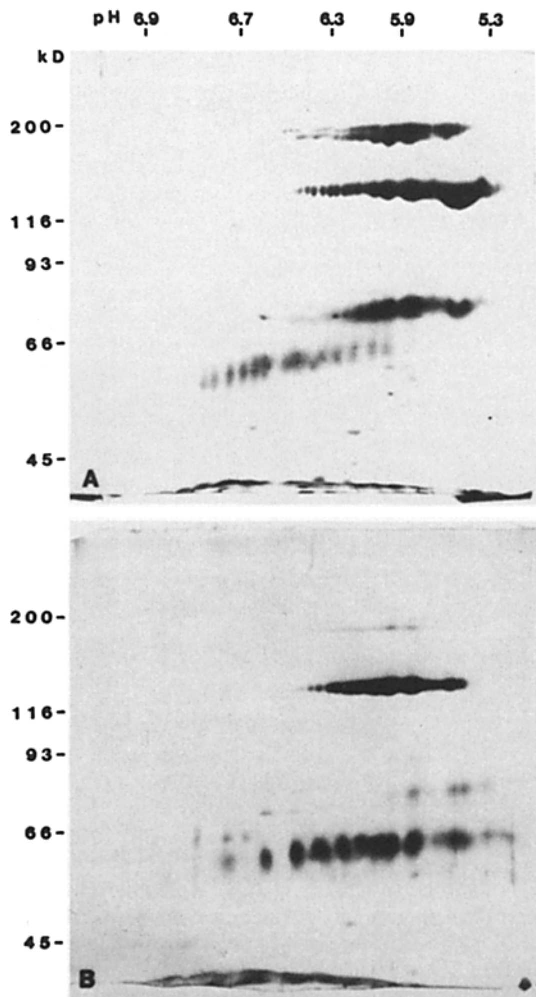


Figure 2. Comparison of mouse L1 antigen and chick G4 antigen by IEF and SDS PAGE. L1 antigen (*A*) and G4 antigen (*B*) were purified from adult mouse and chicken brains, respectively. Purified material was resolved by IEF followed by SDS PAGE (7% acrylamide) in the second dimension by the method of O'Farrell (34). Position of the indicated protein standards is marked to the left of each gel (see Materials and Methods) and the pH values of control gels are shown at the top. Proteins were visualized by silver staining (2).

munoaffinity chromatography was used to prepare the antigen (Fig. 1 *A*, lane *2*). Spleen cells obtained from these immunizations were used to prepare hybridomas. Hybridoma clone culture supernatants were first screened by ELISA for antibodies binding to the glycoprotein fraction used for immunizations. To identify L1-like antigens, positive clones were then tested for (*a*) staining of the surface of living retinal or tectal monolayer cells, (*b*) prominent staining of fiber-containing regions in cryostat sections of embryonic chick brain, and (*c*) their ability to recognize or isolate components with molecular masses similar to those of mouse L1 antigen (Fig. 1 *B*, lane *4*).

In the second step, antibodies were generated using a lentil lectin glycoprotein fraction after removal by immunoaffinity chromatography of L1-like antigens identified in the first group of hybridomas (Fig. 1 *A*, lane *3*). In addition, these mice were boosted twice with material eluted from a 135-kD band obtained by SDS PAGE of glycoproteins obtained using lentil lectin (Fig. 1 *A*, lane *4*). The clones produced from this procedure were then screened according to the criteria described above. The majority of the mAb's produced from these two protocols could be divided into two groups according to the SDS PAGE pattern of the antigens they recognize. The mAb's G4 and F11 were selected as their representatives and their antigens are described in the following sections.

Polypeptides Isolated and Recognized by mAb G4 and mAb F11

mAb G4 was used to isolate G4 antigens from NP-40-solubilized adult chick brain membranes by immunoaffinity chromatography. The resulting column eluate included a major 135-kD component and a very weakly stained doublet at 190 kD. Diffuse migrating minor components were also seen at 80 kD and 65 kD (Fig. 1 *B*, lane *1*). Lane *2* (Fig. 1 *B*) shows an isolate obtained by immunoaffinity chromatography using mAb F11. It was also composed of a major 135-kD component with additional components at 170 kD and 120 kD. Minor components of 95 kD were also detected (Fig. 1 *B*, lane 2). The relative amount of the lower molecular mass components present in the immunoaffinity eluates of mAb G4 and mAb F11 varied from isolation to isolation (compare Fig. 1 *B*, Fig. 2 *B* and Fig. 6). In immunotransfers using the immunoaffinity-purified antigens, mAb G4 recognized the major 135-kD and the minor 190-kD components (Fig. 1 *C*, lane *1*), but not the 80-kD and 65-kD components. In immunotransfers using plasma membrane proteins from adult chicken brains, only the major 135-kD component is recognized by mAb G4 (Fig. 1 *D*, lane 2). However, components isolated by mAb F11 were not recognized by mAb G4 (Fig. 1 *C*, lane 2). MAb F11 was not used in these immunotransfer studies, since its antigenic site is destroyed by SDS PAGE sample buffer. For comparison, adult chick NCAM and adult mouse L1 antigen are shown in lanes *3* and *4*, respectively (Fig. 1 *B*).

Chick G4 Antigen Is Homologous to the Mouse L1 Antigen

The antigen isolated using mAb G4 is composed of similar but not identical molecular mass components in SDS PAGE as mouse L1 antigen (compare lanes *1* and *4* in Fig. 1 *B*). The major difference seen between the two antigens is that the mAb G4 immunoaffinity eluate has only a very weakly stained doublet at 190 kD. The use of additional protease inhibitors in the solubilization buffer (leupeptin and pepstatin) or the direct analysis of plasma membrane proteins in immunotransfer did not increase the amount of 190-kD material recognized by mAb G4.

IEF of purified mouse L1 antigen and purified chick G4 antigen followed by SDS PAGE in the second dimension also suggests that the G4 antigen is similar to mouse L1 antigen (Fig. 2, *B* and *A*, respectively). The major components of both molecules show a considerable degree of charge heterogeneity with mainly overlapping regions. In comparison to chick G4 antigen, mouse L1 antigen has an additional acidic spot. Interestingly, the lower molecular mass components of L1 antigen (80 kD and 65 kD) and of G4 antigen (65 kD) express a similar degree of charge heterogeneity as the major 140-kD or 135-kD components (Fig. 2, *A* and *B*).

To confirm the relationship between G4 antigen and L1 antigen, the NH_2-terminal amino acid sequence of both antigens was determined by gas phase sequence analysis. Edman

G4: H_2N-Ile-Thr-Ile-Pro-Pro-Glu-Tyr-Gly-Ala- ? -Asp-Phe-Leu-Gln-Pro-Pro-Glu-

L1: H_2N-Ile-Gln-Ile-Pro-Asp-Glu-Tyr-Lys-Gly- ? - ? -Val-Leu-Glu-Pro-Pro-Val-

Figure 3. Comparison of the NH_2-terminal amino acid sequence of the 140-kD component of mouse L1 antigen and of the 135-kD component of chick G4 antigen. Immunoaffinity-purified mouse L1 antigen and chick G4 antigen were resolved by SDS PAGE; the 140-kD (mouse L1 antigen) and the 135-kD (chick G4 antigen) bands were excised from the gel and electroeluted; and the NH_2-terminal amino acid sequence was analyzed on a gas phase sequenator. The NH_2 termini are shown at the left. Conserved amino acid residues are boxed.

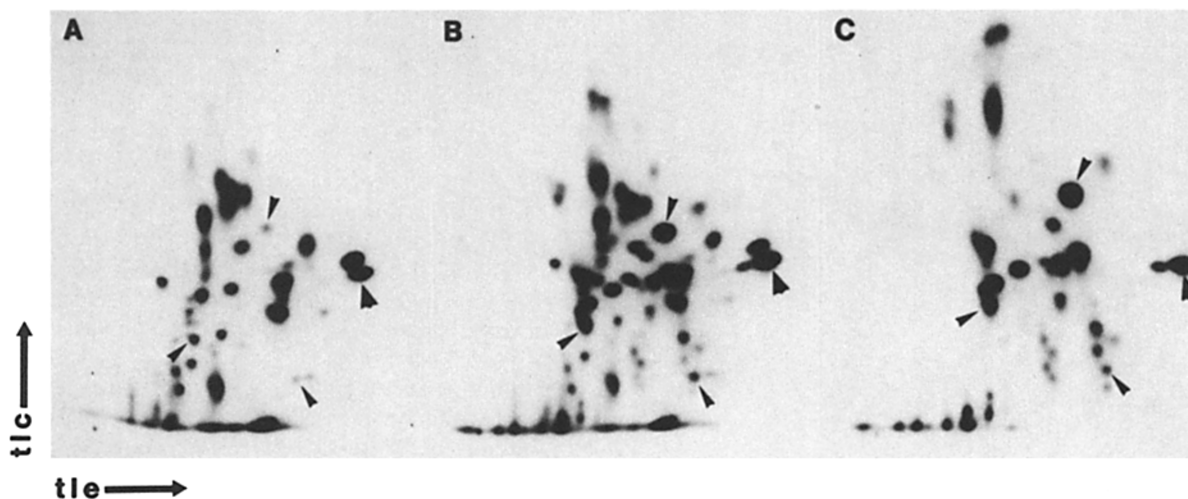


Figure 4. Comparison of tryptic fingerprints of mouse L1 antigen and chick G4 antigen. Immunoaffinity-purified mouse L1 antigen and chick G4 antigen were labeled with ^{125}I and fractionated by SDS PAGE. The 135-kD component of G4 and the 140-kD component of L1 were excised from the gel and trypsinized. Peptides were resolved by thin layer electrophoresis (*tle*) followed by thin layer chromatography (*tlc*) and visualized by autoradiography. (*A*) Tryptic peptides of mouse L1 antigen (140-kD component); (*C*) tryptic peptides of chick G4 antigen (135-kD component); (*B*) a mixture of peptides of both components. Arrows indicate co-migrating peptides, present in unequal (*small arrowheads*) or in approximately equal amounts (*bold arrowheads*) in both L1 and G4 antigen.

degradation of the electroeluted 140-kD component of mouse L1 antigen, and of the electroeluted 135-kD component of chick G4 antigen yielded sequences of 15 and 16 amino acids, respectively (Fig. 3). Comparison of these sequences revealed a considerable homology, with 8 of the 15 residues compared being identical.

If the 50% identity seen at the amino terminus is characteristic of the remainder of the polypeptides, then it would be expected that while the two proteins are related, they would not generate similar fingerprints. In fact, tryptic digests separated by a two-dimensional thin layer system gave very few common peptides. Only 4 out of ~70 resolved peptides apparently co-migrated, and out of these only one peptide was present both in L1 and G4 antigen in comparable amounts (Fig. 4). Thus, major parts of the peptide backbone are not completely shared by both molecules and the overall homology between the two proteins is not likely to be much greater than suggested by their NH_2-terminal comparison. Nevertheless, on the basis of the striking similarities in electrophoretic properties and highly significant sequence homology, the chick G4 antigen will be considered as a chick homologue of the mouse L1 antigen.

The Relationship of G4 Antigen to the 135-kD and 170-kD Glycoproteins Isolated by mAb F11

To analyze the relationship between G4 and F11 glycoproteins, polyclonal antibodies were prepared against the affinity isolate of mAb G4 and mAb F11 and tested in terms of their immunochemical properties. In immunotransfers of neural membrane proteins resolved by SDS PAGE, both polyclonal antibodies to the affinity isolates of mAb G4 and mAb F11 recognized a major 135-kD component (Fig. 5 *A*, lane *2* and *3*). Polyclonal antibodies to mAb G4 isolate reacted with the G4 antigen, including all G4 components but not with components of mAb F11 isolate (Fig. 5, *B* and *C*, lane *2*). Polyclonal antibodies to mAb F11 isolate recognized the 135-kD component of mAb F11 isolate, but not the 170-kD component and not G4 components (Fig. 5, *B* and *C*, lane *1*). These results indicate that the major 135-kD component of mAb F11 isolate has no detectable immunological relationship to that of mAb G4 antigen using rabbit antisera. However, both 135-kD components share with NCAM the HNK-1/L2 carbohydrate epitope (30) (Fig. 5 *D*). In contrast, the 170-kD component of mAb F11 isolate is not recognized by mAb HNK-1 (Fig. 5 *D*, lane *3*).

When analyzed by IEF followed by SDS PAGE, the 135-kD component of mAb F11 isolate ranges in its isoelectric points from 7.0 to 6.1 (Fig. 6 *A*). This region is adjacent to the more acidic components of G4 antigen (6.4 to 5.3, Fig. 6 *B*). The 170-kD component seen in the immunoaffinity isolate of mAb F11 expresses less charge heterogeneity than the 135-kD component and has an isoelectric point of 7.0 to 7.2 (Fig. 6 *A*). The two-dimensional gel analysis also shows an additional minor component of 140 kD with an isoelectric

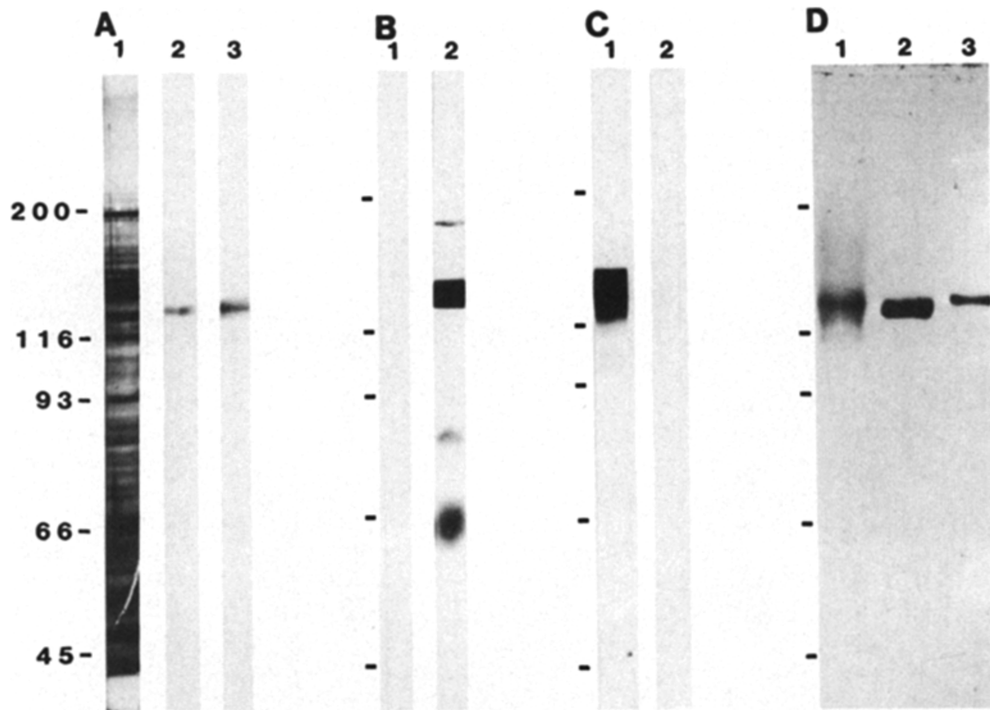


Figure 5. Immunotransfer analysis using polyclonal antibodies to the isolates of mAb G4 and mAb F11 (*A–C*) and using mAb HNK-1 (*D*). (*A*) Analysis of immunotransfers of chick brain plasma membrane extract with polyclonal antibodies to the immunoaffinity isolate of G4 (lane *2*) and mAb F11 (lane *3*). Adult chicken brain plasma membranes were resolved by 7% SDS PAGE, transferred to nitrocellulose, and stained either with India ink (lane *1*) or subjected to antibody analysis (lanes *2* and *3*). (*B* and *C*) The immunoaffinity isolate of mAb G4 (*B*) and mAb F11 (*C*) were fractionated by SDS PAGE (7% gels), transferred to nitrocellulose, and analyzed with polyclonal antibodies to mAb F11 isolate (lane *1*) and to mAb G4 isolate (lane *2*). (*D*) NCAM (lane *1*) and the immunoaffinity isolates of mAb G4 (lane *2*) and mAb F11 (lane *3*) were fractionated by SDS PAGE (7% gels), transferred to nitrocellulose, and analyzed with mAb HNK-1. Binding of antibodies was visualized by peroxidase-conjugated second antibodies. The position of molecular mass standards is indicated at the left of each panel.

point similar to that of the 170-kD component. In the one-dimensional SDS PAGE, this component is probably hidden by the 135-kD component and therefore not seen.

G4 and F11 Antigens Are Associated with Axonal Surfaces

Cell surface expression of G4 and F11 antigens was determined by indirect immunofluorescence on viable dissociated embryonic retinal cells that had been cultured in monolayers and had grown neuritic processes or on retinal explant cultures (Fig. 7). In monolayer cultures, F11 and G4 antigens were mainly associated with process-bearing cells. While F11 antigen is expressed on most retinal cells (Fig. 7 *A*), the G4 antigen was found only on a small percentage of cultivated retinal cells with long processes (∼10%, Fig. 7 *G*). However, in retinal explant cultures, where mainly axons from retinal ganglion cells grow out, F11 and G4 antigens were present on the majority, if not on all of the fibers (Fig. 7, *B* and *H*). The association of F11 and G4 antigens with process-bearing cells was further supported by the finding that immunohistology with mAb G4 and mAb F11 of cryostat sections of chick embryonic brains gave mainly strong staining of fiber-containing regions. As an example, cryostat sections of embryonic day 16 cerebellum are shown (Fig. 7, *C* and *I*). Both antibodies do not stain the proliferating zone of the external granular layer. The G4 antigen, but not the F11 antigen, becomes expressed on the postmitotic, premigratory external granular cells. The nascent molecular layer was strongly stained, while the internal granular layer was only weakly stained by both antibodies. In addition the F11 antigen was intensely expressed in the Purkinje cell layer.

Fab Fragments of Polyclonal Antibodies to mAb G4 and mAb F11 Isolate Alter Fasciculation of Retinal Axons

Since the 135-kD cell surface glycoprotein isolated by mAb F11 and mAb G4 are expressed mainly in fiber-containing regions, we asked if these components are also related to L1 antigen with respect to function. The first test of function was a short-term reaggregation assay using dissociated neural cells. When Fab fragments of the polyclonal antisera against F11 and G4 antigens were added to a Ca^{2+}-independent reaggregation assay of embryonic retinal or tectal cells, the rate of aggregation was only slightly reduced (∼10%). In contrast, polyclonal antibodies to NCAM in the same assay inhibit by ∼90% (Table I). This finding reflected the fact that unlike NCAM, the F11 and G4 antigens were not detected by immunofluorescence staining on either the trypsinized or mechanically dissociated cells used in the assay (not shown).

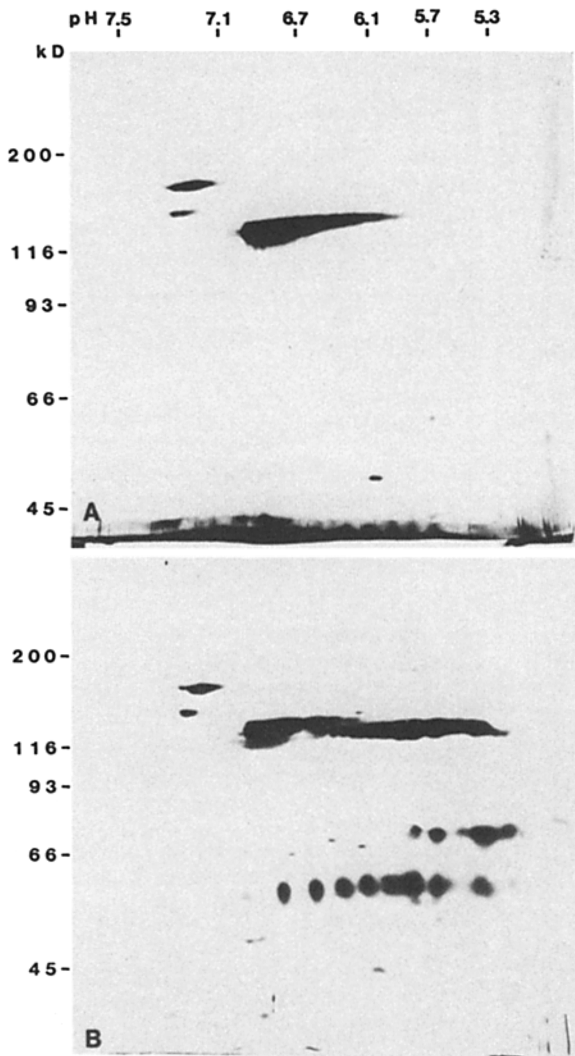


Figure 6. Analysis of the immunoaffinity isolate of mAb Fll (*A*) and an equal mixture of isolates of mAb Fll and mAb G4 (*B*) by IEF and SDS PAGE. The immunoaffinity isolates of mAb G4 and mAb Fll from adult chicken brains were resolved by IEF followed by SDS PAGE (7% acrylamide) in the second dimension by the method of O'Farrell (34). Position of the indicated protein standards is marked to the left of each gel (see Materials and Methods) and the pH values of control gels are shown at the top. Proteins were visualized by silver staining (2).

In view of the selective expression of the G4 and Fll antigens on axon fascicles, we tested the influence of Fab fragments of polyclonal antibodies against these proteins on the fasciculation pattern of retinal axons. In the assay system used, tectal membrane vesicles were used as substrate for axonal growth. The vesicles were arranged in stripes on Nuclepore filters. These membrane-coated stripes, 90 μm in width, were separated from each other by noncoated stripes of the same width. These culture conditions lead to a high degree of easily observed fasciculation of retinal fibers and facilitate the analysis of neurite–neurite interaction. With Fab fragments of polyclonal antibodies to Fll or G4 antigen, strong defasciculation (Fig. 8, *B* and *D*) was observed when compared to control cultures (Fig. 8, *A* and *C*). Although not easily quantified, inhibition of fasciculation of retinal fibers

Table I. Ca^{2+}-independent Aggregation of Retinal Cells in the Presence of Fab Fragments of Polyclonal Antibodies to NCAM, to mAb F11, or to mAb G4 Isolate

	Percent aggregation	
Antibody	Tectal cells	Retinal cells
Nonimmune	48 ± 4.2	69.5 ± 4.9
Anti-NCAM	7 ± 1.4	4.5 ± 0.7
Anti-mAb F11 isolate	42 ± 4.2	63.5 ± 0.7
Anti-mAb G4 isolate	44 ± 1.4	67 ± 1.4

Tectal (from 7-d-old embryos) or retinal (from 9-d-old embryos) cell suspensions were incubated for 40 min at 37°C with rotation at 90 rpm and aggregation was quantitated in terms of percentage decrease in particle number (7). Fab fragments were used at 1 mg/ml per sample. The standard deviation in duplicate assays is shown.

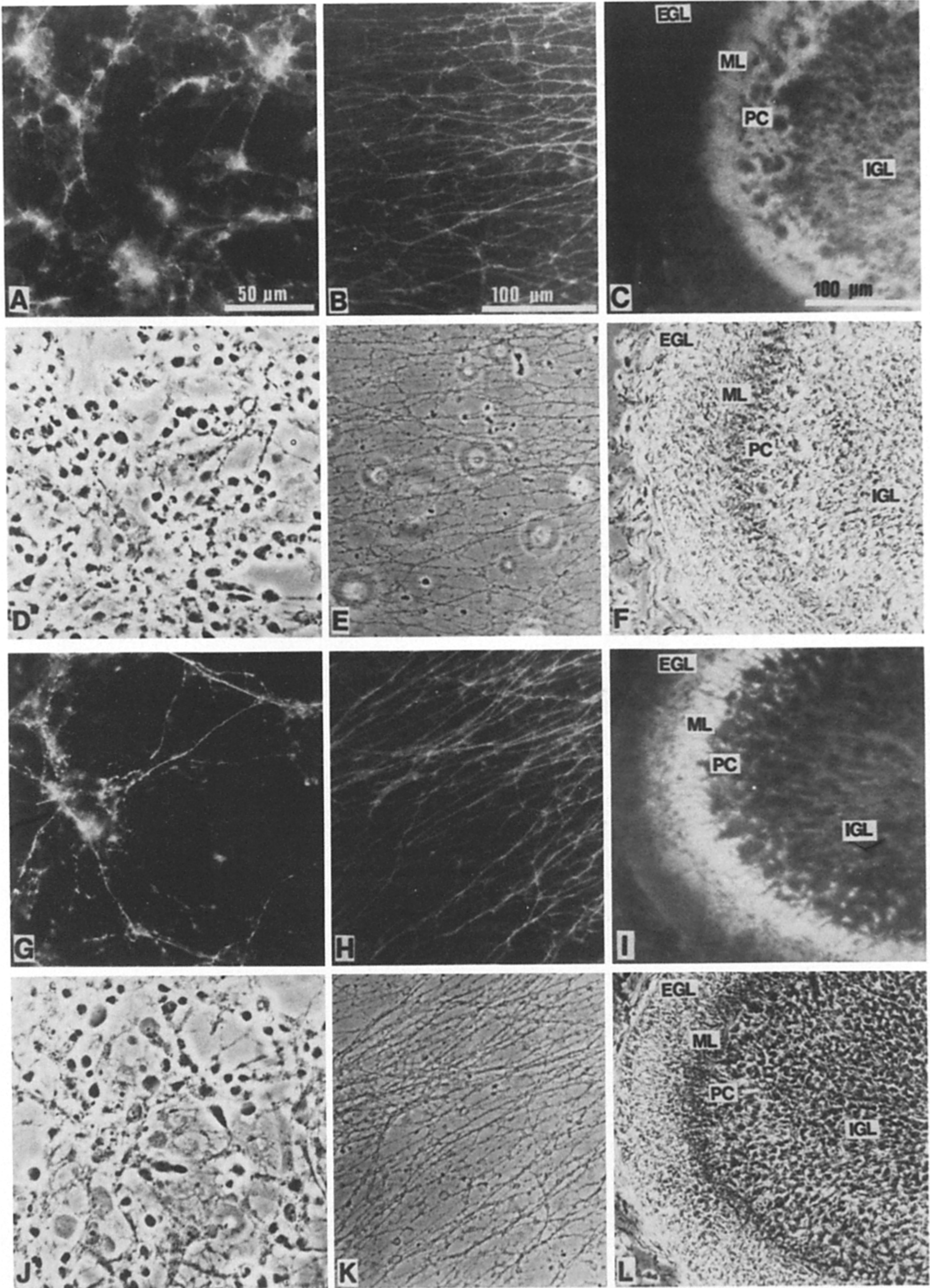
by anti-G4 Fab (Fig. 8 *B*) appeared to be stronger than that obtained with anti-Fll Fab (Fig. 8 *D*). Specificity of the defasciculating effect could be evaluated by using Fab fragments of polyclonal antibodies to C5, an unrelated cell surface glycoprotein (unpublished data). Although C5 antigen is present in similar amounts on fibers, as judged by immunofluorescence, anti-C5 Fab fragments had no effect on the fasciculation behavior of retinal axons (Fig. 8 *F*).

Discussion

The purpose of these studies was to identify molecules in the chick which are homologous to mouse Ll antigen (38); that is, neural cell surface glycoproteins with biochemical properties similar to mouse Ll antigen and which are both associated with fiber tracts and participate in adhesion-related processes. In establishing a strategy for identification of chick Ll antigen, our initial experiments focused on the production of antibodies with interspecies cross-reactivity. However, even when polyclonal antibodies to mouse Ll antigen were produced in different species using different immunization protocols, only a very weak reaction close to background values was obtained against chick brain membrane proteins. A second approach was to use conventional biochemical purification methods such as gel filtration, ion-exchange and hydrophobic chromatography to isolate molecules with chromatographic properties similar to those of mouse Ll antigen. However, these methods did not provide adequate resolution in separating the complex mixture of brain plasma membrane proteins.

The third, successful strategy for identification of chick Ll antigen, combining lectin affinity chromatography and monoclonal antibody production, is described here. This procedure yielded a series of monoclonal antibodies recognizing several high molecular mass neural cell surface glycoproteins.

One of the antigens, prepared using mAb G4, is at present the most clearly related to mouse Ll antigen. Both the chicken G4 and mouse Ll antigens have similar molecular mass components, isoelectric points, histological distributions in the developing brain, and homologous NH_2-terminal amino acid sequences. Although the G4 and Ll NH_2-terminal sequences were identical in about half of their residues, and therefore display highly significant homology, the NH_2-terminal sequences of mouse and chicken NCAM are



A
50 µm
B
100 µm
C
EGL
ML
PC
IGL
100 µm
D
E
F
EGL
ML
PC
IGL
G
H
I
EGL
ML
PC
IGL
J
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L
EGL
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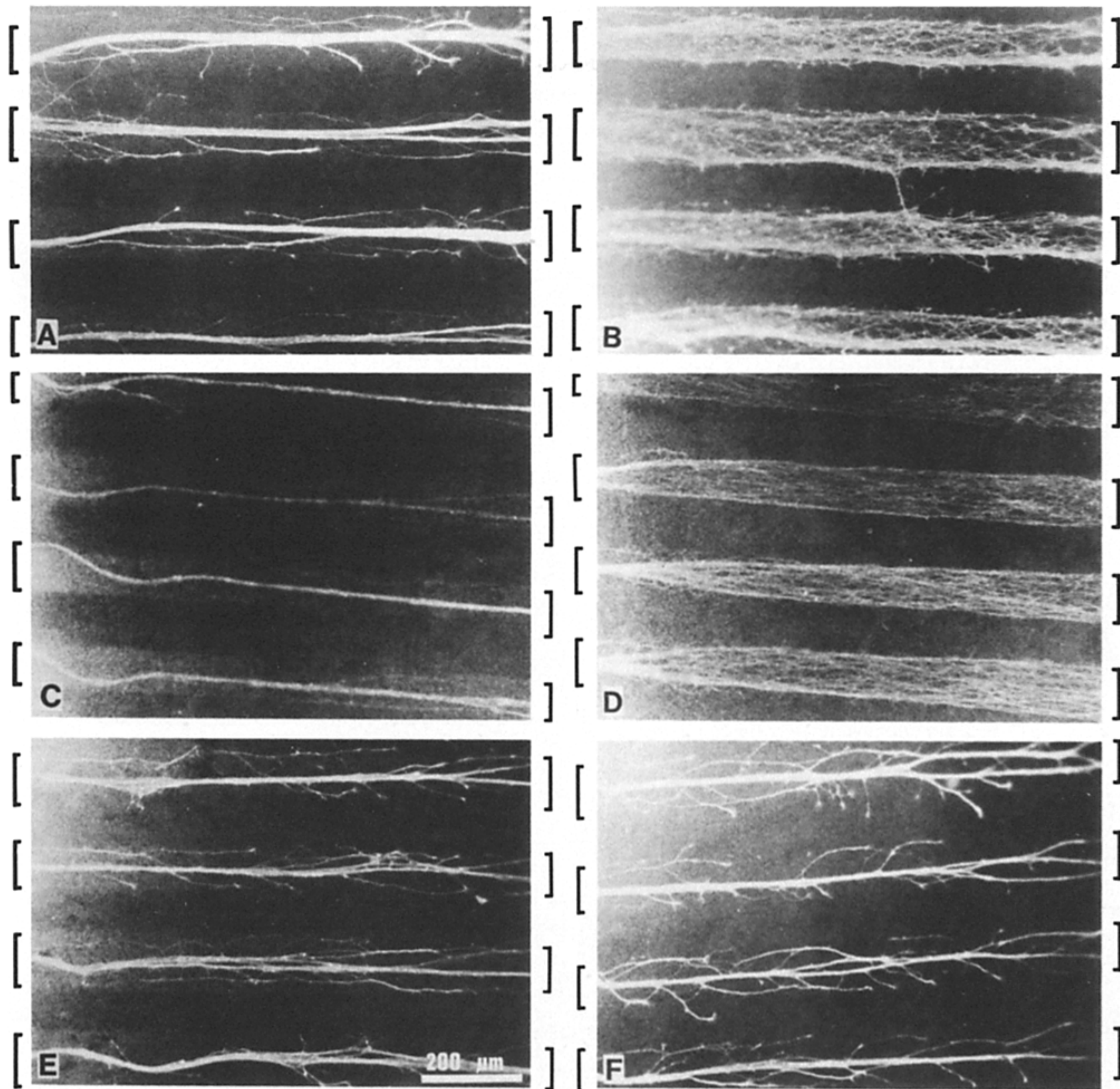

Figure 8. Inhibition of fasciculation of retinal axons by Fab fragments of polyclonal antibodies to mAb G4 immunoaffinity isolate (*B*), to mAb Fl1 immunoaffinity isolate (*D*), and to mAb C5 immunoaffinity isolate (*F*). Rhodamine-labeled retinal explants–extending from the nasal to the temporal part of the retina of a 6-d-old chick embryo–were placed on nuclepore filters coated with anterior tectal plasma membrane vesicles. Nuclepore filters were coated in strips ~90 μm in width (indicated by brackets) interspersed with noncoated strips (not marked) of the same width. Fluorescent photomicrographs of the outgrown, rhodamine-labeled fascicles of temporal axons are shown. The retinal explant is at the left of each photomicrograph and not shown. *A*, *C*, and *E* show comparable fields of the control cultures of explants originally adjacent in the retina to those shown in the test cultures of *B*, *D*, and *F*, respectively.

Figure 7. Localization of mAb G4 and mAb Fl1 antigenic sites on retinal axons and in sections of embryonic chicken cerebellum. (*A*, *D*, *G*, and *J*) Dissociated retinal cells from 9-d-old chick embryos were grown on poly-L-lysine–coated glass slides for 6 d in vitro and stained indirectly by mAb Fl1 (*A*) and polyclonal antibodies to mAb G4 isolate (*G*). *D* and *J* are phase-contrast micrographs of the same field shown in *A* and *G*, respectively. (*B*, *E*, *H*, and *K*) Retinal explants from 6-d-old chick embryos were cultivated on laminin-coated glass slides for 36 h and stained indirectly by affinity-purified polyclonal antibodies to mAb Fl1 isolate (*B*) and by polyclonal antibodies to mAb G4 isolate. *E* and *K* are phase-contrast micrographs of the same field shown in *B* and *H*, respectively. (*C*, *F*, *I*, and *L*) Cryostat sections (12 μm thick) of cerebellum from a 16-d-old chick embryo were stained indirectly by mAb Fl1 (*C*) and mAb G4 (*I*). Fluorescence micrographs (*C* and *I*) of comparable fields are shown. *F* and *L* are phase-contrast micrographs of the same field shown in *C* and *I*, respectively. Note that staining of mAb G4 but not of mAb Fl1 can be observed in the internal part of the external granular layer (*EGL*). Prominent staining is seen for both antibodies in the molecular layer (*ML*), whereas the internal granular layer (*IGL*) is weakly stained. Fl1 antigen is strongly expressed in the Purkinje cell (*PC*) layer.

~90% identical (unpublished data). Thus, either NCAM is more highly conserved between those species or G4 antigen represents a glycoprotein related but not completely homologous to chicken L1. In this respect, it is notable that our initial strategy using cross-species immunoreactivity, which generally requires ~70% identity in sequence (9), was not successful. This failure suggests that the L1 antigens in mouse and chick are not as highly conserved as are NCAMs, and argues that G4 and L1 are probably the homologous protein in these two species. Therefore, although we cannot exclude the possibility that another chicken protein exists with a higher degree of homology to mouse L1, we tentatively designate the G4 antigen as chicken L1.

It has been suggested that another chick cell adhesion molecule, called neuron–glia cell adhesion molecule (NgCAM), is related or identical to mouse L1 antigen (21). Both L1 and NgCAM comprise three polypeptides of similar molecular mass and antigenic properties, with the two smaller components being antigenically related to the largest component but not to each other (14, 21). Our findings therefore raise the question whether chicken G4 antigen is the same as or closely related to NgCAM. However, one reason against this assumption is that polyclonal antibodies to chick G4 antigen, and to the F11 antigen, do not inhibit adhesion of neurons to glial cells (our unpublished data), and a similar observation has been made for mouse L1 antigen (29). On the other hand, antibodies against NgCAM are known to inhibit neuron–glia adhesion (20). On the basis of these results, it would appear that either NgCAM is not completely identical to L1, or that the in vitro demonstration of neuron–glial cell adhesion for NgCAM is not an accurate reflection of the molecule's function in vivo. In this respect, it is noteworthy that the predominant expression of L1 on axon fascicles, which during early development primarily contain neurites in direct contact with each other, is consistent with a prominent role for this molecule in fiber–fiber interactions.

The applied cascade steps for mAb production used in our studies also led to the identification of other high molecular mass neural cell surface glycoproteins associated with fiber tracts. Among these we have described components isolated by mAb F11 with molecular masses of 120, 135, and 170 kD. The biochemical and immunological data presented suggest that the major 135-kD component of F11 antigen is not related to G4 antigen components. The 170 kD component of mAb F11 isolate is not recognized by polyclonal antibodies to G4 or F11 antigen, suggesting that this component does not share antigenic determinants with the 135-kD components and might therefore co-isolate during immunoaffinity chromatography on mAb F11 columns. However, at present it is not known whether the mAb F11 itself recognizes the 170-kD component, since its antigenic site is SDS labile and immunotransfers can therefore not be analyzed by mAb F11. To choose definitively between these possibilities, a detailed biochemical characterization of each component is being carried out to establish their structural relationships directly.

The fiber-associated distribution of mAb F11 antigenic sites and the defasciculation activity of polyclonal antibodies to F11 antigen suggests that this component may have a function similar to that of the L1 antigen. The mouse L1 antigen has been linked to cell–cell adhesion on the basis of the strong inhibition of neuroblastoma N2A cell aggregation by antibodies against L1 antigen (38). Inhibition of aggregation by Fab in an in vitro short-term assay has proven so far to be a reliable criterion for direct involvement of an antigen in adhesion. However, G4 and F11 antigens described in this study are not expressed in significant amounts on dissociated cells, and therefore it has not been possible to carry out adhesion studies of this type. To circumvent this problem, we have turned to the more complex fasciculation assay using retinal explant cultures to evaluate function. Under the culture conditions used retinal fibers show a strong tendency to fasciculate in the absence of antibodies; that is, they appear to adhere to each other more readily than to the substratum. Defasciculation should then occur if side-to-side adhesion among neurites is inhibited by antibodies, while neurite–substratum adhesion is relatively unaffected. Extrapolating from our studies and similar results obtained for mouse L1 (16) and rat NILE proteins (40), it appears that alteration of fasciculation patterns obtained by Fab fragments in the retinal culture system primarily reflects a perturbation of fiber–fiber adhesion. The present studies therefore suggest that axons have a group of distinct cell surface glycoproteins on their surfaces that contributes to fasciculation. The possibility that these molecules may provide a basis for selective axon behavior during formation of major fiber tracts is addressed experimentally in the following paper (8).

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