Nerve Growth Cones Isolated from Fetal Rat Brain. IV. Preparation of a Membrane Subfraction and Identification of a Membrane Glycoprotein Expressed on Sprouting Neurons

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ABSTRACT This study describes the preparation of a membrane subfraction from isolated nerve growth cone particles (GCPs) (see Pfenninger, K. H., L. Ellis, M. P. Johnson, L. B. Friedman, and S. Somlo, 1983, *Cell*, 35:573–584) and the identification in this fraction of a glycoprotein expressed during neurite growth. While ~40 major polypeptides are visible in Coomassie Blue–stained SDS polyacrylamide gels of pelleted (partially disrupted) GCPs, a salt-washed membrane fraction prepared from lysed, detergent-permeabilized GCPs contains only 14% of this protein and has an unusually simple polypeptide pattern of seven major bands. Monoclonal antibodies have been generated to GCP membranes isolated from fetal rat brain. These antibodies have been screened differentially with synaptosomes from adult rat brain in order to identify those which recognize antigens expressed selectively during neurite growth. One such antibody (termed 5B4) recognizes a developmentally regulated membrane glycoprotein that is enriched in GCP membranes and expressed in fetal neurons sprouting in vitro. The 5B4 antigen in fetal brain migrates in SDS polyacrylamide gels as a diffuse band of ~185–255 kD, is rich in sialic acid, and consists of a small family of isoelectric variants. Freezing-thawing and neuraminidase digestion result in the cleavage of the native antigen into two new species migrating diffusely around 200 and 160 kD. Prolonged neuraminidase digestion sharpens these bands at about 180 and 135 kD, respectively. In the mature brain, antibody 5B4 recognizes a sparse polypeptide migrating at ~140 kD. As shown in the following paper (Wallis, I., L. Ellis, K. Suh, and K. H. Pfenninger, 1985, *J. Cell Biol.*, 101:1990–1998), the fetal antigen is specifically associated with regions of neuronal sprouting and, therefore, can be used as a molecular marker of neurite growth.

The biochemical analysis of growth cones of sprouting neurons has been limited by the inability heretofore to isolate these structures in sufficient quantity. A further difficulty is the structural complexity of the growth cone: in addition to the plasma membrane, it contains several types of organelle including endomembrane systems (4, 10, 58, 67). In previous publications, we have described a subcellular fraction from fetal rat brain highly enriched in particles that (*a*) have all the cytological characteristics of nerve growth cones (40), (*b*) co-purify with nerve growth cones microdissected from cultures (40), and (*c*) contain a complement of phosphoproteins characteristic of neurons (for review see, e.g., reference 36), including synapsin I and its kinase (11, 28).

We describe here the preparation of a membrane subfrac-

tion (GCM)[1] of the nerve growth cone particle (GCP). These membranes contain a simplified set of polypeptides and can be prepared in sufficient quantity (100-μg amounts) for the generation of polyclonal and monoclonal antibodies (mAb's) against their components. The preparation of such antibodies was based on the following rationale. During embryogenesis, the neuron undergoes dramatic changes in phenotype as it differentiates from a dividing neuroepithelial cell into a post-mitotic, sprouting neuron and, finally, into a neuron with synaptic connections with the appropriate target cells. One experimental approach to the analysis of growth-specific molecules is the immunochemical comparison of components expressed during specific stages of neuronal development, e.g., components present in GCPs vs. synaptosomes (their mature counterpart from adult brain). The availability of substantial quantities of GCPs and synaptosomes makes it feasible to use these subcellular fractions as antigens for the generation of mAb's. One can then select the antibodies that recognize developmentally regulated neuronal antigens by screening differentially with both fractions. In a report to be published shortly we will describe an antigen expressed in synaptosomes but not in GCPs (cf. reference 62). In the present study, we describe a mAb which recognizes a developmentally regulated neuronal antigen of GCP membranes that is present only in very small amounts in synaptosome fractions. The antigen exhibits biochemical properties consistent with those of a polysialylated membrane glycoprotein. Preliminary reports of these findings have been presented in abstract form (62, 63).

MATERIALS AND METHODS

Materials

Ultrapure sucrose was from Schwarz/Mann Div., Cambridge, MA. *N*-Tris-[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), Tris(hydroxymethyl)aminomethane (Tris, as Trizma base), aminopterine, thymidine, soybean trypsin inhibitor, DNAase I, and all protease inhibitors were from Sigma Chemical Co., St. Louis, MO. Trypsin was from Worthington Biochemical Corp., Freehold, NJ. Protein standards for SDS PAGE were from Pharmacia Fine Chemicals, Piscataway, NJ, and Bio-Rad Laboratories, Richmond, CA. Calbiochem-Behring Co., La Jolla, CA, supplied neuraminidase and hypoxanthine. SDS was from British Drug House (obtained through Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY). Ampholytes were from LKB Instruments, Inc., Paramus, NJ. All chemicals for SDS PAGE were electrophoresis grade. 4-Chloro-1-naphthol was obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were reagent grade. Nitrocellulose was from Schleicher & Schuell, Inc., Keene, NH (BA85, 0.45 μm). Immunochemicals were from Boehringer Mannheim Biochemicals, Indianapolis, IN. Culture media were from Gibco Laboratories, Grand Island, NY, and fetal bovine serum was from Flow Laboratories, Inc., McLean, VA.

Subcellular Fractions and Membrane Preparations

All membrane fractions were prepared from adult or developing brains of Sprague-Dawley rats. The gestational age of fetal rats is indicated counting the plug date as day 0.

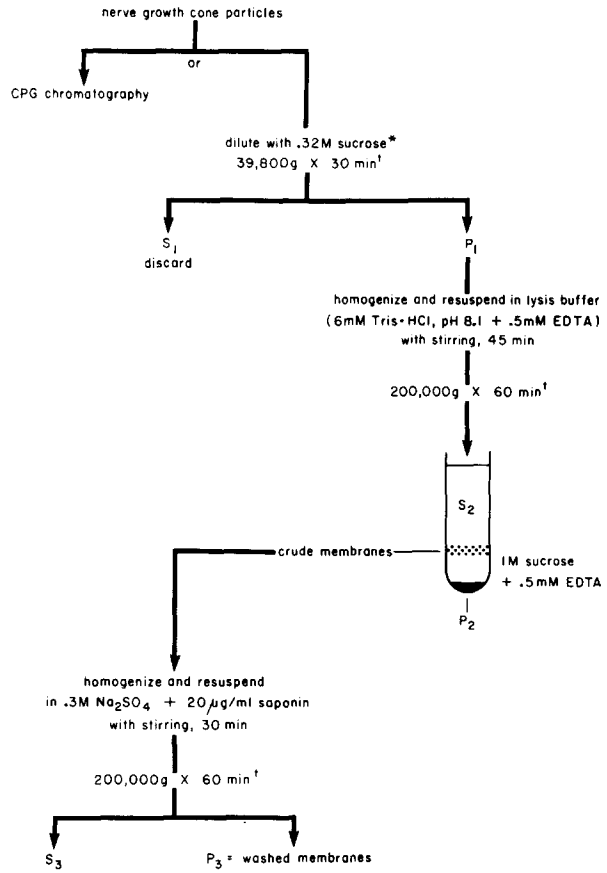
CRUDE MEMBRANE PREPARATIONS: Crude membrane preparations were generated by homogenizing the brains gently (Teflon/glass homogenizer) in ~6 vol of 0.32 M sucrose containing 1 mM $MgCl_2$ and 1 mM TES buffer (pH 7.3). Homogenates were spun at 1,660 g_{max} for 15 min in a Beckman JA-17 rotor, the pellet was discarded, and the supernatant pelleted at 100,000 g_{max} for 60 min in a Beckman SW55Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellets were then resuspended in the appropriate buffer system for dot-immunobinding assay (DIA) or gel electrophoresis as indicated below.

GROWTH CONE PARTICLES (GCPS): GCPs were prepared from 17–18-d fetal brains according to the two-step procedure described by Pfenninger et al. (40). The resulting "A fraction" was diluted three- to fourfold with 0.32 M sucrose (with 1 mM $MgCl_2$ and 1 mM TES buffer, pH 7.3) and pelleted at 39,800 g_{max} for 30 min at 4°C. This GCP pellet (P1) was resuspended for use as described or processed further for the generation of GCP membranes as indicated below. In many situations (see text), GCPs and membranes were prepared in the presence of the following protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), soybean trypsin inhibitor (type 1-S; 100 μg/ml), pepstatin A (1 μg/ml), and leupeptin (30 μM).

GCP MEMBRANES (GCMS): GCMs were prepared from the "A-fraction" pellet as follows (see Fig. 1): GCPs were lysed by resuspending the pellet (P1) in 0.5 ml of 6 mM Tris-HCl, pH 8.1, containing 0.5 mM EDTA, adding Tris/EDTA to a volume of 4 ml, and then stirring at 4°C for 45 min (cf. reference 9). The lysed material was loaded onto a 1.0-ml cushion of 1 M sucrose containing 0.5 mM EDTA and spun at 200,000 g_{max} for 60 min at 4°C in a SW55Ti rotor (Beckman L8-55).

SOLUBLE PROTEINS (S2): S2 were precipitated from the lysate by the addition of 50% ice-cold trichloroacetic acid to a final concentration of 8.3% and collected by centrifugation (11,200 g_{max} for 20 min at 4°C). The resulting pellet was washed three times with ice-cold diethylether to remove the trichloroacetic acid (15) and solubilized in sample buffer for electrophoresis (see below).

MEMBRANE FRACTION: The crude membrane fraction was collected at the Tris/sucrose interface and treated with 5 ml of 0.3 M Na_2SO_4 plus 20 μg/ml saponin for 30 min at 4°C, with stirring (6). The washed membranes were



* Solution contains 1mM $MgCl_2$ + 1mM TES·NaOH, pH 7.3

† All g values are maximum centrifugal forces

FIGURE 1 Flow diagram of the preparation of growth cone membranes.

[1] *Abbreviations used in this paper:* BSA-P, 1% bovine serum albumin–phosphate buffer; DIA, dot-immunobinding assay; GCMs, growth cone particle membranes; GCPs, growth cone particles; IEF, isoelectric focusing; mAb's, monoclonal antibodies; N-CAM, neural cell adhesion molecule; SS, synaptosome; TBSA, 3% bovine serum albumin in 50 mM Tris-HCl, pH 7.3, plus 150 mM NaCl.

collected by centrifugation at 200,000 g_{max} for 60 min at 4°C. The pellet (P3) was washed twice with water, resuspended in 50 μl of water by homogenization in a glass-glass grinder (Bees-Knees; Pierce Chemical Co., Rockford, IL), and either prepared immediately for SDS PAGE by adding an equal volume of twice-concentrated sample buffer (31) or stored frozen at −20°C until used. Proteins in the final supernatant (S3) were precipitated and collected as above for S2. In some experiments, all solutions contained the protease inhibitors mentioned above.

For a series of experiments, GCMs were incubated at 37°C for various periods of time with neuraminidase (from *Vibrio cholerae*; 0.125 IU/ml). The incubation medium consisted of 0.05 M sodium acetate buffer, pH 5.0, 2 mM $CaCl_2$, and 0.2 mM EDTA, with the protease inhibitors listed above. Approximately 100 μg of sample proteins was incubated in a total volume of 200 μl. Control membranes were incubated in the same solutions, but without neuraminidase. After incubation, membranes were pelleted and then dissolved in sample buffer for electrophoresis.

SYNAPTOSOMES (SS's): SS's (16) were prepared from adult rat brain essentially according to the procedure of Cohen et al. (9). Cortices were homogenized in 0.32 M sucrose containing 1 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$ (solution A), at a ratio of 10% wet wt/vol, and filtered through a 100-μm nylon mesh screen. The 1,400 g_{av} (10 min, Beckman JA-17 rotor) supernatant of this filtrate was centrifuged at 13,800 g_{av} for 10 min (JA-17 rotor). The pellet was homogenized in 1.5 ml of 0.32 M sucrose plus 1 mM $NaHCO_3$ (solution B), and layered onto a three-step sucrose density gradient (2.6 M:7 ml; 1.2 M:15 ml; 1.0 M:15 ml). This gradient was spun at 131,700 g_{av} using a vertical rotor (Beckman VTi50) for 40 min. The band formed at the 1.0/1.2 M sucrose interface was collected, diluted in 4 vol of solution A, and pelleted for 30 min at 27,200 g_{av}. The pelleted SS fraction was resuspended in phosphate-buffered saline (PBS), homogenized, and frozen away in aliquots for later use.

Generation of mAb's

The technique used for the production of mAb's was based on the original work of Kohler and Milstein (30), as modified by Sharon et al. (52). BALB/c mice were given an initial intraperitoneal (i.p.) injection of pelleted GCPs (400 μg protein) in PBS, mixed 1:1 with Freund's complete adjuvant. Over a period of months mice were boosted with i.p. injections of GCPs plus Freund's incomplete adjuvant, and then given a final intravenous (i.v.) injection with salt-washed GCMs in PBS. 4 d after the i.v. injection, the spleen was aseptically removed from an immunized mouse, and splenocytes were prepared. Splenocytes were fused with a k-chain-secreting myeloma cell line (45.6.TG.1.7.5) using PEG-1000 (J. T. Baker Chemical Co., Phillipsburg, NJ). Cells were fused at a ratio of 10 splenocytes to 1 myeloma and plated overnight in Iscove's medium plus 20% fetal calf serum at 37°C, in the presence of 4% CO_2 in air. The following day, the cells were pelleted through a cushion of 100% fetal calf serum (140 *g* for 10 min) and resuspended in a 1:1 mixture of Iscove's medium with 20% fetal calf serum and myeloma-conditioned medium, plus HAT components (10^{-4} M hypoxanthine, 10^{-5} M aminopterine, and 3.0×10^{-5} M thymidine). The resuspended cells were plated into 96-well microtiter plates at a density of 125,000 cells/well. The cells were maintained at 37°C in air with 4% CO_2 added. Hybrids were fed on days 5 and 10 after the fusion and then switched to HAT medium minus the aminopterin. Wells containing >50% confluent hybridoma growth were assayed for antibody production.

DIFFERENTIAL SCREENING OF HYBRIDOMAS: Selected wells were screened for the presence of antibodies that would differentially recognize GCPs but not synaptosomes in either an enzyme-linked immunosorbent assay (see reference 12) or a DIA (see reference 19). Briefly, 1 μl of GCPs or SS's in Tris-buffered saline (50 mM Tris-HCl, pH 7.3, plus 150 mM NaCl) were spotted onto a nitrocellulose filter grid at a concentration of 1 μg protein/μl. Remaining reactive areas of the filter were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBSA), and the individual filters were incubated with supernatants from selected hybridoma wells diluted two- to fourfold in TBSA. After washing with TBSA, the filters were incubated with an immunoaffinity-purified goat anti-mouse-IgG conjugated to horseradish peroxidase. Filters were again washed with TBSA and reacted with the chromogenic substrate 4-chloro-1-naphthol. Supernatants binding to the spotted antigens on the filter develop a purplish spot after the substrate incubation. Hybridomas of interest were then cloned in semi-solid agarose (52) once or twice, grown in mass culture, and then used for the preparation of ascites fluid. Aliquots of the cloned hybrids were also frozen away in liquid nitrogen.

Biochemical Methods

Protein concentrations were determined according to the method of Bradford (see reference 3; Bio-Rad reagent), using bovine plasma gamma-globulin as standard. Phospholipid phosphorus was determined by a modification of the method of Ames and Dubin (1).

PAGE: One-dimensional SDS PAGE was carried out according to Laemmli (31, as described in reference 46). All samples were boiled for 3 min in the presence of SDS and beta-mercaptoethanol prior to electrophoresis. Slab gels were 140 × 240 × 0.75 mm, with a 20-mm stacking gel of 3% acrylamide. They consisted either of a 5–15% linear acrylamide gradient (run for a distance of 200 mm in the resolving gel) or of 5% acrylamide (run for a distance of 100 mm in the resolving gel). Gels were stained with Coomassie Blue (65) or ammoniacal silver (66). The molecular mass standards were as follows: myosin (200 kD), beta-galactosidase (116 kD), phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and alpha-lactalbumin (14.4 kD). Molecular mass values of blotted proteins were determined by extrapolation from the position of blotted protein standards; the values indicated are based on separation on 5% acrylamide gels and are averages (rounded to the nearest 5 kD) of at least two independent determinations.

Two-dimensional gels with isoelectric focusing (IEF) in the first dimension were prepared as described by O'Farrell (37), with the following modifications: samples contained about 100 μg protein in 60 μl; gels were 130 × 2 mm; ampholytes of pH 5–7 (0.8%), pH 3.5–5 (0.8%), and pH 3.5–10 (0.4%) were included in the sample buffer and IEF gels. Second dimension gels were the slab gels prepared as described above.

WESTERN BLOT ANALYSIS: Proteins were transferred electrophoretically from SDS PAGE or two-dimensional gels to nitrocellulose paper in an electroblot apparatus (E-C Apparatus Corp., St. Petersburg, FL) at 300 mA, for 1 h as described by Towbin et al. (60). The resulting blots were processed according to the procedure of Hawkes et al. (19). Initially, blots were incubated with hybridoma culture supernatant (diluted 1:1 with TBSA. In later experiments, different batches of 5B4 ascites fluid, diluted 1:2,000, were used. mAb's bound to the blot were detected with a horseradish peroxidase–conjugated goat anti-mouse-IgG (immunoaffinity-purified, light chain– and heavy chain–specific). The peroxidase reaction was carried out with 4-chloro-1-naphthol as a substrate. Control blots were incubated with either myeloma supernatant or with ascites fluid from myeloma-injected mice.

Morphological Methods

ELECTRON MICROSCOPIC ANALYSIS: Samples for electron microscopic analysis were prepared as described in a previous paper (40), except that high-speed pellets P1 and P3 were fixed and embedded in toto in the ultracentrifuge tube.

CELL CULTURE AND IMMUNOFLUORESCENCE: Brains from fetal rats at day 18 of gestation were used for the preparation of neural cultures. Regions of the cortex were divided into smaller pieces and then incubated in a trypsin solution (500 U/ml) at ambient temperature for 8 min. Soybean trypsin inhibitor and DNAase I (4 mg/ml and 0.025 mg/ml, respectively) were added, and the dissociated cells were pelleted from solution at ~100 *g* for 8 min. Cells were resuspended in medium and plated into 35-mm poly-D-lysine-coated plastic dishes. The cells were grown in F-12 medium containing 10% fetal calf serum, insulin, transferrin, progesterone, putrescine, selenous acid, and estradiol (all from Sigma Chemical Co., St. Louis, MO) at 37°C, with ~3% CO_2 in humidified air (2). Cultures were maintained for 3 d to 1 wk.

Cultured cells were fixed by slowly infusing, directly into the culture medium, a 2% solution of paraformaldehyde in phosphate buffer (0.1 M, pH 7.3). After infusion, 10 drops of fixative were added to the plates. After 5 min the cultures were drained and fresh 2% paraformaldehyde–phosphate buffer was added with a pipette. After 15 min in the fresh fixative, the cultures were carefully washed with phosphate buffer. They were then incubated in 1% BSA–phosphate buffer (BSA-P) to block any residual aldehyde groups; some cultures were also permeabilized by incubating in BSA-P containing 0.02% saponin. Antibodies used for incubation were all diluted in BSA-P (all incubation media and washes of the permeabilized cultures contained 0.02% saponin in BSA-P). Primary antibody incubation was with 5B4 ascites fluid diluted 500- to 1,000-fold or, as a control, with myeloma ascites fluid diluted to the same extent. Incubation was for 2 h at room temperature. Cultures were then washed six times for 5 min each time followed by the addition of the secondary antibody. An immunoaffinity-purified goat anti-mouse-IgG conjugated to fluorescein isothiocyanate, diluted 40-fold, was used as the secondary antibody. Incubation was as above. After 2 h, the cultures were washed six times for 5 min each time and partially drained. The sides of the 35-mm dishes were then broken off. Two drops of 50% glycerol in phosphate buffer were placed on the cultures, followed by a round coverslip which was sealed in place with nail polish. The cultures were observed using a Leitz Ortholux microscope equipped with epifluorescence optics.

RESULTS

Preparation of GCMs

GCPs consist of sheared-off, membrane-bound cellular fragments that have the ultrastructural features of nerve growth cones (cf. Fig. 2): they contain an abundance of microfilaments, large dense-core vesicles, large clear vesicles, elongated agranular reticulum and occasional mitochondria, but no ribosomes (40). If GCPs are separated from soluble proteins of the "A-fraction" by chromatography on a column of controlled pore glass (40), they are ultrastructurally intact and exclude both the extracellular tracer [^{3}H]inulin (Clark, R., and K. H. Pfenninger, unpublished observations) and γ-^{32}P-ATP (Hyman, C., and K. H. Pfenninger, unpublished observations). More efficient for the large-scale preparation of GCPs and for the isolation of membranes from them is a second strategy (Fig. 1). The A-fraction is diluted with 0.32 M sucrose and pelleted (P1). As illustrated in Fig. 3, P1 contains the GCPs, but many of them have been broken during pelleting (cf. Fig. 1 in ref. 40). The A-fraction represents ~5% of homogenate protein (5.4 ± 0.8%; mean ± SEM, n = 13; see ref. 40). However, P1 contains only ~8% (8.2 ± 2.3%; mean ± SEM, n = 6) of the protein of the A-fraction, or ~0.4% of homogenate protein.

In initial experiments, P1 was treated with a hypotonic buffer at basic pH (6 mM Tris-HCl, pH 8.1), rapidly frozen in liquid nitrogen, and then thawed to assist in the lysis of the GCPs. Finally, the membranes were collected as a high-speed pellet (200,000 g_{max} for 60 min). However, the membranes collected in this manner are frequently resealed and often have electron-dense fibrillar material attached to them (data not shown). Furthermore, aggregates of electron-dense material and organelles, such as mitochondria, contaminate this membrane preparation. Therefore, the protocol has been modified as follows (cf. Fig. 1): P1 is resuspended in hypotonic Tris-HCl as before, but with 0.5 mM EDTA added to assist with lysis of the particles; furthermore, the crude membrane fraction is spun onto a 1.0-M sucrose cushion. While heavier elements, such as mitochondria and lysosomes, are pelleted (P2), the lighter membrane sacs band at the 1.0-M sucrose interface. The crude membranes are then washed in a solution of high ionic strength (0.3 M Na_2SO_4) containing a low concentration of the non-ionic detergent saponin (20 μg/ml) to permeabilize any resealed membrane vesicles. The washed membranes are collected as a high speed pellet (P3). Electron microscopic analysis of P3 reveals a highly homogeneous membrane preparation (Fig. 4). At higher magnification (Fig. 5), the bilayer of the membranes is seen to be intact, the membrane surfaces are essentially free of adsorbed electron-dense protein, and the pellet is devoid of any identifiable organelles or amorphous debris. The protein yield of the GCM preparation, P3, is 13.7 ± 1.1% (mean ± SEM, n = 7) with respect to P1. The removal of >85% of the protein of the already disrupted GCPs (i.e., P1) during this procedure is accompanied by an increase in the ratio of phospholipid to protein (based on phospholipid phosphorus) in the final GCM pellet (P3). The protein-to-phospholipid ratio is decreased from ~3.5 in P1 to ~0.95 in P3 (average of two determinations; data not shown).

Polypeptides of the GCP and GCMs

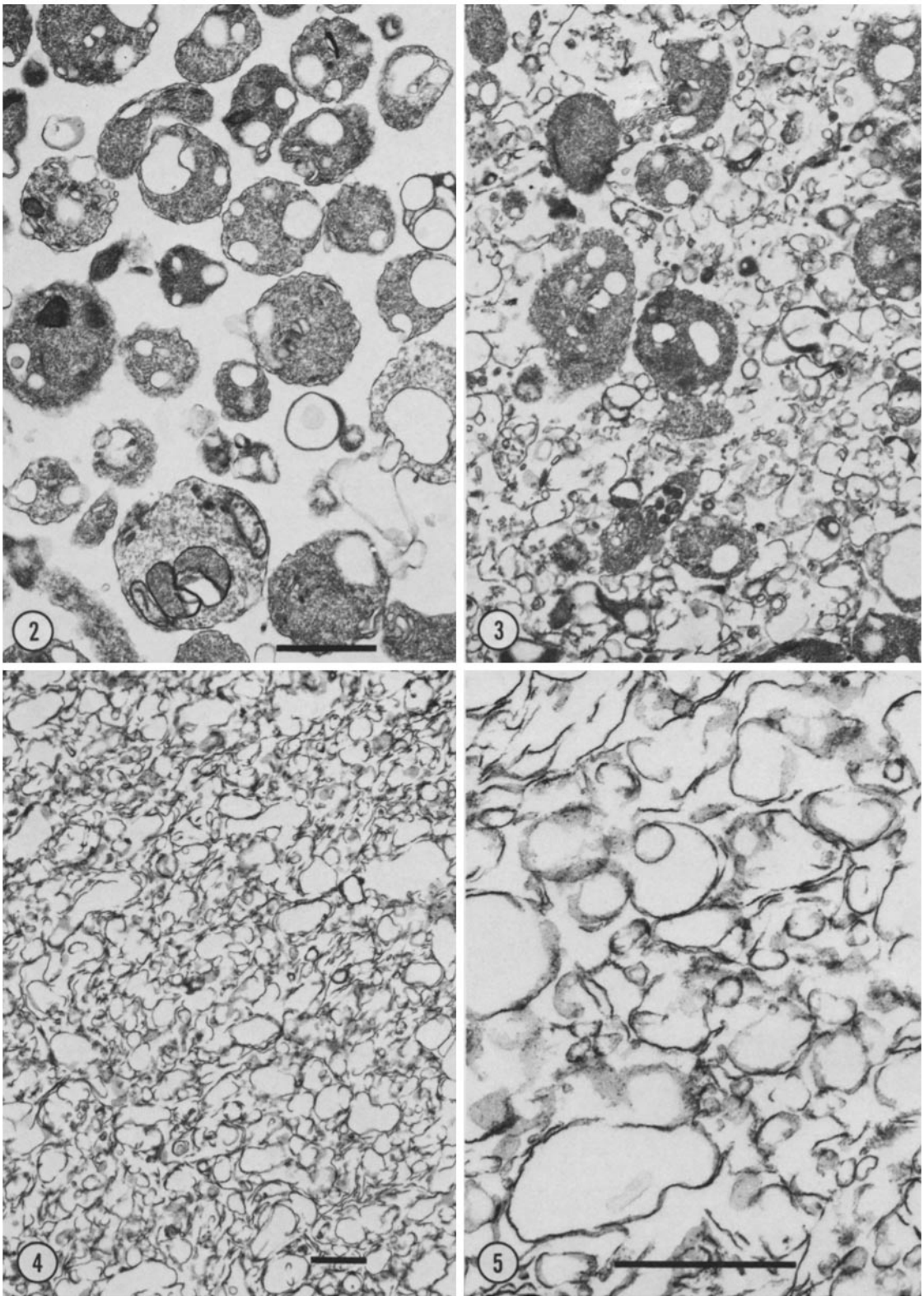
The polypeptide pattern of pelleted GCPs (P1) on one-dimensional SDS polyacrylamide gels is comprised of 40 or 70 bands when stained with Coomassie Blue or silver, respectively (Fig. 6, *GCP*). Upon hypotonic lysis of the particles at basic pH, the majority of the bands visible in the GCP lane are also present in the lysate (S2) (*LYS* in Fig. 6), including prominent bands of 52 and 42 kD. Under one-dimensional gel electrophoresis, tubulin purified from adult rat brain (cf. ref. 55) forms a single, broad band (the α and β subunits are not well resolved) that co-electrophoreses with the major GCP and lysate band visible at 52 kD. Furthermore, actin (prepared as actomyosin from mouse heart; see reference 17) co-migrates with the 42-kD major band (data not shown).

In contrast to the GCP lysate, the washed GCMs (Fig. 6, *GCM*) contain a simple polypeptide pattern, with major bands of 52, 46, and 42 kD, and doublets at 38 and 34 kD. Certain of these major bands (38 and 34 kD) are substantially enriched compared to the GCP pattern and are represented minimally, if at all, in the lysate. A similar pattern of GCM polypeptides is observed when gels are stained with silver (Fig. 6).

Selection of Monoclonal Antibodies and Subcellular Localization of the 5B4 Antigen

The fusion of myeloma cells with splenocytes from GCP/GCM-immunized mice resulted in ~200 hybridomas. Each hybridoma supernatant was screened differentially by an enzyme-linked immunosorbent assay of GCP vs. SS protein. This technique identified a total of ~50 hybridomas whose antibodies recognized either GCPs or synaptosomes or both. A family of such hybridomas, six subclones of 5B4, secreted an mAb which reacted with the GCPs but not the SS's. The result of one of these initial screening tests is shown in Fig. 7. Even using a 10-fold amount of protein (5 μg) for SS vs. GCP and 5B4 ascites fluid, we failed to detect antibody binding to SS. Subclone 5B44, forthwith called 5B4, was selected for further analysis. This mAb is of the type IgG_1 as determined by double-diffusion tests with antisera to specific IgG subclasses of the mouse. mAb 5B4 was tested on immunoblots of GCP polypeptides separated by SDS PAGE (on either 5–15% gradient or 5% acrylamide gels). 5B4 stains a unique band that is considerably larger than the 200-kD standard (not shown), but not visible after Coomassie Blue or silver staining of companion gels.

Two approaches were used to study whether the 5B4 antigen is present in GCMs and nerve growth cones. These consisted (*a*) of assaying for the enrichment of the 5B4 antigen in the GCP and GCM fractions from fetal brain and (*b*) of studying by immunofluorescence its distribution in central nervous system neurons sprouting in vitro. The distribution of the 5B4 antigen in vivo is described in the following paper (64). For the biochemical analysis, we loaded on SDS polyacrylamide gels (5% acrylamide) equal amounts of protein from each of the various fractionation steps used to prepare GCMs (cf. Fig. 1), prepared immunoblots, and stained them with mAb 5B4. The results of this study are shown in Fig. 8. Homogenate (*H*), low-speed supernatant and pellet (*LSS* and *LSP*, respectively) exhibit quite low levels of immunoreactivity, in the form of a broad, sometimes heterogeneous band of ~185–255 kD (cf. Figs. 10 and 13). The particulate subfractions of the LSS (*GCPs*, *B*, and *C*; see reference 40) contain considerably more antigen. A dramatic further increase in antigen content is found in GCM. It is important to note that these membranes were washed at a high salt concentration



FIGURES 2–5 (Figs. 2 and 3) Electron micrographs of GCPs fixed in suspension (Fig. 2) or after pelleting (Fig. 3). Note that many of the GCPs in Fig. 3 are broken and/or lysed whereas those fixed in suspension are intact. (Figs. 4 and 5) Electron micrographs of salt-washed membrane pellet (P3). Note that the field is homogenous (Fig. 4) and consists entirely of membranes. At higher magnification (Fig. 5), the membrane bilayers can be seen to be intact and essentially free from contamination. Bars, 1 μm.

prior to electrophoresis in order to minimize the amount of adsorbed non-membrane proteins. In the GCM preparation, we often find broadening of the stained band and, sometimes, an additional weak band of considerably smaller molecular weight (see below). Omission of protease inhibitors during the fractionation procedure and repeated freeze-thaw steps in-

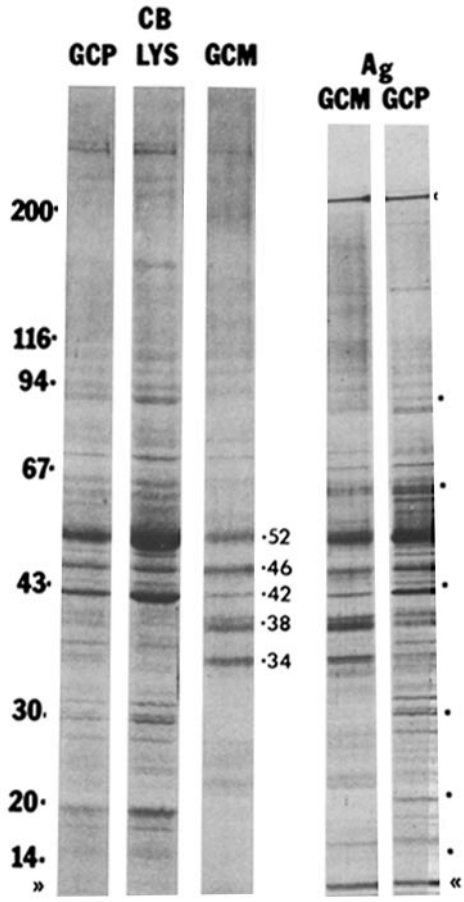


FIGURE 6 Analysis of polypeptides at different stages of GCM preparation. Gels are 5–15% polyacrylamide gradients, stained with Coomassie Blue (lanes *1–3*; *CB*) or silver (lanes *4* and *5*; *Ag*). All lanes are from a single gel. They show: *GCP*, pelleted GCPs (P1); *LYS*, lysate (S2); *GCM*, GCP membrane pellet (P3). The position of molecular mass standards for the Coomassie Blue–stained gels is shown on the left; for the silver-stained gel, the positions of the same standards (from 14 to 94 kD) are indicated by the marks on the right. Apparent molecular masses calculated for the major polypeptide bands of the GCM are also indicated ($\times 10^3$). A staining artifact in the silver-stained gels is indicated by the single arrowhead, and the dye front by double arrowheads.

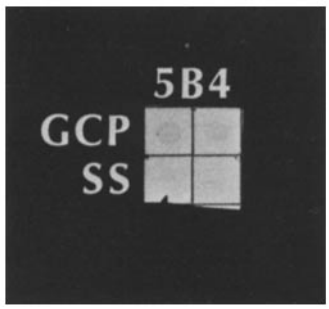


FIGURE 7 DIA with mAb 5B4, comparing synaptosomes (*SS*) and GCPs. Note selective staining of GCP material.

crease the appearance of the lower molecular weight species stained in immunoblots.

The distribution of the 5B4 antigen in the intact neuron was studied by indirect immunofluorescence, using dissociated cells from fetal cerebral cortex grown in culture for several days. In pilot experiments, the antigen was found to be highly sensitive to glutaraldehyde but not denatured by paraformaldehyde. The patterns of immunolabeling of paraformaldehyde-fixed neurons, intact or permeabilized with saponin, are shown in Fig. 9. No fluorescence can be observed in controls with myeloma-conditioned medium (in the presence or absence of saponin) or in labeling experiments in which the cells were not permeabilized with saponin. However, specific fluorescence is seen if labeling with the 5B4 antibody is performed in the presence of a permeabilizing agent. As will be reported in the following paper in detail (64), only neurons are stained in these cultures. Their perikarya are outlined by fluorescence along the plasma membrane. Neurite shafts are also fluorescent. The brightest fluorescence is often seen at the nerve growth cone, especially in its basal region.

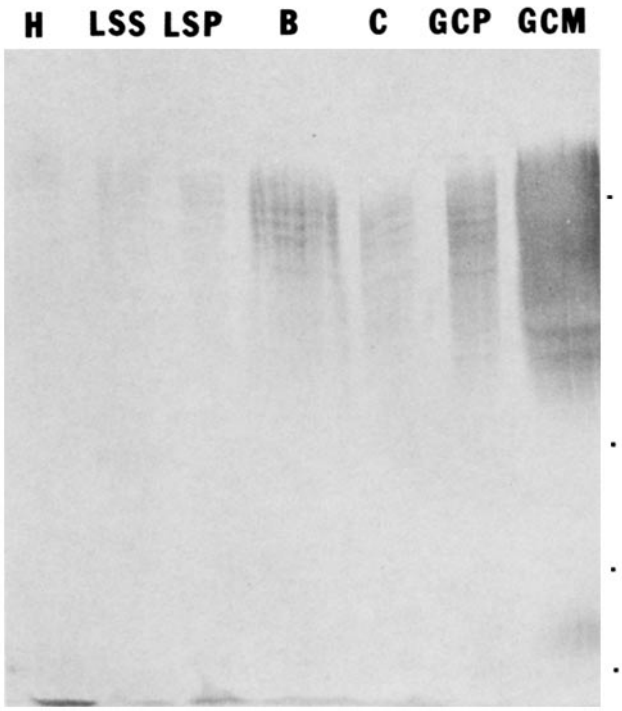


FIGURE 8 Immunoblot to show enrichment of the 5B4 antigen in GCPs and GCP membranes (*GCM*). Equal amounts of protein (100 μg) were loaded in each lane and electrophoresed on 5% acrylamide gels before being blotted and immunostained. The designation of the fractions is as in Pfenninger et al. (40): *H*, homogenate of F17 fetal brain; *LSS*, low-speed supernatant (parent fraction of *GCP*, *B*, *C*); *LSP*, low-speed pellet; *B*, *C*, heavy, heterogeneous subfractions of LSS (0.78:1.0 M sucrose and 1.0:2.6 M sucrose, respectively), known to contain neurite shafts and GCP fragments; GCM, prepared from GCPs as described. The top of this gel (not visible) would be just above the lettering. Dots on the right indicate position of molecular mass markers: 200, 116, 94, and 67 kD. Note the enrichment of the 5B4 antigen in GCM. As described further below, extensive processing of the material (as for GCMs) leads to broadening of the reactive band. In the heavily overloaded GCM lane, a trace of a small degradation product is seen at ~90 kD.

Biochemical Characterization of the 5B4 Antigen

The appearance of the 5B4 antigen on immunoblots as a broad smear suggests that it may be microheterogenous in form, possibly due to differences in glycosylation. To investigate the possible involvement of sialic acid residues, salt-washed GCP membranes were subjected to neuraminidase digestion prior to electrophoresis and immunoblotting. First we examined the commercially obtained neuraminidase for

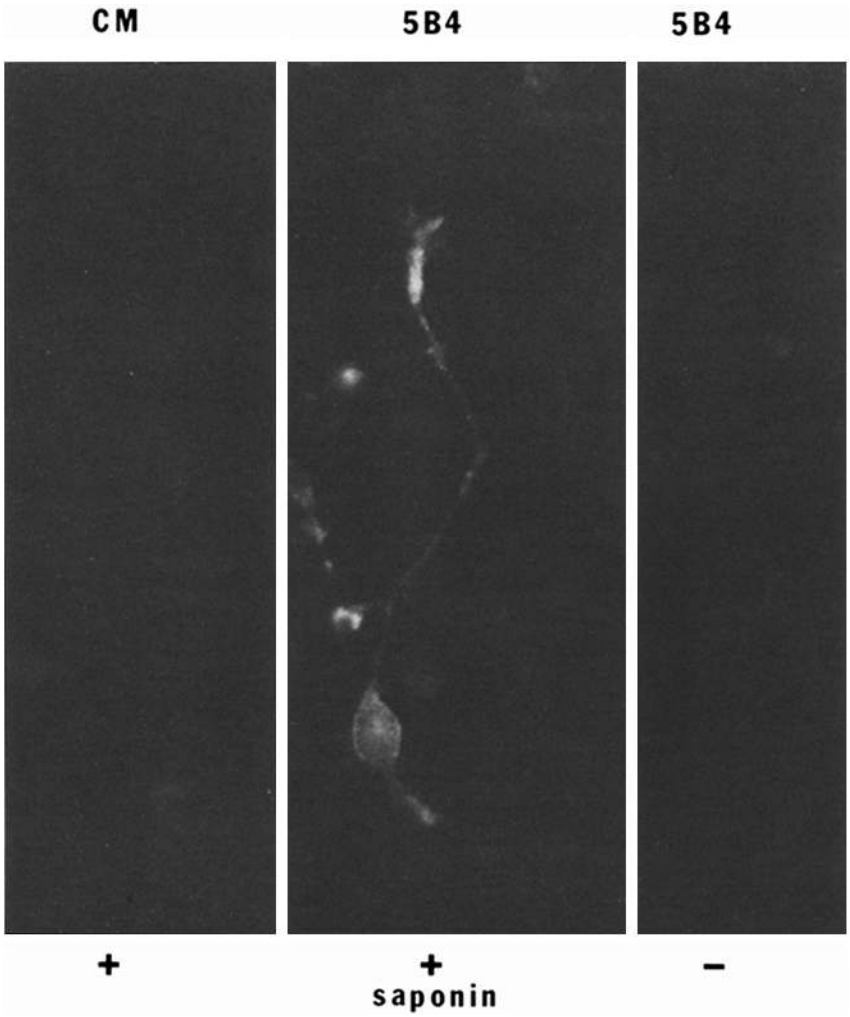


FIGURE 9 Indirect immunofluorescence with mAb 5B4 of dissociated neurons grown in vitro. (*Left*) Staining with control ascites fluid (*CM*), after cell permeabilization (+ saponin). (*Center*) 5B4 staining of permeabilized neuron. (*Right*) 5B4 staining without permeabilization (− saponin). Demonstration of the 5B4 antigen necessitates membrane permeabilization. Note, in the center, ring-like fluorescence around the perikaryon, the fluorescent outline of growth cone lamellopodia and neurite shaft, and the bright fluorescence at the base of the growth cone. Non-neuronal cells present in this culture are not stained.

protease activity using the azo-albumin assay (7, 59). Even after 16 h of incubation at 37°C, proteolysis could not be detected in this neuraminidase preparation (data not shown). Neuraminidase digestion was carried out usually on salt-washed GCP membranes, freshly prepared and incubated in the presence of a cocktail of protease inhibitors (see Materials and Methods). Such membranes, if electrophoresed without prior freezing and thawing, generate the single, diffuse band between 185 and 255 kD described earlier and shown in Fig. 10 (*left panel*). Incubation of these GCMs for up to 8 h at 37°C in the absence of neuraminidase does not lead to major changes in the immunoblot pattern. By 16 h of incubation, the intensity of the stained band is somewhat decreased and a second, faint band appears near 160-kD (data not shown). In contrast, incubation with neuraminidase for as little as ½ h leads to the appearance of two stained bands of significantly greater electrophoretic mobility. Both of these bands are still diffuse but, with increasing neuraminidase digestion, they become gradually sharpened at ~135 and 185 kD, respectively. Note that we observe the highest staining intensity in the samples digested for 16 h, indicating that the material diffusely distributed over a broad molecular weight range in controls has become concentrated to form the sharper bands, and that most of the antigenic material has been retained during the incubation period. The smaller, neuraminidase-induced band appears to be similar in size to the 5B4 antigen of the mature brain (see below). However, co-electrophoresis of the two samples has consistently indicated that the neuraminidase-treated fetal antigen migrates slightly faster on 5% or 5–15% acrylamide gels (cf. Fig. 11 *D*).

The appearance of two bands after neuraminidase treatment and the diffuse nature of the antigen in control gels raise the question of whether two or more protein species are recognized by the 5B4 antibody. This question was investigated using two-dimensional gel electrophoresis. Because of the apparent lability of the fetal 5B4 antigen, we used crude GCP membranes (high speed pellet from lysed GCPs, prepared in the presence of protease inhibitors) in order to avoid its alteration by elaborate processing. The freshly prepared membranes were immediately solubilized and their proteins separated by IEF in the first dimension and SDS PAGE in the second dimension. The immunoblot of such a gel is shown in Fig. 11 *A* and reveals a series of vertical streaks indicating the presence of differently charged species of heterogenous molecular weight.The pI's range from ~5.1 to ~5.3, and the molecular mass varies from ~185 to ~255 kD. Next, GCMs were prepared in the presence of protease inhibitors, incubated for 16 h in the absence of neuraminidase (but with protease inhibitors present), and again analyzed by two-dimensional gel electrophoresis and immunoblotting (Fig. 11 *B*). Again, a series of vertical streaks is evident, but their pI's range from ~4.7 to ~4.9, and their molecular masses from ~150 to ~210 kD. Neuraminidase treatment drastically changes the appearance of the GCM antigen in two-dimensional gels (Fig. 11 *C*). The family of vertical steaks observed in the controls just described has been shifted to a more

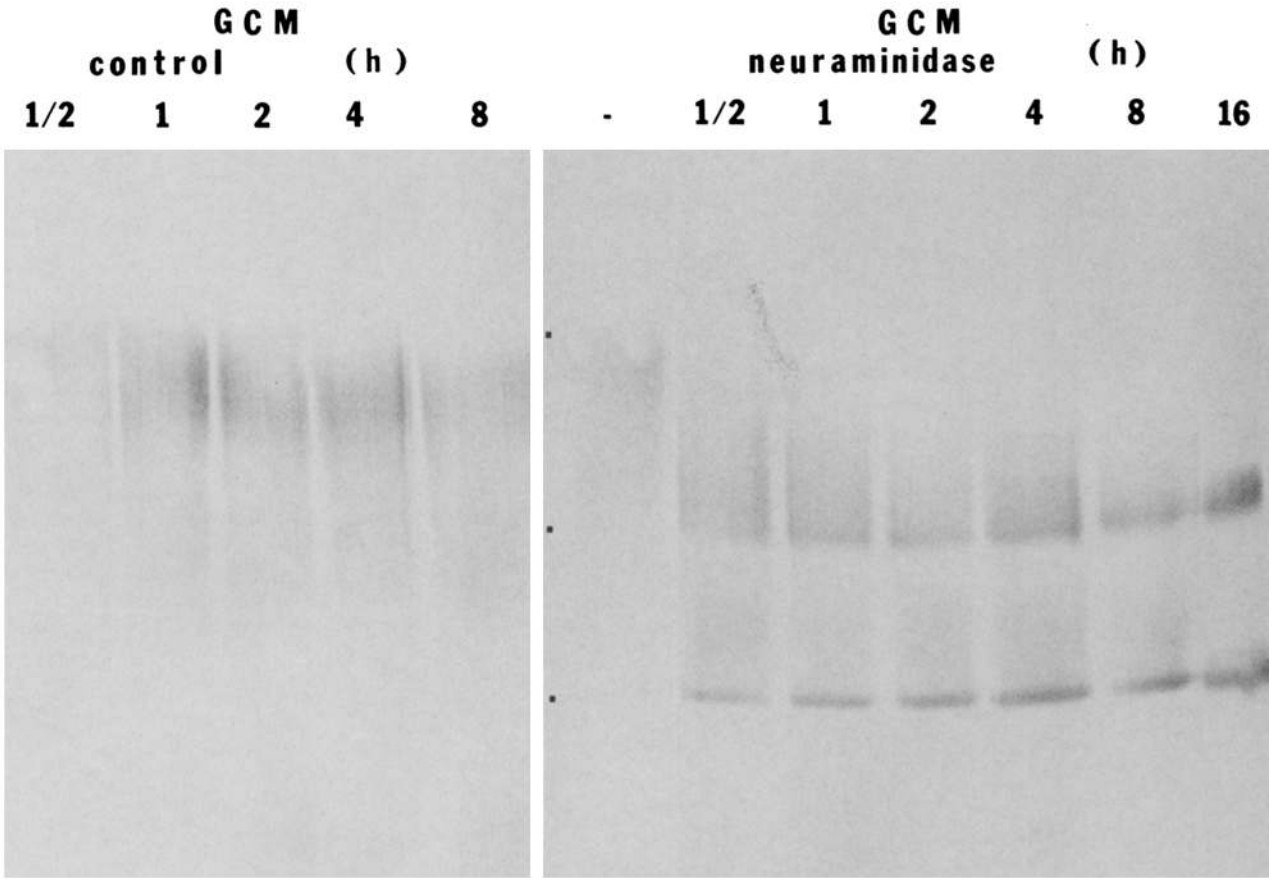


FIGURE 10 The effect of neuraminidase treatment on the fetal 5B4 antigen, in the presence of protease inhibitors. GCM were prepared in the presence of the inhibitors, and the incubated samples (equal aliquots of 100 μg membrane protein) were immediately processed for electrophoresis and immunoblotting. The marker dots indicate 250, 185, and 140 kD. Unincubated and control-incubated GCMs show a single but diffuse band between 185 and 255 kD. By 8 and 16 h (not shown) of control-incubation, there is slight fading of the original band, with appearance of diffuse 5B4-reactive material between 140 and 185 kD. However, neuraminidase treatment for as little as one half an hour cleaves the native fetal antigen into two diffuse bands with relatively sharp lower margins at 135 and 185 kD, respectively. Extended neuraminidase digestion slowly sharpens these bands, without apparent loss of antigenicity.

alkaline pI and condensed to form two series of horizontally arranged spots (at 135 and 185 kD). In each series, the mobility in SDS PAGE is more or less constant for the majority of protein, but there are four recognizable isoelectric variants for the 135-kD group and three such variants for the 185-kD group. Interestingly, the pI's for the three most acidic species in each group seem to be identical (from ~5.1 to 5.2). However, the 135-kD group exhibits a further, slightly more basic variant.

Developmental Regulation of the 5B4 Antigen

To assess the expression of the 5B4 antigen in brain development, we first used a DIA of crude membrane prepared from whole rat brains at successive stages of development. Increasing amounts of protein (12.5 to 1,000 ng) from fetal brains at days 13, 16, 19, and 22 of gestation (Fig. 12, *F13–22*) as well as from adult brain (*A*) were spotted onto nitrocellulose and then reacted as described. The 5B4 antigen is first weakly detectable on membranes prepared from brain on fetal day 13 (*F13*) and increases steadily towards the time of birth (*F22*). As illustrated in Fig. 12, the 5B4 antigen can be detected with this method in as little as 25 ng membrane protein prepared from 19- or 22-d fetal brains. Membranes prepared from adult rat brain exhibit very low levels of immunoreactivity with the 5B4 antibody. Controls carried out in parallel, using myeloma-conditioned medium, do not exhibit any nonspecific binding.

To study whether mAb 5B4 recognizes the same antigen during brain development, immunoblots were prepared from 5% SDS polyacrylamide gels of crude membranes isolated from developing rat brain. Such a developmental series is shown in Fig. 13. Immunoreactivity in the range of ~185–255 kD becomes detectable with this method near day 16 of gestation. An increase in 5B4 staining is observed at day 19 of gestation, and the antigen persists at least until postnatal day 13 (*P13*), albeit in diminished quantity. However, at this stage of development a second antigen of ~140 kD is clearly detectable and remains visible in the mature brain (adult, *A*). In heavily overloaded immunoblots of membranes from mature brain (≥400 μg protein/lane), one can see a faint second band of ~185 kD. As already mentioned, the 140-kD antigen from mature brain migrates slightly but significantly more slowly in SDS PAGE than the smaller antigen in neuraminidase-treated GCP membranes. In two-dimensional gels (IEF in the first dimension), the mature 5B4 antigen appears as a

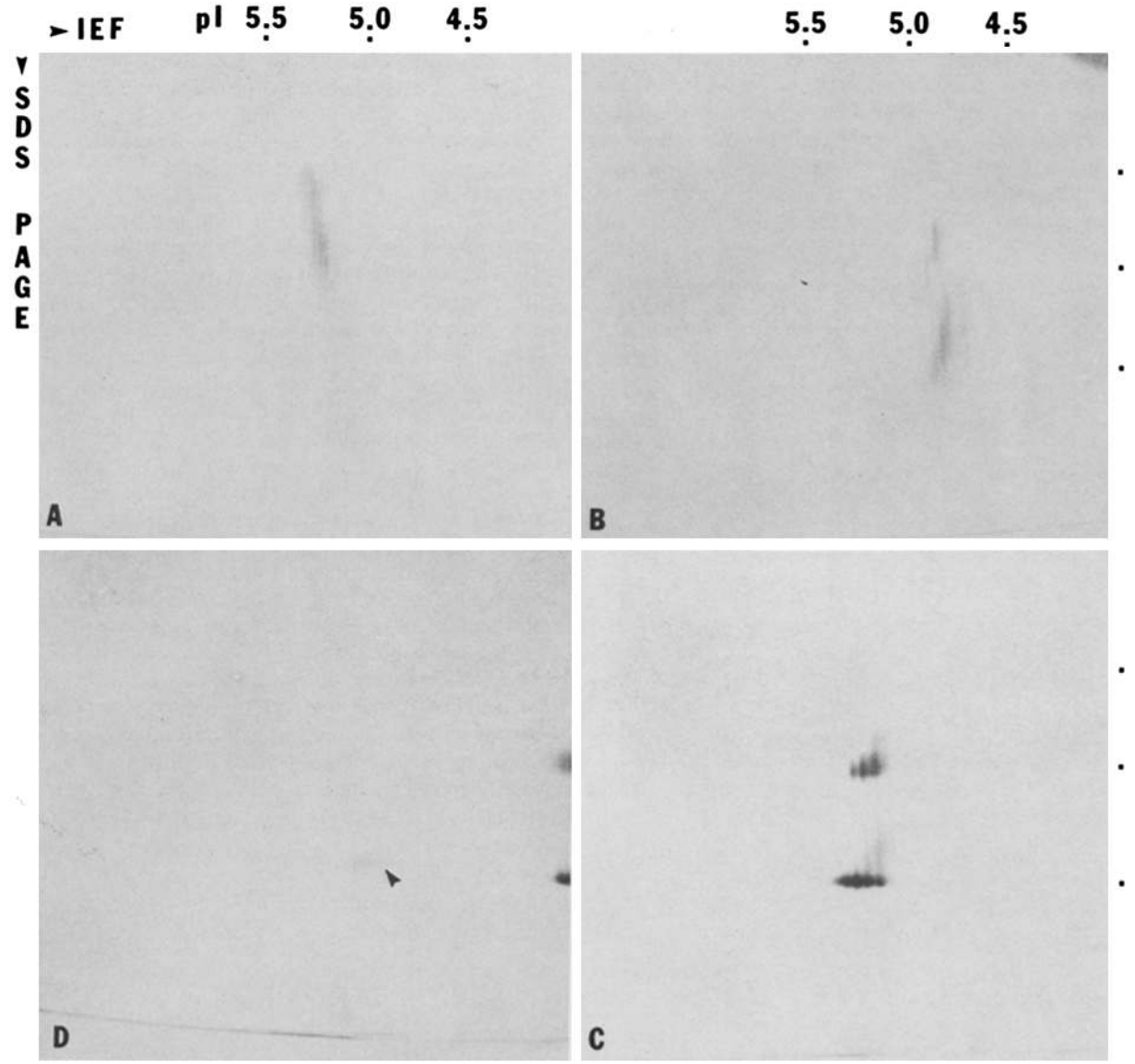


FIGURE 11 Two-dimensional gel electrophoresis of the 5B4 antigen as localized by immunoblotting. (*A*) Crude GCP membranes (see text); (*B*) GCM, once frozen and thawed and control-incubated for 16 h; (*C*) GCM as in *B*, but incubated with neuraminidase for 16 h; (*D*) crude adult membranes. The two spots on the right margin in D are from a neuraminidase-treated sample (as in C), run only in the second dimension; the arrowhead points at the mature antigen. Note the distinctive vertical streaks indicating isoelectric variants in *A–C*, the shift in pI to the right during "spontaneous" breakdown (compare *A* and *B*) and to the left during neuraminidase digestion (compare *B* and *C*). Also note that the mature antigen migrates with a slightly larger apparent molecular mass than the smaller product of neuraminidase digestion. The dots on the right margin indicate approximate molecular masses: 250, 185, and 135 kD.

horizontal band between pI 4.8 and 5.0 (cf. Fig. 11 *D*). Discrete isoelectric variants have not been resolved.

DISCUSSION

At present, our knowledge of the biochemical composition of the nerve growth cone is very limited. The majority of information available is derived from labeling studies on sprouting neurons or neuroblastoma cells grown *in vitro*. Cytoskeletal proteins such as tubulin (33, 34, 53), actin (32–34), myosin (32), neurofilament subunits (53, 54), clathrin (24), and fimbrin (53) have been identified by immunofluorescence. Microtubules, some neurofilaments, and an abundance of microfilaments are evident in electron micrographs (e.g., references 22, 23, 54). Furthermore, a few membrane proteins have been localized on growth cones: Na^+, K^+-ATPase (51), the neural cell adhesion molecule, designated N-CAM (8), and the receptor for nerve growth factor (5, 29, 48). In addition, lectin receptors have been mapped in the growth cone region (42, 43, 45). Yet, none of these components appear to be unique to the nerve growth cone, so that biochemical markers specific for it are not known at the present. The availability of milligram quantities of native nerve growth cone fragments, however, now renders the biochemical analysis and the search for growth cone– or sprouting-specific markers possible.

The GCM Fraction

The protocol for GCM preparation includes a step for the

removal of dense elements, such as mitochondria and lysosomes, and a step for the release of adsorbed proteins (wash at high ionic strength in the presence of non-ionic detergent). As determined in a previous paper (40) by stereologic analysis, 80% of the membranes of the GCP fraction consist either of plasma membrane or of large clear vesicles. After removal of mitochondria (5%) and lysosomal elements (2%), the remaining membranes (endomembranes exclusive of large clear vesicles) represent ~13% of GCP membrane. It has been suggested that the large clear vesicles are the growth cone plasmalemma's precursor (38, 39, 41, 42, 44; however, see also references 18 and 47, which argue that the vesicles may be fixation artifacts). Therefore, the GCM preparation, predominantly composed of plasmalemma and large clear vesicles, may in fact represent a closer view of growth cone plasmalemma than would be expected a priori.

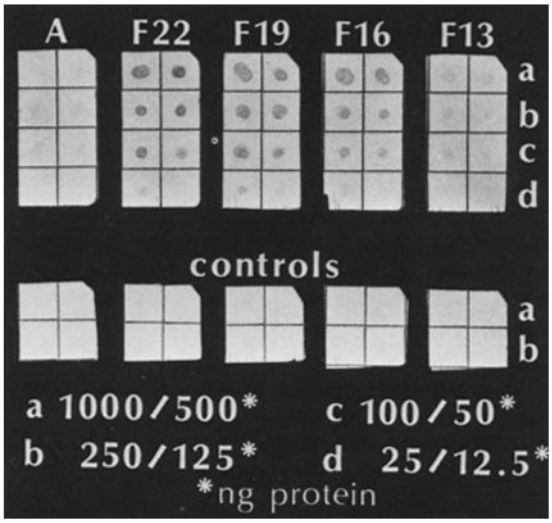


FIGURE 12 DIA to determine 5B4 binding to crude membranes isolated from developing forebrain. Brains were from rat fetus at 13 to 22 days of gestation (*F13–F22*) and from an adult (*A*) animal. Different concentrations of membrane protein were spotted for each stage (12.5–1,000 ng/μl). Binding was carried out with hybridoma supernatant, diluted 1:4, and controls involved the use of myeloma supernatant. For further description, see text.

The protein composition of the intact GCP, when analyzed by SDS PAGE, is complex. However, most of the GCP polypeptides can be released by hypotonic lysis and, therefore, are soluble or cytoskeletal components. Thus, the preparation of GCMs is accompanied by a marked reduction in protein content (14% of P1) and a relative increase in phospholipid phosphorus. The protein-to-phospholipid ratio for the washed GCMs is ~1.0. This value is probably an overestimate considering the presence of actin and tubulin residues in the fraction (see below). A relatively low protein content of GCMs is consistent with the results of freeze-fracture studies of nerve growth cones in vivo and in vitro, which have demonstrated a low density of intramembrane particles in both plasmalemma and large clear vesicles (39, 57).

A particularly striking feature of the GCM preparation is the simple pattern of seven major Coomassie Blue–stained polypeptides. The identity of most of these major bands is not known at present. However, two major polypeptides of the GCM, p52 and p42 (which are also major components of the intact GCP, the lysate, and the homogenate of fetal brain), co-migrate with tubulin and actin, respectively. A more detailed analysis of the major GCM polypeptides will be presented separately (Simkowitz, P., L. Ellis, and K. H. Pfenninger, manuscript in preparation; see also reference 56).

Specificity of mAb 5B4

The generation of mAb's to GCPs and GCMs has led to the identification of a large glycoprotein. Based on its relative

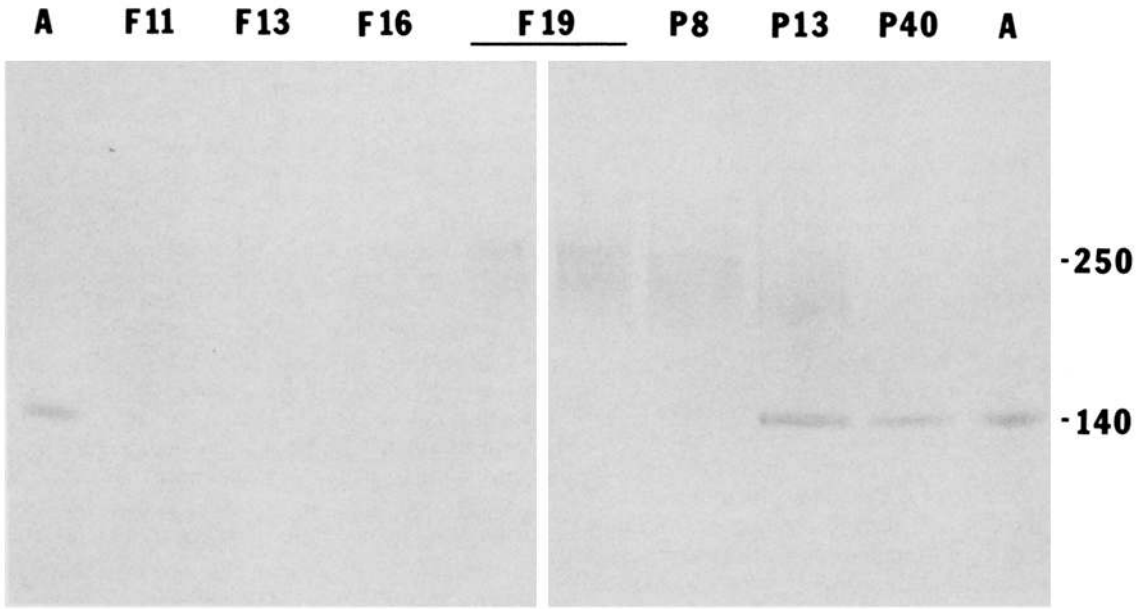


FIGURE 13 Immunoblot of crude membranes prepared from developing rat brain (except for F11, which contains membranes from whole 11-d-gestation embryos). Equal amounts of membrane protein (100 μg) were loaded in each lane of two 5% acrylamide gels, electrophoresed, blotted, and then reacted with the 5B4 antibody. Because two gels had to be run and blotted for this series, the F19 material (from fetal brains at day 19 of gestation) is shown twice. *A*, adult forebrain material; *P8–P40*, material from forebrain of the respective postnatal ages (in days). The markers on the right side indicate 250 and 140 kD, respectively. For further description, see text.

abundance in immunoblots of fetal brain subfractions, this antigen is enriched in, but not unique to, GCMs. However, based on Coomassie Blue and silver staining, the antigen is a minor GCM component. As determined by our DIA using up to 1 μg protein, mAb 5B4 does not bind at detectable levels to crude membranes prepared from rat liver, kidney, or muscle (data not shown). Immunofluorescence studies on cultures of cells dissociated from fetal brain and on frozen sections of rat brain at various developmental stages (cf. following paper [64]) indicate that 5B4 staining is confined to neurons. However, relatively small amounts of antigen (its mature form, see below) have been detected in cell lines with immunochemical but not with immunofluorescence methods (Rosenberg, J., L. Ellis, and C. Kaylar, unpublished observations).

The expression of central nervous system components binding mAb 5B4 is developmentally regulated. In the forebrain, antigenicity peaks just prior to, and right after birth, when axonal growth is extensive and many synapses have yet to be formed. This applies specifically to the large, 185–255kD antigen, which is present in the developing brain in much greater amounts (on a protein basis) than the cross-reacting, 140-kD antigen in the mature brain. The large, fetal antigen is the molecule enriched in GCMs but essentially absent from synaptosomes. Yet, this antigen is not restricted to the growth cone because immunofluorescence analysis of isolated growing neurons reveals antigenic sites on perikarya and neurites as well. Nevertheless, the data presented here and the immunofluorescence studies described in the following paper (64) demonstrate that the large 5B4 antigen is associated with neurite formation and, therefore, can be used as a marker for this stage of neuronal differentiation.

It is important to note that the definition of the specificity of the large 5B4 antigen is not dependent on the identity or the purity of our GCP fraction but rests on the results of immunochemical assays involving total membranes from developing brain and on data obtained by immunofluorescence. Therefore, the enrichment of the large 5B4 antigen in GCP membranes supports our contention that GCPs are derived from growing neurons.

The Molecular Nature of the 5B4 Antigen

FETAL ANTIGEN; The appearance of this antigen as a broad, diffuse band on immunoblots suggests that it is composed either of a family of polypeptides that share the 5B4 epitope and/or carries a large, heterogenous carbohydrate portion. The enrichment of the antigen in GCMs washed in the presence of high ionic strength—and its absence from soluble fractions—strongly suggests that it is a membrane-associated molecule, most likely an integral membrane glycoprotein. This interpretation is supported by immunofluorescence data, i.e., ring-like fluorescence around the perikaryon and outline of growth cone filopodia, as well as by the results of neuraminidase digestion. Treatment with this enzyme leads to a shift in electrophoretic mobility (sharpening of the bands) as well as pI of the antigen, suggesting that the antigen is extensively sialylated. The long period of incubation required for complete digestion of the 5B4 antigen has also been observed by other investigators, for fetal brain antigens containing α-2,8-polysialic acid (see below).

Even in the presence of protease inhibitors, neuraminidase induces the appearance of two sets of isoelectric variants of significantly different molecular weight. This observation suggests that the fetal 5B4 antigen consists of two different, but co-migrating glycoproteins, and/or that neuraminidase treatment of the 5B4 antigen makes a cleavage site available to either an endogenous protease (resistant to the inhibitors used in our experiments) or to an (endo)glycosidase (cf. ref. 20). Such a cleavage site would be present and/or become accessible in only a part of the antigen population, shifting it to a considerably lower molecular weight. It is of interest to note that the neuraminidase-digested 5B4 antigen has three isoelectric variants of 185 kD that seem to have the same pI's as three of the four 135-kD variants.

The neuraminidase digestion experiment also shows that antibody binding is resistant to desialylation. This observation is consistent with the finding that the labeling of the 5B4 epitope requires permeabilization of intact cells. This suggests that the 5B4 epitope is on an intracellular, subplasmalemmal portion of the antigen, whereas the oligosaccharide chains containing sialic acid are almost certainly located at the cell surface (Pfenninger, K. H., and E. Abreu, unpublished observations). We conclude, therefore, that the 5B4 antigen consists of a heterogenous family of polysialylated, integral membrane glycoproteins.

MATURE ANTIGEN: mAb 5B4 recognizes in membranes from the mature brain a protein that is only slightly larger (140 kD) and more acidic than the smaller product of the neuraminidase-treated fetal antigen. However, the mature antigen is much sparser (on a protein basis) than its fetal counterpart and is virtually absent from the synaptosome fraction (detectable in blots only when >400 μg SS protein are used). Its biochemical relationship to the fetal antigen remains to be investigated.

COMPARISON WITH OTHER NEURAL ANTIGENS: Fetal brain has recently been shown to contain a novel class of α-2,8-polysialosyl glycoproteins (13, 14, 35, 61). Some of the properties characteristic of these molecules have been observed in a number of closely related or identical, developmentally regulated neuronal antigens. These include: chick N-CAM (21, 49), rat and mouse N-CAM (8), rat D2 (25–27), and mouse BSP-2 (14, 20, 50). The 5B4 antigen shares with these glycoproteins the following properties: all are developmentally regulated neuronal membrane glycoproteins of large molecular weight, and in the case of N-CAM/D2/BSP-2, neuraminidase treatment causes a shift in electrophoretic mobility, approximately to the size of an immunochemically similar antigen of the mature brain. BSP-2 has been demonstrated to contain α-2,8-polysialic acid (14), and the 5B4 antigen has also been shown to contain such oligosaccharide chains (Rosenberg, J., L. Ellis, F. A. Troy, and C. Kaylar, manuscript submitted for publication). However, there are significant differences in the number and apparent size (*a*) of the neuraminidase cleavage products and (*b*) of the mature cross-reacting antigens. Furthermore, the 5B4 antigen of the mature brain is a very sparse molecule whereas the other mature antigens seem to be as abundant as their fetal counterparts. A more detailed comparison of 5B4 with these antigens has to await immunochemical studies with the appropriate reagents.

Conclusion

The preparation of a GCP membrane fraction, the generation of mAb's to GCP components, and the use of a differ-

ential screening assay have enabled us to identify a large membrane glycoprotein that seems to be associated specifically with neurite growth. While the close correlation between the expression of the fetal 5B4 antigen and neurite elongation suggests that this glycoprotein is involved in a mechanism related to or coincident with neurite growth, its function is unknown. Even so, the fetal 5B4 antigen is likely to prove very useful as a molecular marker of neurite growth.

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