Membrane Glycoproteins of the Nerve Growth Cone: Diversity and Growth Regulation of Oligosaccharides

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Abstract. A subcellular fraction prepared from fetal rat brain and enriched in growth cone membranes is analyzed for its lectin-binding proteins. Growth-associated glycoproteins are identified by comparing the growth cone glycoproteins with those of synaptosomes. Protein was resolved in one- or two-dimensional gels, electroblotted, and blots probed with radioiodinated concanavalin A, wheat germ agglutinin, and *Ricinus communis* agglutinins I and II. In one-dimensional gels, each lectin recognizes ~20 polypeptides (with substantial overlap) most of which migrate diffusely and have relatively high molecular masses (range 30–200 kD). The seven major Coomassie-staining proteins of the membrane fraction (34–52 kD) are not the major lectin-binding proteins. In two-dimensional gels, the lectin-binding proteins are either streaked across the pH gradient or exist as multiple spots, indicating broad charge heterogeneity.

Seven wheat germ agglutinin- and *Ricinus communis* agglutinin II-binding glycoproteins are present in greater abundance in growth cone fractions compared with synaptosomes. Most notably, an acidic, sialic acid–rich protein (27–30 kD, pI 4.0; termed gp27–30) is most abundant at postnatal day 4, but absent from adult brain. The protein's very acidic isoelectric point is due, at least in part, to its high sialic acid content.

Growth regulation of specific protein-linked oligosaccharides suggests that they play a special role in growth cone function. In addition, the great diversity of growth cone glycoproteins from whole brain suggests glycoprotein heterogeneity among growth cones from different neuron types.

During nervous system development, newly formed neurons become highly polarized and send out neurites. The advancing axon can select a specific path and target site from many of possible choices. At the tip of the axon is the nerve growth cone (for review, see Landis, 1983). The growth cone, in particular, its cell surface, is the first element of the advancing neurite to encounter a novel environment. Thus, the growth cone is likely to play a fundamental role in neuron–substrate and/or neuron–target cell interaction. The molecular bases of such neuronal interactions remain undefined.

Cell surface carbohydrates have been implicated in processes involving recognition phenomena such as morphogenesis, cell differentiation, and oncogenesis (Feizi, 1985), as well as in receptor–ligand interactions (Ashwell and Harford, 1982). Membrane glycoconjugates have also been implicated in neuronal cell–cell interaction. Three such molecules are retinal cognin (Moscona and Hausman, 1977), a family of neural cell adhesion molecules (N-CAM)[1] (Brackenbury et al., 1977), and a nerve growth factor–inducible large external glycoprotein (NILE) (Stallcup and Beasley, 1985); they have been shown to be involved in neural cell adhesion. Of further importance are those studies that have demonstrated differences in the surface glycoconjugates between perikaryon and perikaryal projections (Hatten et al., 1979; Pfenninger and Maylié-Pfenninger, 1981) and between different neuron types (Pfenninger and Maylié-Pfenninger, 1981; Braun et al., 1981; Schwab and Landis, 1981; Pfenninger et al., 1984; Dodd et al., 1984; Dodd and Jessel, 1985). The lectin mapping studies performed in this laboratory are of particular interest because they demonstrated the neuron-type specificity of surface oligosaccharides on nerve growth cones (Pfenninger et al., 1984). These findings are consistent with the concept that surface carbohydrates may be involved in neuronal recognition.

To further explore the possible role of carbohydrates in growth cone–mediated events such as axonal path selection and synaptogenesis, it is necessary to isolate growth cone membrane in bulk. Recently, Pfenninger and collaborators

1. *Abbreviations used in this paper*: Con A, concanavalin A; 1D, one dimensional; 2D, two dimensional; D-NANA, 2-3-dehydro-2-deoxy-*N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; N-CAM, neural cell adhesion molecule; PVP, polyvinylpyrrolidone; RCA I and II, *Ricinus communis* agglutinins I and II; *Vc*, *Vibrio cholerae*, WGA, wheat germ agglutinin.

(1983) have described a procedure for the subcellular fractionation of growth cones from fetal rat brain. The fraction of interest is highly enriched in so-called growth cone particles, which are ultrastructurally similar to growth cones and co-purify with radiolabeled growth cones microdissected from neurons sprouting in vitro. Biochemical characterization of the growth cone particles has shown that they are enriched in a variety of neuron- and synapse-specific markers (Ellis et al., 1985*b*; Katz et al., 1985). Most notably, growth cone particles are enriched in the antigen of a growth-regulated neuronal glycoprotein related to N-CAM (Ellis et al., 1985*b*), and one of the growth cone particles' major membrane proteins, pp46, is identical to the growth-associated protein GAP43 (Meiri et al., 1986).

The ability to obtain microgram amounts of growth cone–enriched fraction makes it possible to ask what is the glycoprotein composition of the growth cone membrane. This information, reported here, will serve as a guide for future studies directed at determining the nature of the glycoconjugates that distinguish growth cones from different neuron types. Furthermore, comparison of the glycoproteins of growth cone particles with their closest adult counterpart, synaptosomes, allows identification of developmentally regulated species. Part of this work has appeared in abstract form (Greenberger and Pfenninger, 1985).

Materials and Methods

Materials

N-Acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), analine, chloramine T, leupeptin, α2-macroglobulin, α-methylmannoside, neuraminidase from *Clostridium perfringens*, phenylmethylsulfonyl fluoride, pepstatin A, polyvinylpyrrolidone (PVP; pharmaceutical grade, 40,000 av mol wt), Sephadex G-100, sodium bisulfite, sodium cyanoborohydride, sodium-*m*-periodate, soybean trypsin inhibitor, *N*-Tris-(hydroxylmethyl)methyl-2-aminoethane sulfonic acid (TES), Tris (hydroxymethyl) aminomethane (Tris as Trizma base), Tween-20, and wheat germ (untreated), were purchased from Sigma Chemical Co., St. Louis, MO. *N*-Succinmidyl 3-(4-hydroxy,5-^{125}[I]iodophenyl) propionate (Bolton-Hunter reagent) and free ^{125}I-iodine were obtained from New England Nuclear, Boston, MA. High molecular mass protein standards for SDS gel electrophoresis and Affigel-*N*-acetylglucosamine were obtained from Bio-Rad Laboratories, Richmond, CA. Low molecular mass standards and prestained standards were purchased from Pharmacia Inc., Piscataway, NJ, and Bethesda Research Laboratories Inc., Gaithersburg, MD, respectively. Ultra-pure urea and sucrose were obtained from Schwarz/Mann, Spring Valley, NY. Ampholines were from LKB Instruments, Inc., Gaithersburg, MD. *Vibrio cholerae* neuraminidase (protease-free) was from Calbiochem-Behring Corp., La Jolla, CA. Concanavalin A (Con A) was obtained from Miles Scientific Div., Miles Laboratories Inc., Naperville, IL, while *Ricinus communis* agglutinins I and II (RCA I and RCA II) were extracted and purified from castor beans (George W. Park Seed, Inc., Greenwood, SC) on a "Lactose II" affinity column (Pierce Chemical Co., Rockford, IL). Nitrocellulose paper (0.45-μm pore size) was purchased from Schleicher & Schuell, Keene, NH. 2-3-Dehydro-2-deoxy-*N*-acetyl-neuraminic acid (D-NANA) was from Boehringer Mannheim Biochemicals, Indianapolis, IN. Biotinylated goat anti-mouse IgG and avidin D-horseradish peroxidase were from Vector Laboratories, Inc., Burlingame, CA. 4-Chloro-1-naphthol was obtained from Aldrich Chemical Co., Milwaukee, WI. Bovine serum albumin (BSA) was purchased from Gibco, Grand Island, NY. Trasylol (aprotinin) was from Mobay Chemical Corp., Pittsburgh, PA. India ink was the Pelican brand. Monoclonal antibody 5B4 was from this laboratory (see Ellis et al., 1985*b*). All other chemicals were reagent grade and purchased from Sigma Chemical Co. or Fisher Scientific Co., Pittsburgh, PA.

Subcellular Fractionation Methods

Growth Cone Material. All biological material used in this study was from whole brains of adult or fetal Sprague-Dawley rats. Growth cone membranes were prepared according to previously described methods (see Pfenninger et al., 1983; Ellis et al., 1985*b*). Briefly, embryonic day 17–18 rat brains were homogenized in ~6 vol of 0.32 M sucrose with 1 mM TES (pH 7.3) and 1 mM $MgCl_2$. A protease inhibitor cocktail consisting of pepstatin A (1 μg/ml), soybean trypsin inhibitor (0.1 mg/ml), leupeptin (30 μM), and phenylmethylsulfonyl fluoride (1 mM) was present throughout the isolation protocol. (In some of the later experiments Trasylol, 1,000 kallikrein inactivator units/ml, replaced the inhibitor cocktail.) The homogenate was centrifuged and the resultant low-speed supernatant was loaded onto a discontinuous sucrose density gradient. After centrifugation, the uppermost interface (A band) was removed, diluted with 0.32 M sucrose, and pelleted by centrifugation. The resultant pellet, designated growth cone particles, was resuspended in Tris-lysate buffer (6 mM Tris, 0.5 mM EDTA, pH 8.1). After particle lysis, the material was washed in the presence of high salt and a small amount of neutral detergent (0.3 M $NaSO_4$ containing 20 μg/ml saponin) and pelleted by centrifugation. The final pellet is called growth cone membrane. The entire fractionation was conducted at 4°C.

Synaptosomes. Synaptosomes were prepared from adult rat brains based on a procedure modified after that of Cohen et al. (1977), and described in detail by Ellis et al. (1985*b*).

Crude Membrane Preparation. Crude membrane from embryonic, newborn, and adult rats were prepared from the rostral cortex. Brains were quickly removed, placed on ice, and their front halves collected. The underlying thalamic tissue and corpus callosum were crudely removed with the aid of a dissecting microscope. Crude membranes were prepared from the homogenate according to the procedures outlined for growth cone membranes except that the sucrose density gradient segment was skipped and the membranes were not salt-washed.

Biochemical Methods

Neuraminidase Digestion. Samples (50–125 μg) were digested with 0.125 U/100 μl neuraminidase from either *Vibrio cholerae* or *Clostridium perfringens* at 37°C for the times specified. The *Vibrio cholerae* incubation medium contained 2.0 mM $CaCl_2$, 0.2 mM EDTA, 0.1 M sodium acetate buffer (pH 5.0), and 0.1 mg/ml α2-macroglobulin. The *Clostridium perfringens* incubation medium contained 0.1 M sodium acetate buffer, 0.1 mg/ml α2-macroglobulin, 100 U/ml Trasylol, and 1 μg/ml pepstatin A. In some experiments, D-NANA or sialic acid (1–5 mM) was added to the incubation solution. All reactions were terminated by adding 3× concentrated Laemmli (1970) sample buffer followed by boiling for 3 min (for one-dimensional [1D] analysis) or adding 1× solubilization buffer (O'Farrell, 1975) and solid urea to bring the final concentration to 9.0 M (for two-dimensional [2D] analysis).

PAGE. 1D gel electrophoresis was done according to the methods of Laemmli (1970). Resolving gels consisted of a 17-cm long, 5–15% linear polyacrylamide gradient. Stacking gels, 2-cm long, consisted of 3% polyacrylamide. The molecular mass standards were as follows: myosin (200 kD), beta-galactosidase (116 kD), phosphorylase *b* (94 kD), BSA (68 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), and chymotrypsinogen (25.7 kD). Samples for SDS PAGE were prepared by boiling the sample for 3 min in the presence of 2% SDS and 5% β-mercaptoethanol. 50 μg of protein, determined by the method of Lowry et al. (1951), was run per gel lane unless noted otherwise. Gels were stained with 0.04% Coomassie Blue or transferred to nitrocellulose (see below).

2D gel electrophoresis isoelectric focusing followed by SDS PAGE was done according to O'Farrell (1975) with the modifications described previously (Ellis et al., 1985*b*). In some gels, a sample of nerve growth cone particles (5 μg) was run at the side of the gradient gel only in the second dimension. This sample was prepared by placing material in 1% hot agarose containing sample buffer and gelling the mixture in a 130 × 2-mm tube. After gelling, the tube gel was removed and cut into 0.5-cm pieces (5 μg/0.5 cm). Pieces were used immediately or frozen at −80°C for later use. After 2D separation, the proteins were either stained with ammoniacal silver (Wray et al., 1981) or transferred to nitrocellulose.

Western Blotting. Resolved proteins were electrophoretically transferred to nitrocellulose according to published procedures (Towbin et al., 1979), with the following modifications. After rinsing the gel in tank blot buffer, it was mounted against nitrocellulose; the area surrounding the gel was blocked off with Mylar foil. Electro-transfer was performed with an 830 Electro-Blot system (E-C Apparatus Corp., St. Petersburg, FL). The time and current were adjusted to the size of the gel to maximize transfer (Lin and Kasamatsu, 1983). The current was 300 mA for a time specified by the following equation: $t = k$ (area of gel)$/I$, where t is time in minutes, k is

an empirically determined constant of 0.234, I is current in amperes, and the area of the gel is in square centimeters. After transfer, the nitrocellulose paper was either stained with Amido black, dried, and stored at −80°C, or immediately quenched in preparation for lectin incubation.

Lectin Purification and Iodination. Lectins were either purchased (Con A) or prepared from seeds (wheat germ agglutinin [WGA], RCA I, and RCA II). WGA was prepared according to published methods using a GlcNAc affinity column (Shaper et al., 1973). RCA I and RCA II were purified according to the procedures of Nicolson et al. (1974) except that the final extract was run over a lactose affinity column. RCA II was selectively eluted from the column by 0.01 M GalNAc and did not require further purification. RCA I was eluted with 0.1 M galactose; trace contaminants of RCA II were then eliminated by chromatography on a Sephadex G-100 column. Both WGA and the ricins agglutinated red blood cells and were electrophoretically pure.

Lectins were iodinated in the presence of the hapten sugar by the method of Bolton and Hunter (1973; Con A) or by the chloramine T method (Hunter and Greenwood, 1962; WGA, RCA I, and RCA II). After iodination, free iodine and sugar were removed by extensive dialysis, and the lectin was repurified on an affinity column as described above for WGA, RCA I, and RCA II. Con A was affinity purified on a Sephadex G-100 column and eluted with 0.1 M α-methylmannoside. Specific activities were ~0.07 mCi/mg protein for Con A, 10 mCi/mg for WGA, and 3.5 mCi/mg for RCA I and RCA II.

Lectin Binding to Blotted Proteins. ^{125}I-Lectin labeling of blotted proteins was done based on previously published methods (Gershoni and Palade, 1983; Bartles and Hubbard, 1984). After electrotransfer, the blot was quenched overnight in phosphate-buffered saline (PBS; 140 mM NaCl, 3.7 mM KCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.02% NaN_3, pH 7.4) containing 1% hemoglobin, 2% PVP, or 2% PVP plus 0.5% hemoglobin. Then the blot was incubated in fresh quenching agent for 1 h followed by incubation with the radiolabeled lectin for 8 h. The total incubation volume was 4 ml/gel lane or 32 ml/2D gel. In control experiments, incubation solutions contained the appropriate hapten sugar. Then the blot was washed in PBS three times for 15 min/wash, dried overnight, and radioautographed with x-ray film (Kodak X-Omat AR) at −80°C. All procedures were done at 4°C and all solutions contained the quenching agent except the final wash. For Con A incubations, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ were added to PBS. Multiple x-ray exposures were prepared to detect minor radiolabeled species. In all experiments, lectin concentrations were submicromolar; hence all lectin binding is likely to be to high-affinity species.

Lectin binding conditions were optimized for glycoprotein detection and identification. Variables tested were: lectin concentration, quenching agent (PVP vs. hemoglobin, with or without 0.1% Nonidet P-40), and pre-staining the blot (for protein detection) before lectin incubation. When lectin concentration was increased above the amounts indicated, lectin binding increased but no new glycoprotein species were detected. Hemoglobin and PVP were equally effective in their quenching capacity (an advantage of PVP is that, subsequent to lectin incubation, the blot can be stained with Amido black or India ink). Lectin incubation in the presence of detergent increased the signal-to-noise ratio, but eliminated lectin binding to certain glycoprotein species. Therefore, detergent was not present in any lectin incubations whose results are reported here. Amido black staining did not alter lectin binding to blotted glycoproteins. However, India ink staining (Hancock and Tsang, 1983) can only be done after lectin incubation.

In one series of experiments, WGA binding to blotted proteins was enhanced by pre-treating the blots with the oxidative/reductive phenylamination procedure done exactly as described by Bartles and Hubbard (1984).

Antibody Labeling of Western Blots. Labeling with monoclonal antibody "5B4" (mouse IgG) was done by first quenching the blot in 3% BSA in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.3, 0.15 M NaCl) overnight at 4°C. All subsequent procedures were done in the presence of the quenching agent at room temperature. Blots were then incubated in the following sequence: 5B4 ascites supernatant (dilution 1:2,000) for 1 h, wash (three times for 5 min), biotinylated goat anti-mouse IgG (dilution 1:500) for 1 h, wash, avidin conjugated to horseradish peroxidase (dilution 1:500) for 1 h, and wash. Horseradish peroxidase was detected by using the chromogen 4-chloro-1-naphthol (Hawkes et al., 1982). Control blots were incubated in myeloma supernatant in place of the primary antibody. In the experiments shown, immunoblots were done on blots that had been previously reacted with ricin. The same results were obtained if native blots were used.

Results

Growth Cone Membranes Contain Many Lectin-binding Proteins: The Major Coomassie Blue-staining Proteins Are Not the Major Glycoproteins

Four fractions collected during the growth cone isolation procedure were sampled, each fraction being a subset of the latter. The fractions were: low-speed supernatant (LSS), "A" band (0.32–0.75 M interface from the sucrose density gradient), pelleted "A" band (P; growth cone particles), and growth cone membranes (M; salt-washed membranes from lysed particles).

As has been previously described (Ellis et al., 1985*b*), growth cone particles contain 40–70 Coomassie Blue–staining polypeptides resolved by SDS PAGE. Upon removal of cytoplasmic and peripheral proteins, the pattern simplifies to seven major polypeptides which are 52, 46, and 42 kD, and doublets at 38 and 34 kD (see Ellis et al., 1985*b* and Fig. 1; Amido black–stained lane). These polypeptides can be transferred efficiently to nitrocellulose, although the 46- and 34-kD polypeptides do not transfer as well as other species (data not shown). Glycoproteins within the growth cone–enriched fractions were detected by incubating the blot with iodinated lectins. The lectins used were Con A, WGA, RCA I, and RCA II (Fig. 1 and 2). In general, the results are as follows: (*a*) the lectin-binding proteins within growth cone membranes are similar to those in the low-speed supernatant, but the quantity of lectin binding increases as one proceeds through the fractionation scheme up to membranes; (*b*) most of the lectin-binding proteins are of relatively high molecular mass (range 50–200 kD); and (*c*) the major Coomassie-binding proteins in growth cone membranes are not major lectin-binding proteins.

Con A binds to ~20 generally diffuse bands. The molecular masses of the major species are ~204, 198, 192, 146, 127, 115, 110, 94, 80, 52, and 42 kD. The 52- and 42-kD Coomassie-staining proteins (presumably tubulin and actin, respectively) co-migrate with Con A–binding proteins. The Con A binding is specific since it can be blocked by incubation of the blot in the presence of 0.25 M α-methylmannoside.

WGA, RCA I, and RCA II also bind to ~20 bands, with considerable homology between these three lectins. Major species are observed at 240 (sharp band), 200–170 (triplet), 124, 112, 94, 68, and 27–30 kD. When the blots were incubated in similar amounts of RCA I and RCA II (~0.7 μg protein, 60,000 cpm/gel lane), more RCA II binds than RCA I. RCA I labels an 80-kD protein that is prominent in the "A" fraction but decreases in intensity when growth cone particles and membranes were prepared. This suggests that the 80-kD RCA I–binding protein is a soluble glycoprotein. Similar to the Con A–binding analysis, the 52 and 42-kD Coomassie-staining proteins co-migrate with (relatively minor) WGA- and ricin-binding proteins.

When Con A and RCA II (or WGA) patterns are compared, the major lectin-binding proteins that do not overlap migrate at 240 kD (RCA II-positive), 146 kD (Con A-positive), and 27–30 kD (RCA II-positive). Other subtle differences between these two lectins cannot be clearly distinguished unless a side-by-side comparison between gel lanes is performed.

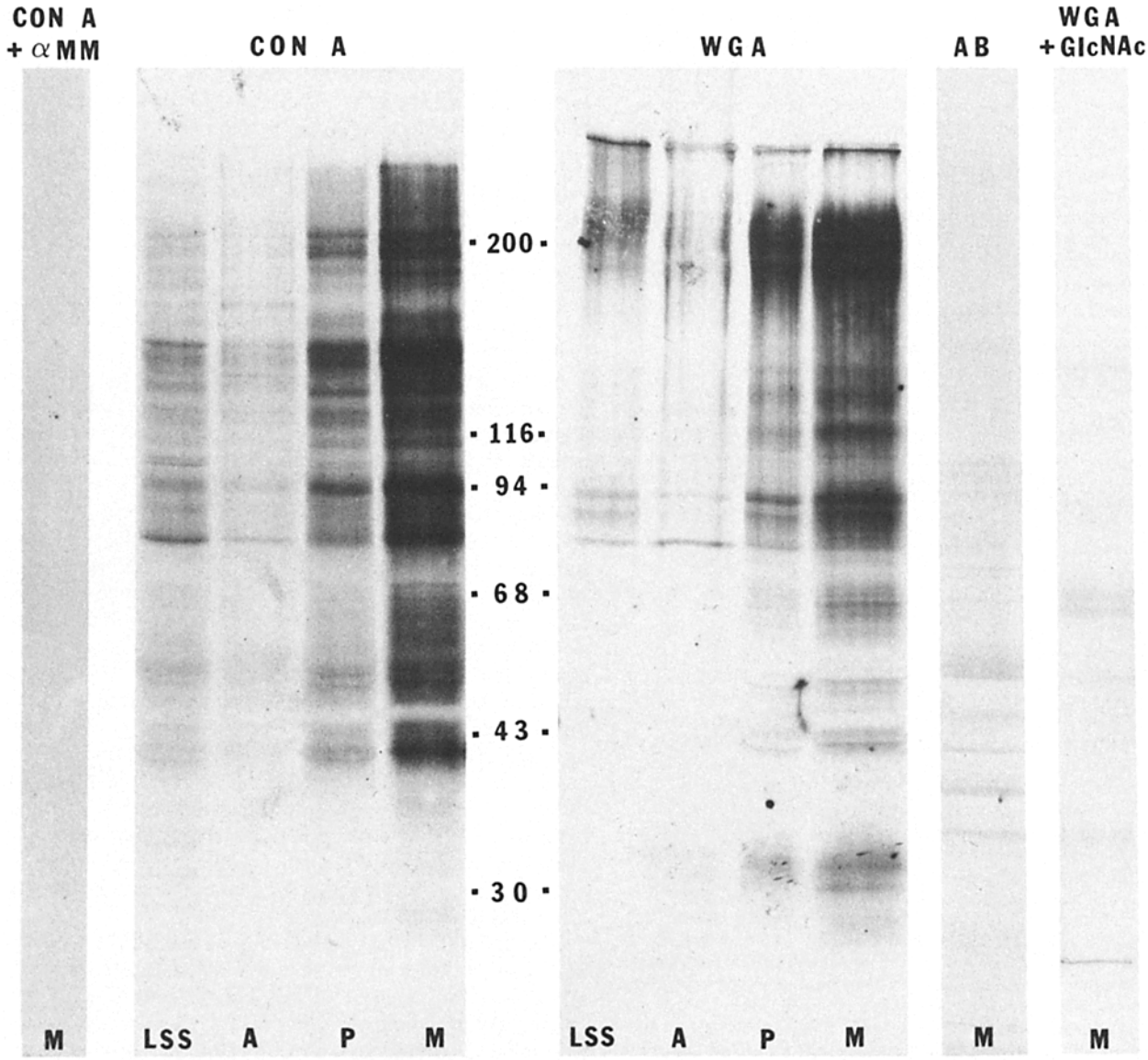

Figure 1. Con A– and WGA-binding proteins of the nerve growth cone. Subcellular fractions were run on SDS PAGE, transferred to nitrocellulose, and stained with Amido black (*AB*). For Con A binding, blots were quenched in 1% hemoglobin and incubated in 3.0×10^5 cpm (10 μg) ^{125}I-Con A per gel lane, in the presence or absence of 0.25 M α-methylmannoside (α*MM*). For WGA binding, blots were quenched in 2% PVP and incubated in 2.6×10^6 cpm (0.25 μg) ^{125}I-WGA per gel lane, in the presence or absence of 0.25 M *N*-acetylglucosamine (*GlcNAc*). Subcellular fractions from fetal brain were as follows: low-speed supernatant (*LSS*); fraction A, consisting of growth cone particles and soluble protein of the homogenate (*A*); growth cone particles (*P*); and growth cone membranes (*M*). The position of the molecular mass standards ($\times 10^{-3}$) are shown. x rays were exposed 16 d and 0.625 d for Con A and WGA blots, respectively. Note that lectin binding is enriched in membranes. The major Amido black–staining bands at 52, 46, 42, 38, and 34 kD are not the major Con A– or WGA-binding proteins.

Charge Heterogeneity of WGA-binding Proteins

To gain further insight into the complexity of the glycoproteins of the growth cone membrane, WGA-binding proteins were resolved by charge (isoelectric focusing) and molecular mass (SDS PAGE). As shown in Fig. 3, silver-staining proteins resolve as distinct spots (Fig. 3 *A*) and therefore are focused well. The molecular masses of the major species are indicated; the 52-kD protein is overloaded and gives a negative image upon silver staining, whereas the 46-kD protein stains an anomalous bright yellow (on an amber background) and does not photograph well.

In electroblots, successful transfer of the proteins to the blot is confirmed by the detection of numerous India ink–stained proteins on the blot (Fig. 3 *B*) as well as the paucity of silver-stained proteins on the electro-eluted gel (not shown). Note that the 46-kD protein is seen on the India ink–stained blot. This blot was treated with the periodate-analine-cyanoborohydride method to enhance WGA binding (Bartles and Hubbard, 1984), and then incubated with ^{125}I-WGA in the absence or presence of 0.2 M GlcNAc (Fig. 3, *C* and *D*). This treatment enhances the binding of WGA to all species resolved in one dimension, particularly the 27–30-kD protein, but no new WGA-binding species are observed (data not shown). WGA specifically binds to the same

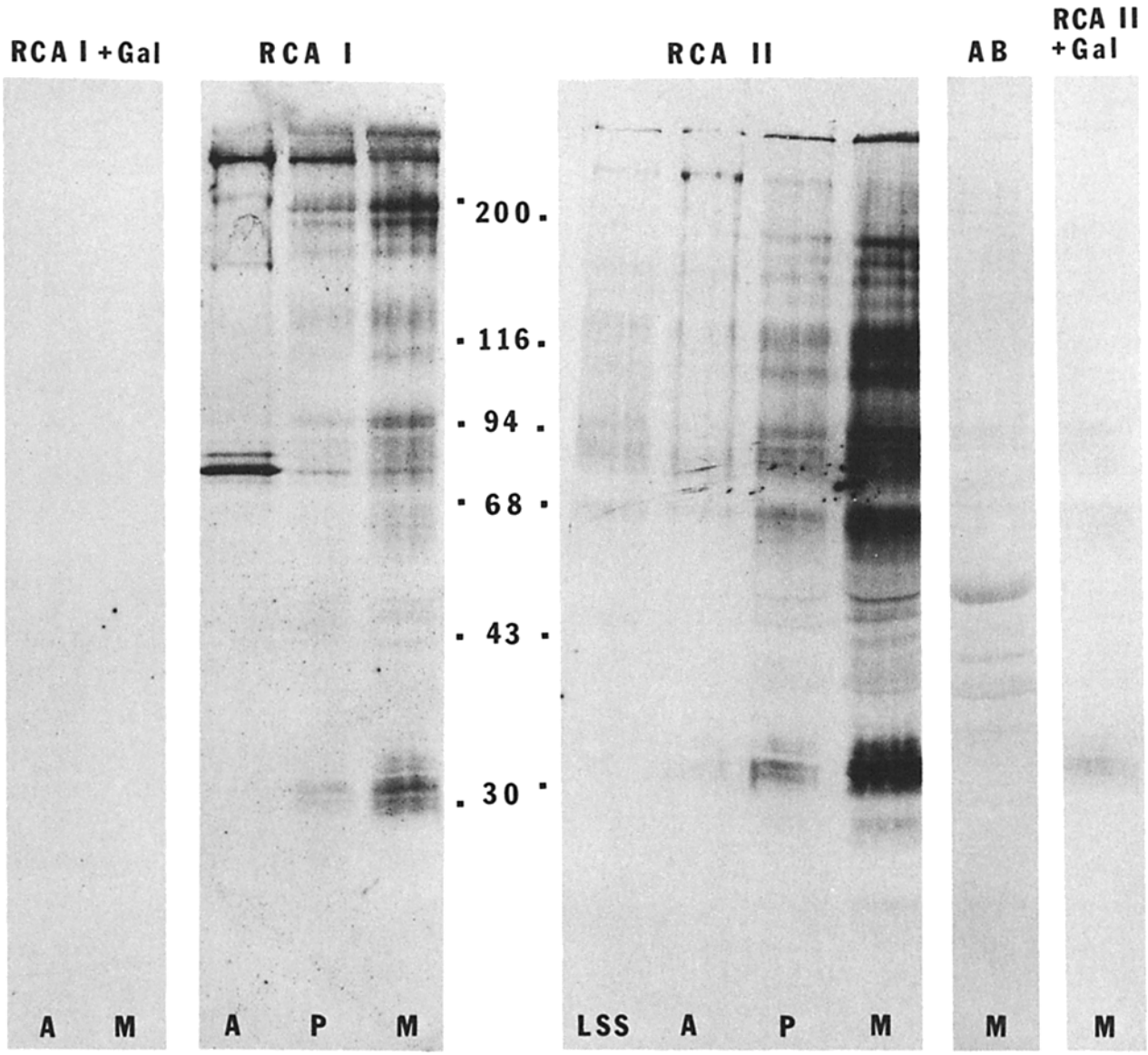

Figure 2. RCA I- and RCA II-binding proteins of the nerve growth cone. Western blots were prepared as in Fig. 1, quenched in 2% PVP, and incubated in 6×10^5 cpm (0.70 μg) ^{125}I-RCA I or ^{125}I-RCA II per gel lane, in the presence or absence of 0.05 or 0.20 M galactose (*Gal*), respectively. x ray of blots were exposed for 18 d (*RCA I*) and 3 d (*RCA II*). For further definition of lane abbreviations see Fig. 1. Note that the ricins bind to similar proteins compared with WGA. Most ricin-binding proteins are enriched in membranes, except an 80-kD RCA I-binding protein, which is most prominent in the A fraction. As with WGA-binding proteins, the major Amido black-staining proteins are not the major ricin-binding proteins.

molecular weight regions as described above, but now the polypeptides are spread across a wide pH range (4.0–6.5), suggesting that the WGA-binding proteins possess highly heterogeneous charges. The 27–30-kD protein is exceptional because it has an extremely acidic isoelectric point (pI 4.0) and migrates as a distinct spot just at the acidic end of the pH range of the gel. WGA does not bind to the position at which tubulin is known to migrate (Fig. 3 *C*, arrowhead).

Compared with Synaptosomes, Growth Cone Particles Contain an Overlapping But Distinct Set of Glycoproteins

To determine which, if any, of the glycoproteins present in growth cones might be developmentally regulated, growth cone particles prepared from whole fetal rat brain (18 d) were compared with their closest adult counterpart, synaptosomes. The Coomassie Blue–staining profile of growth cone particle polypeptides is similar to that found in synaptosomes (Fig. 4, left), although doublets at 250 and 90 kD are clearly more abundant in growth cone particles. In contrast, striking differences are observed when a comparison is made between the lectin-binding proteins of the two fractions (Fig. 4, center and right). The WGA- and ricin-binding glycoproteins present in greater abundance in growth cone particles compared with synaptosomes are summarized in Table I, and indicated in Fig. 4 (arrowheads).

The WGA- and RCA II–binding species of the growth cone particle which overlap are 250, 116, and 27–30 kD. The 27–30-kD species is outstanding since it is a major RCA

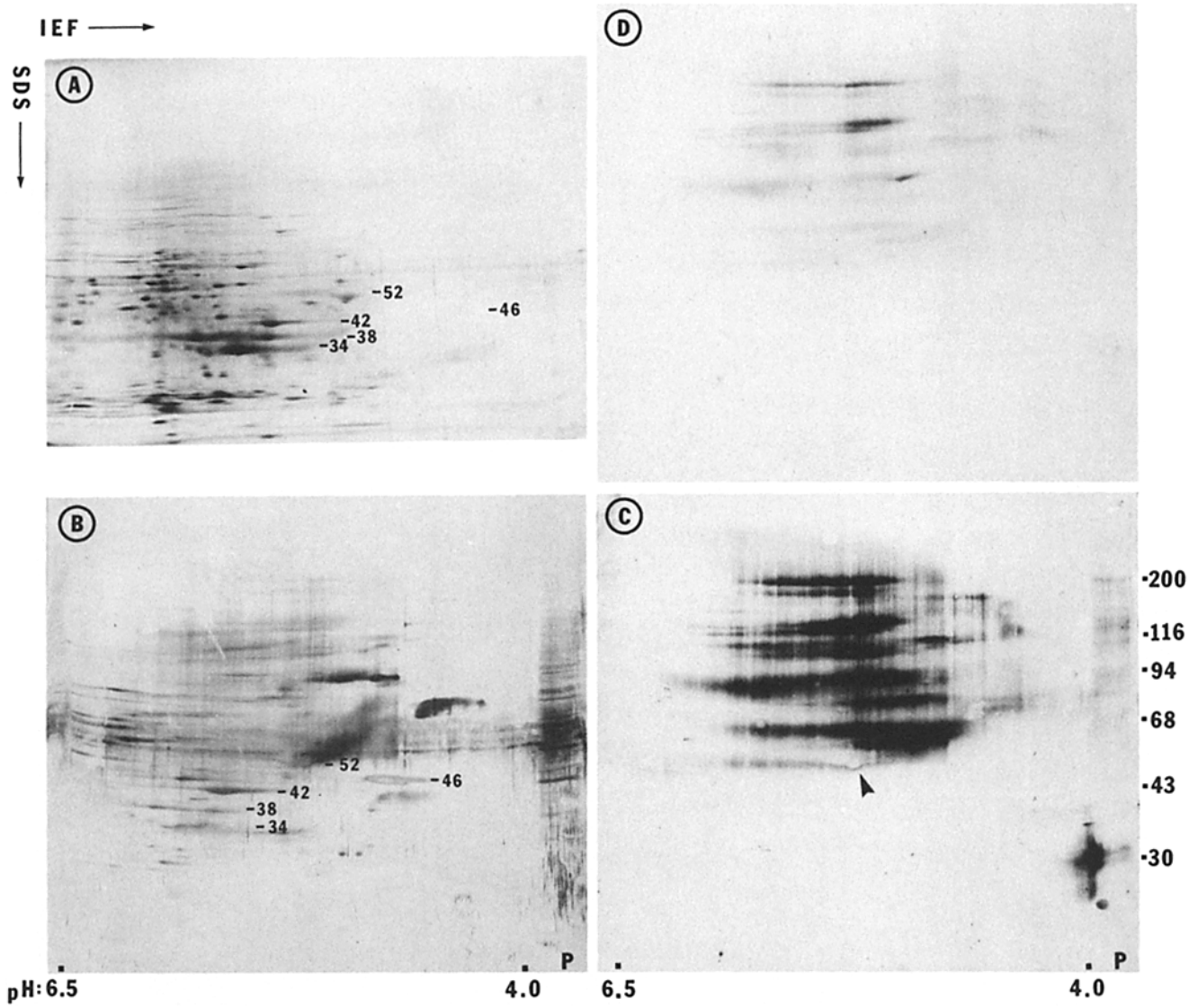

Figure 3. WGA-binding proteins of the nerve growth cone resolved in two dimensions. Growth cone membrane protein, 50 or 125 μg, were resolved in two dimensions and silver-stained (*A*) or blotted (*B–D*), respectively. Blots were first quenched with 2% PVP and subjected to periodate oxidative/reductive phenylamination to enhance WGA binding. Then, they were incubated in 2.6×10^7 cpm (3.1 g) ^{125}I-WGA. In the silver-stained gel five major membrane proteins are indicated. The 46-kD protein produced a bright yellow negative image after silver staining and is not visible in the photograph. (*B*) India ink–stained blot. The lane on the right (*P*) contained growth cone particle protein (5 g) resolved in the second dimension only. (*C*) x ray of blot shown in *B* incubated in WGA (2-d exposure). The positions of the molecular mass standards ($\times 10^{-3}$) are indicated on the right. Arrowhead indicates the position of the 52-kD protein (presumably tubulin). (*D*) x ray of blot incubated in WGA + 0.25 M GlcNAc. Note that, while the silver-stained proteins are well focused, the WGA-binding proteins are spread in the isoelectric focusing axis, indicating highly heterogeneous charge. The 27–30-kD WGA-binding protein has an extremely acidic isoelectric point (pI 4.0).

II–binding protein in growth cone particles but appears to be completely absent from synaptosomes. Crude membranes prepared from adult brain also do not contain a lectin-binding protein at 27–30 kD (see below). Therefore, the absence of the protein in synaptosomes cannot be attributed to fractionation procedures that would select against purification of the protein. This protein is designated as gp27–30.

Synaptosomes contain a few lectin-binding proteins which are present in greater abundance than in growth cone particles (Table I, right).

Neuraminidase Enhances RCA II Binding to Growth Cone Particles and Synaptosomes: gp27–30 Is Not Detected in Synaptosomes after Neuraminidase Treatment

RCA II binds with high affinity to certain oligosaccharides containing galactose or GalNAc. The affinity constant for RCA II binding to glycopeptides is markedly decreased if the carbohydrate tree is terminated with sialic acid, thereby masking penultimate galactose or GalNAc residues (Baenziger and Fiete, 1979*b*). Therefore, if many of the glycoproteins are initially capped with sialic acid, RCA II binding should increase upon removal of sialic acid with neuramini-

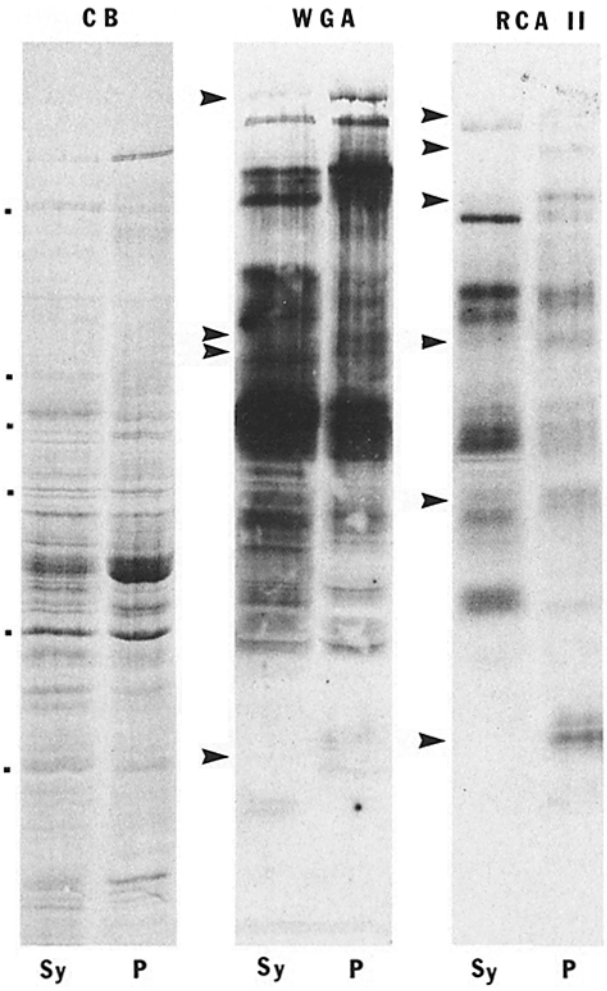

Figure 4. Comparison of lectin-binding proteins of nerve growth cone particles versus synaptosomes. Growth cone particle (*P*) and synaptosomal (*Sy*) protein were resolved by SDS PAGE and stained with Coomassie Blue (*CB*) (*left*) or blotted (*center* and *right*). Blots were incubated in 6×10^5 cpm (0.4 μg) ^{125}I-WGA or 6×10^5 cpm (0.7 μg) ^{125}I-RCA II per gel lane. x rays were exposed for 2.2 d and 3.8 d for WGA and RCA II, respectively. Position of molecular mass standards are, from top to bottom, 200, 116, 94, 68, 43, and 30 kD. Arrowheads indicate lectin-binding proteins that are present in greater abundance in nerve growth cone particles compared with synaptosomes. Control blots incubated in the appropriate hapten sugar plus lectin were blank and are not shown.

dase, and perhaps new lectin-binding polypeptides would be identified.

Neuraminidase obtained from *Vibrio cholerae* (*Vc*) or *Clostridium perfringens* (*Cp*) was used for enzyme digestion. After incubation of the sample with neuraminidase for 2–16 h, the proteins were subjected to electrophoresis, blotted, and the blot was incubated with ^{125}I-RCA II or ^{125}I-RCA I. While incubation in buffer alone has little or no effect on lectin binding (Fig. 5, compare no treatment [–] and control [*c*]), neuraminidase digestion markedly enhances RCA II and RCA I binding to glycoproteins of growth cone particles and synaptosomes (Fig. 5; enzyme treatment [*Vc* or *Cp*]). The lectin binding is specific since ricin binding is effectively competed by 0.2 M galactose. Sialidase digestion not only enhances RCA II binding to bands that are only weakly detected without enzyme treatment, but also results in the appearance of a few new RCA II–binding species. Compared with 2-h digestion, treatment for 16 h does not significantly change the results qualitatively or quantitatively (data not shown).

Table I. Glycoproteins of Growth Cone Particles vs. Synaptosomes

	WGA	RCA II
	kD	
Greater abundance in growth cone particles	27–30	27–30
	–	68
	110	–
	116	116
	–	200
	–	220
	250	250
Greater abundance in synaptosomes	–	46
	77	77
	90	90
	–	126
	135	135
	175	175
	240	240

Two notable results are observed concerning gp27–30. First, while neuraminidase digestion enhances RCA II binding to many polypeptides of synaptosomes, no species is detected at or around the 27–30-kD position. Therefore, it is unlikely that synaptosomes contain a glycoprotein with a carbohydrate tree similar to that of gp27–30 found in growth cone particles. Second, neuraminidase treatment causes a striking change in RCA II binding near the 27–30-kD region within growth cone particles. After neuraminidase digestion, three prominent RCA II–binding bands are detected at 25, 27, and 29 kD (Fig. 5, arrowhead triplet).

D-NANA has been reported to block the action of sialidases (Gottschalk and Drzeniek, 1972). In these experiments, D-NANA does not generally block the enhancement of ricin binding to the glycoprotein species (Fig. 5; compare enzyme alone [*Vc*] and enzyme plus 1 mM D-NANA [*Vc* *]). (This may be due to the high neuraminidase concentration present during the incubation and/or the affinity of neuraminidase for various sialo-proteins.) However, 1 mM D-NANA does block the appearance of the 25-kD ricin-binding protein. Similar results were observed when 5 mM D-NANA or 5 mM sialic acid was added with the enzyme during incubation.

gp27–30 Has a High Sialic Acid Content

To test if sialic acid might be responsible, at least in part, for gp27–30's unusually acidic isoelectric point (pI 4.0), the effects of sialidase on gp27–30's electrophoretic mobility was examined. Growth cone membranes were treated with or without neuraminidase as before, and their proteins were resolved by 2D gel electrophoresis. Western blots were prepared and RCA II–binding analysis was performed (Fig. 6). As expected, neuraminidase enhances RCA II binding to many species (Fig. 6; compare *A* and *B*). Many of the RCA II–binding proteins overlap with those previously identified by WGA-binding analysis. The pattern also confirms the charge heterogeneity of glycoproteins. RCA II binding spares

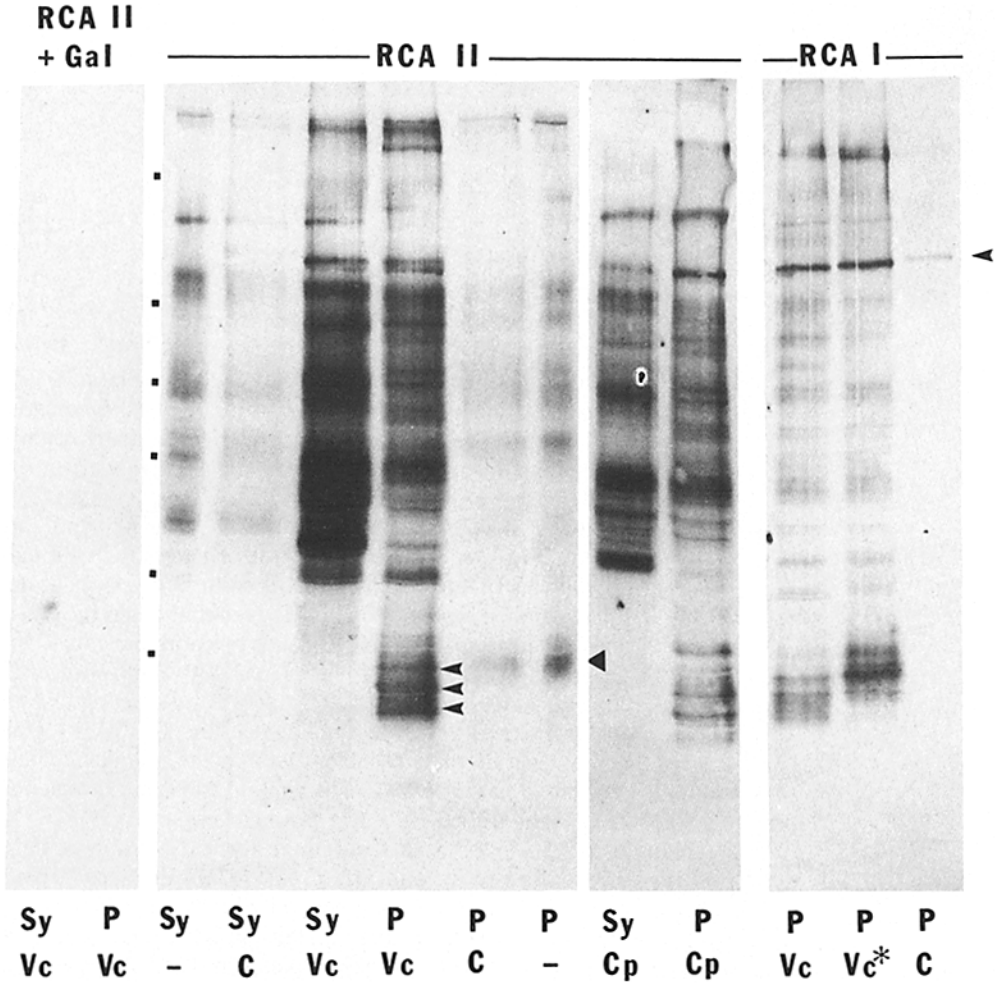

Figure 5. The effects of neuraminidase on RCA binding to glycoproteins of nerve growth cone particles (*P*) and synaptosomes (*Sy*). Protein (35 μg) was incubated with neuraminidase from *Vibrio cholerea* (*Vc*) or *Clostridium perfringens* (*Cp*). Incubations were for 2 h and 16 h for RCA II and RCA I, respectively. Controls: *C*, incubation without enzyme, –, no incubation. The proteins were resolved by SDS PAGE and blotted. Blots were incubated in 1.8×10^6 cpm (0.8 μg) ^{125}I-RCA II per gel lane in the absence or presence of 0.2 M galactose (*Gal*) or 2.0×10^6 cpm (1.1 μg) ^{125}I-RCA I per gel lane. x rays were exposed for 1 d or 3 d for RCA II and RCA I blots, respectively. Molecular mass markers (squares) are as described in Fig. 3. Neuraminidase enhances ricin binding to all proteins. Note that a 27–30-kD protein (*large arrowhead*) is present in growth cone particles but absent from synaptosomes. After neuraminidase digestion three prominent bands (29, 27, 25 kD; *arrowhead triplet*) are found in growth cone particles. Arrowhead at the right indicates the position of exogenous α2-macroglobulin (added as protease inhibitor). Incubation in the presence of 1 mM D-NANA (*Vc* *) blocks the neuraminidase-induced appearance of several bands including the 25-kD RCA I-binding species.

the positions at which tubulin and actin are known to migrate (Fig. 6 *A*, arrowheads).

Consistent with WGA-binding analysis, gp27–30 resides at a pI of 4.0 without prior digestion (Fig. 6 *B*, arrow). After neuraminidase digestion, the isoelectric point shifts. A charge train containing at least five distinct spots extending from 4.1 to 4.5 is seen; the major species present has a pI of 4.5 (Fig. 6 *A*, arrow). More alkaline to the major species, a band of RCA II–binding species is observed at 27–30 kD. The shift in the isoelectric point is demonstrated better in a second experiment where the sample did not achieve equilibrium in the isoelectric focusing axis (Fig. 6, *D* and *E*). The sample not treated with neuraminidase (Fig. 6 *E*) exhibits one major species in the 27–30-kD position. (Note that a more acidic species of gp27–30 than already shown is not seen.) Upon neuraminidase digestion (Fig. 6 *D*), a similar but condensed pattern compared with that of the first experiment is observed. The blot in Fig. 6 *D* also demonstrates an additional, minor species in the untreated samples (see asterisk) which co-migrates with the broad RCA II–binding region upon neuraminidase treatment. The area corresponds to the pI range of 4.7–5.5 in Fig. 6, *A* and *B*.

The Lectin-binding Capacity of gp27–30 Is Developmentally Regulated

Since gp27–30 as detected by lectin binding is present in growth cone particles but not in synaptosomes, this suggests that gp27–30 and/or its oligosaccharides are developmentally regulated. If this were true, it should be possible to demonstrate that RCA II binding to gp27–30 decreases during neural development. To test this hypothesis, crude membranes were prepared from developing cortex and cerebellum. Only the data for the forebrain are presented; cerebellar samples generate similar results in principle concerning gp27–30. RCA II binding to gp27–30 within the crude membrane preparations was enhanced by pretreating the membranes with neuraminidase as described above.

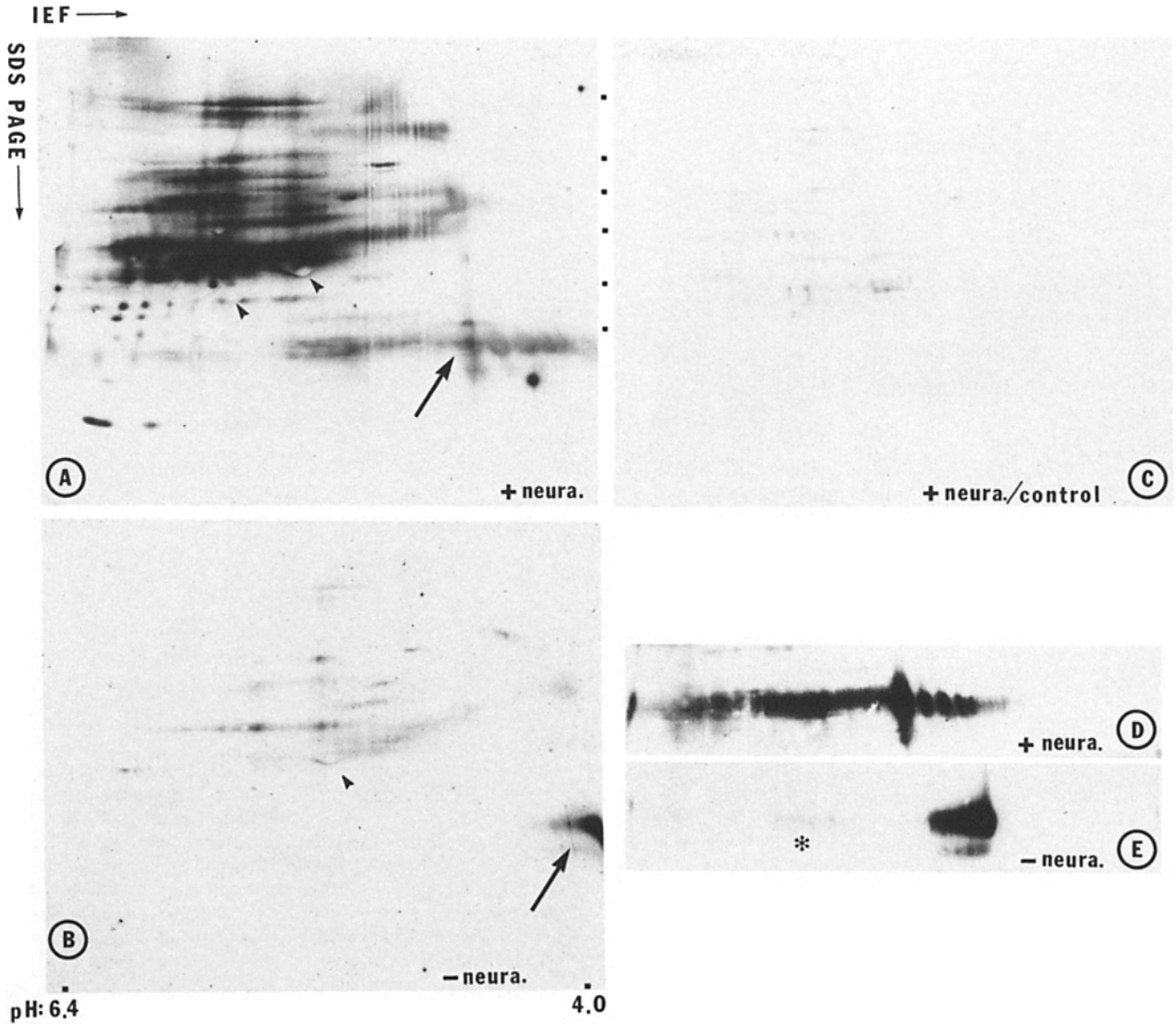

Figure 6. The effects of neuraminidase on the isoelectric point of gp27–30. Growth cone membrane protein (125 μg) was incubated with or without neuraminidase (*Vc*) for 2 h. The proteins were then resolved in two dimensions and blotted. After quenching in 2% PVP + 0.5% hemoglobin, blots were incubated in 1.9×10^7 cpm (10 μg) ^{125}I-RCA II per gel lane in the presence or absence of 0.2 M galactose. To reduce nonspecific binding, after washing and drying the blot, the blot was washed an additional 2 h in 0.025% Nonidet P-40. x-ray film was exposed for 17 d. (*A* and *D*) Neuraminidase-treated membranes; (*B* and *E*) untreated membranes; (*C*) neuraminidase-treated membranes incubated with RCA II + 0.2 M Gal. In *D* and *E*, only the 27–30-kD region of the gel is shown. Molecular mass markers in *A* are 200, 116, 94, 68, 43, and 30 kD. The large arrows in *A* and *B* emphasize the shift in the major isoelectric species at 27–30 kD after neuraminidase treatment. The arrowheads in *A* denote RCA II-sparing of the position of tubulin (52 kD, pI 5.5) and actin (42 kD, pI 6.0). The position of tubulin is also identified in *B*. The neuraminidase-induced shift in the major isoelectric point at 27–30 is emphasized in *D* and *E* where isoelectric focusing was not complete. The asterisk in *E* indicates weak RCA II binding at ~27–30 kD, which is enhanced after neuraminidase treatment.

Fig. 7 (top and bottom) demonstrates that there is a decrease in RCA II binding to gp27–30 during development. RCA II binds to a group of polypeptides extending from 23 to 29 kD (solid arrowhead), which are present at P0, maximal at P4, and then decrease until just above background levels at P18. These polypeptides are not detected with RCA II in adult forebrain samples.

Neuraminidase-treated gp27–30 contained within crude membrane preparations is not exactly the same as its counterpart in growth cone membranes. Crude membranes contain a broader range of molecular mass species extending from 23 to 29 kD compared with the 25–29-kD range for growth cone membranes (compare also with Fig. 5). Furthermore, neuraminidase-treated gp27–30 is enriched in growth cone particles compared with crude membranes prepared from the same developmental stage (compare lanes *GCP* and *EI8* in Fig. 7).

Other RCA II–binding proteins are developmentally regulated besides gp27–30. They include one glycoprotein that decreases (78 kD; solid arrowhead) and five glycoproteins

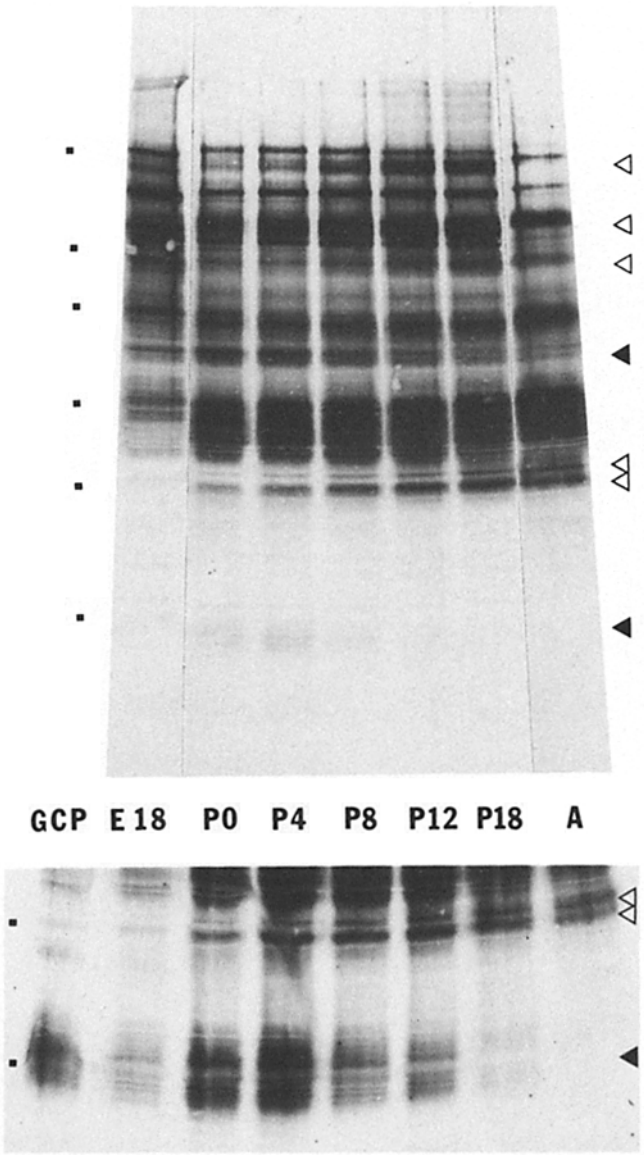

Figure 7. RCA II binding to gp27–30 from the rostral cortex at different developmental stages. Crude membrane protein (40 μg) was incubated in neuraminidase (*Vc*) for 2 h. Then, protein was resolved by SDS PAGE and blotted. Blots were probed with 8.6 × 10^6 cpm (0.8 μg) ^{125}I-RCA II per gel lane. Time points analyzed were E18, postnatal day P0 (birth) to P18, and adult (*A*). Growth cone particles (*GCP*) prepared on E18 are included for comparison with E18 crude membranes. (*Top*) Material freeze-thawed three times before use. (*Bottom*) Material thawed once before use. The position of the molecular mass standards are, from top to bottom, 200, 116, 94, 68, 43, and 26 kD. Only the 52–20-kD range is shown (*bottom*) since results were similar compared with the higher molecular weight region (*top*). During development, there is a decrease in RCA II binding to polypeptides at 23–29 and 78 kD (*closed arrowheads*) and an increase to polypeptides at 44, 46, 115, 135, and 175 kD (*open arrowheads*). Note that while the 44- and 46-kD bands have the same intensity (*top* and *bottom*), the RCA II binding to the 23–29 kD protein is greatly enhanced in material that was not repeatedly freeze-thawed. x rays were exposed for 4 d.

that increase (44, 46, 115, 135, and 175 kD; open arrowheads) during development. These differences are not obvious in the Coomassie-staining profiles of the samples (data not shown).

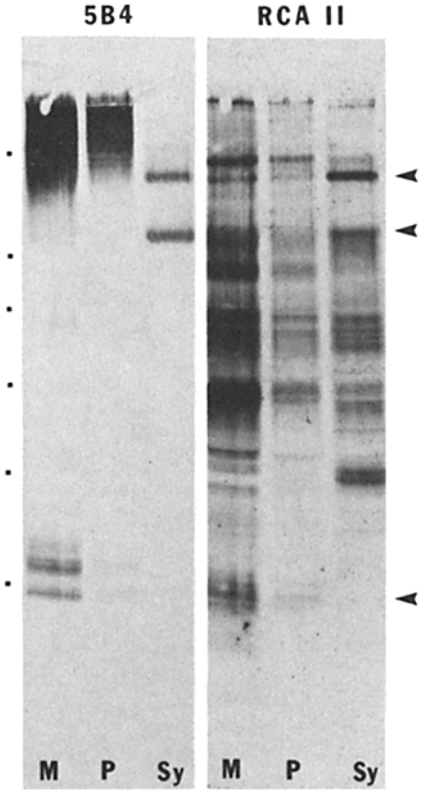

Figure 8. Co-localization of RCA II– and 5B4-binding proteins in 1D gels. Polypeptides were resolved by SDS PAGE, blotted, and the blot was probed with ^{125}I-RCA II. After an autoradiogram was prepared, the blot was re-screened with 5B4 by immunoblot assay methods. Lane abbreviations are described in earlier figures. Molecular mass markers are, from top to bottom, 200, 116, 94, 68, 43, and 30 kD. Arrowheads point to RCA II and 5B4 co-migrating species. Note that only the 5B4-positive species of lowest molecular mass (29 kD) migrates in the 27–30-kD RCA II-positive region. Control blots incubated with ascites supernatant were completely negative and are not shown.

gp27–30 Is Distinct from the 5B4 Antigen

At least in some respects, gp27–30 has characteristics similar to those of 5B4, a glycoprotein related to N-CAM. Both gp27–30 and the 5B4 antigen have a high sialic acid content, are developmentally regulated (Ellis et al., 1985*b*), and are enriched in growth cone particles. Monoclonal antibody 5B4 seems to bind to a cytoplasmic epitope of the glycoprotein (Ellis et al., 1985*b*). To test if gp27–30 might be a proteolytic fragment of 5B4 (fetal molecular mass 180–250 kD), we prepared immunoblots with the monoclonal antibody 5B4 (Ellis et al., 1985*b*) to see if the antibody recognized gp27–30. Pilot experiments showed that blots reacted previously with RCA II could be re-probed for antibody-binding species with the same fidelity as new, unscreened blots.

In 1D blots of growth cone particle and membrane samples, 5B4 binds to three species (Fig. 8). As shown previously, the major species extends from 180 to 260 kD. However, unlike the observation in our previous reports, the sensitive avidin–biotin procedure reveals minor bands in growth cone membranes at 32 (doublet) and 29 kD. Since the blot had previously been incubated with ^{125}I-RCA II, it was possible to superimpose the x ray of the RCA II–binding proteins on the 5B4-reactive polypeptides. This comparison

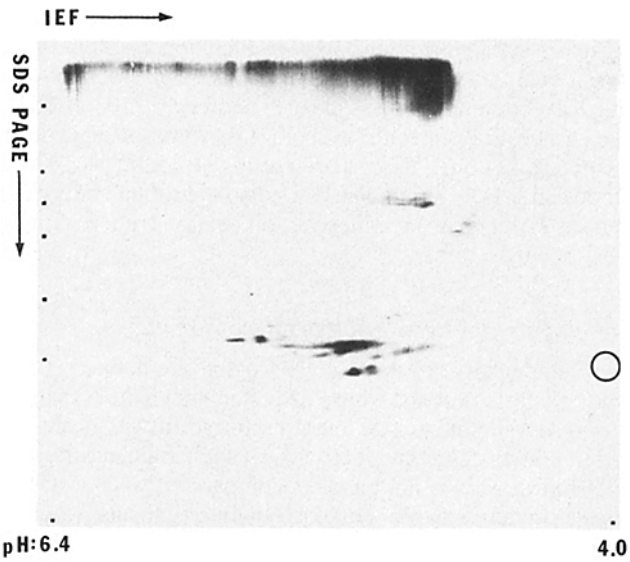

Figure 9. Comparison of gp27–30 and 5B4 antigens by 2D gel electrophoresis. After a 2D blot was screened with ^{125}I-RCA II (Fig. 6 *B*), the exact same blot was re-screened with the 5B4 antibody. Molecular mass standard positions are the same as those in Fig. 6 *A*. The position of the major RCA II–binding species at 27–30 kD, pI 4.0, is indicated by the circle at the right side of the blot. It is distinct from the minor 5B4-species at 29–32 kD, pI 4.7–5.5. Control blots incubated with myeloma supernatant in place of the primary antibody were negative and are not shown.

shows that part of gp27–30 co-migrated with the 5B4-binding species of lowest molecular mass. RCA II does not bind to the 180–260-kD 5B4-positive region, but does recognize a sharp band at 250 kD spared by 5B4. The 5B4-binding pattern changed when samples from mature brain were examined. 5B4 recognizes distinct bands at 135 and 175 kD in synaptosomes but no smaller species. Both the 135- and 175-kD proteins are major RCA II–binding proteins but minor 5B4 antigens relative to the broad, intense band in the growth cone samples.

5B4 binding was also performed on the same blot of a 2D gel used for RCA II–binding analysis (compare Figs. 6 *B* and 9). The major 5B4-binding species of growth cone membranes migrates to a pI of 4.75–5.25 (180–260 kD), although a long tail is present which extends to what corresponded to the top of the tube gel. In the 27–30-kD region, there are several minor 5B4 species present, but they have isoelectric points of 4.7–5.5 and, thus, are considerably more basic than the major RCA II–binding species of gp27–30 (pI 4.0). However, 5B4-postive spots of the lowest molecular weight are in the same region as the minor RCA II species at 27–30 kD, pI 4.7–5.5 (Fig. 6, *A* and *E*), yet there is little overlap at distinct spots between the two binding patterns. Neuraminidase treatment does not remarkably alter the 5B4 staining pattern of small species nor does it result in 5B4 binding to the major RCA II–binding species in the isoelectric range of 4.1–4.5 (data not shown).

Discussion

Glycoproteins have been identified in this study by lectin binding to proteins after electrophoretic transfer from polyacrylamide gels to nitrocellulose. This approach imposes certain restrictions on interpretation of the data. In particular, glycoprotein identification is limited to those species that transfer efficiently. It is known that high molecular weight proteins often do not transfer as well as low molecular weight proteins (reviewed by Gershoni and Palade, 1983). In agreement, we have noted that the lectins do not detect some polypeptides of >200 kD consistently. Furthermore, the 46- and 34-kD proteins of growth cone particles do not transfer efficiently. While we have not characterized the lectin-binding proteins with other methods such as direct overlay of gels (Burridge, 1978), we have determined that electrotransfer was good by examining the staining profile of proteins on nitrocellulose blots and comparing them with the staining patterns of any proteins left behind in the gel. We cannot be certain that our description of lectin-binding proteins within the growth cone fraction is complete, but the omission of major glycoproteins is highly unlikely.

We have also used a novel method whereby blots can be re-screened with a second probe. In particular, antibody binding can be recognized by a biotin-avidin-horseradish peroxidase detection method after a first screening with ^{125}I-RCA II. This method does not generate false-positives or false-negatives, since (*a*) not all RCA II–binding proteins are 5B4-positive and (*b*) 5B4 can bind to some RCA II–binding species (e.g., the 135- and 175-kD proteins in synaptosomes). The dual labeling method has the advantage of (*a*) allowing an exact comparison of two binding profiles and (*b*) reducing the labor and subsequent variability in sample, gel, and blot preparation.

The identification of glycoproteins in this report is limited to those that bind the lectins Con A, WGA, RCA I, and RCA II. Originally, these lectins were thought to bind specifically to mannose, GlcNAc, Gal, and Gal or GalNAc, respectively, since the agglutination of red blood cells by the lectins could be inhibited by these monosaccharides (reviewed by Goldstein and Hayes, 1978). Now it is clear that the lectins bind with far greater specificity to certain glycopeptides. Con A has a high affinity for high mannose and hybrid glycopeptides (Baezinger and Fiete, 1979*a*; Ohyama et al., 1985). WGA binds preferentially to complex glycopeptides that contain a bisecting GlcNac residue linked to the β-mannose residue (Yamamoto et al., 1981). Both ricins bind maximally to bi- and tri-antennary glycopeptides that have multiple exposed galactose residues (Baezinger and Fiete, 1979*b*). RCA II has a very high affinity for O-linked glycopeptides with exposed galactose residues (Baezinger and Fiete, 1979*b*). Our working assumption is that the specificity of lectins for glycoproteins is similar to that for their glycopeptide constituents. (However, the affinity for a glycoprotein may be greater than that for the glycopeptide.) Based on this assumption, the lectins used in this study are likely to identify a broad range of oligosaccharides found on glycoproteins. It is not possible to determine the precise oligosaccharide structure of the glycoproteins based on the lectin-binding properties reported here. The lectins are used to highlight certain glycoprotein families of growth cones and to compare them with those found in synaptosomes.

The Growth Cone Membrane Fraction Contains a Complex Glycoprotein Profile

Previous analysis has demonstrated that a fraction enriched in growth cone membranes contains a relatively simple pat-

tern of major polypeptides. In contrast, Con A, WGA, RCA I, and RCA II bind to a remarkably different set of proteins. These glycoproteins are probably integral membrane proteins, since lectin binding increases when growth cone particles are lysed and salt-washed. The major Con A–binding proteins are 204, 198, 192, 146, 117, 115, 110, 94, 80, 52, and 42 kD, while the WGA and RCA receptors are 204, 198, 192, 124, 112, 94, 68, and 27–30 kD. Many of the RCA-binding proteins have terminal sialic acids, since RCA binding is markedly enhanced after neuraminidase treatment. The striking discrepancy between Coomassie- and silver-staining patterns on the one hand and those obtained by lectin binding on the other hand, could be due to either an inability of Coomassie and silver to detect certain glycoproteins, or the relative paucity of glycoproteins present in amounts below the sensitivity of the protein dyes. (It is known that Coomassie Blue stains some glycoproteins poorly [Zacharius et al., 1969].)

The 52- and 42-kD major Coomassie-staining proteins, which co-migrate with tubulin and actin, respectively (Ellis et al., 1985*b*), do not bind the lectins used. While lectins bind to glycoproteins that co-migrate at these molecular weights, they spare the positions of actin and tubulin in 2D blots. There is a small amount of specific RCA II–binding (after neuraminidase digestion of membranes) to a 46-kD, pI 4.8, species which co-migrates with the most basic region of the (silver-staining) 46-kD protein (pI 4.5–4.8). The 46-kD protein, probably identical to GAP43 (Meiri et al., 1986), has been identified as a major growth cone phosphoprotein (Katz et al., 1985). If it contains carbohydrate, then this protein is likely to span the membrane.

The growth cone membrane fraction contains material pooled from a heterogeneous population of neurons of different developing brain regions. This raises several related questions: (*a*) are any glycoproteins identified here found exclusively at the growth cone, (*b*) are these glycoproteins present on all growth cones or are they the composite of distinct classes of glycoproteins from individual neuron types, and (*c*) is the observed glycoprotein heterogeneity due to contaminants of the growth cone particle fraction? The last question is the most difficult one to answer because subcellular fractions are never entirely pure. However, as described in our original paper (Pfenninger et al., 1983), the growth cone particle preparation is remarkably homogeneous, at least morphologically, with probably <10% contamination. Furthermore, the simple pattern of major membrane proteins is not what one would expect from a significantly contaminated heterogeneous fraction. Glycoproteins primarily present on growth cones might exist: comparisons between growth cone particles (derived from light subfractions) and the heavier subfractions of fetal brain (which contain glial elements, numerous organelles from the perikaryon, and neurite shafts) reveal a few glycoproteins (e.g., the 27–30-kD RCA II–binding protein) that are enriched in the growth cone particle fraction (data not shown). Furthermore, previous morphological and biochemical studies have shown that the plasmalemma of neuronal perikaryon can be distinguished from that of growth cones (Pfenninger and Maylié-Pfenninger, 1981; Sonderegger et al., 1984; Schlosshauer, 1985). Concerning the possibility of type-specific glycoproteins, quantitative lectin-binding analysis on cultured neurons has been used to show that growth cones from individual neuron cell types can be distinguished by their complement of lectin receptors (Pfenninger and Maylié-Pfenninger, 1981; Pfenninger et al., 1984). Furthermore, a few (glyco)proteins distinguish different neuron types (Sonderegger et al., 1984), and carbohydrate-specific antibodies have been used to demonstrate functional classes of neurons (Dodd et al., 1984; Dodd and Jessel, 1985). These results suggest that individual neuron types may be different, especially with regard to growth cones.

Developmental Regulation of Glycoproteins

Developmentally regulated glycoproteins are defined in this study as those proteins whose detection with lectins changes during development. An initial comparison was made between growth cones and their closest adult counterpart, synaptosomes. This is not an exact comparison since synaptosomes contain both pre- and postsynaptic elements, whereas growth cone particles contain only the precursors of the future presynaptic membrane. Therefore, only those changes that result in a loss of lectin binding in synaptosomes relative to growth cone particles can be attributed with certainty to presynaptic changes (negative regulation). Conversely, increased lectin binding to synaptosomal proteins could either reside in the presynaptic membrane or be due to the inclusion of postsynaptic elements (positive regulation).

Seven developmentally regulated lectin-binding proteins in each category have been described (Table I). Similar lectin-binding species have been identified previously in synaptic membranes (reviewed by Mahler, 1979). The positively regulated species at 175 and 135 kD co-migrate with antigens recognized by monoclonal antibody 5B4. Previous work has suggested that both of these species might be due to a reduction in the sialic acid content from the embryonic form (250–180 kD) of the molecule (Ellis et al., 1985*b*; cf. Rothbard et al., 1982).

The 27–30-kD protein is a striking negatively regulated glycoprotein, since it appears to be completely absent from synaptosomes. To test if a cryptic RCA II–binding site might be present in synaptosomes in that molecular weight region, samples were treated with neuraminidase. Neuraminidase treatment can enhance RCA II binding to many proteins by removing sialic acid and exposing galactose residues, and it may increase the electrophoretic mobility of the protein. After neuraminidase treatment, no species at 27–30 kD or any lower molecular mass is detected with RCA II in synaptosomes. Therefore, gp27–30 of growth cone particles has no counterpart with a similar oligosaccharide compared with synaptosomes. Consistent with these results, the amount of gp27–30 varies during neural development. The peak amount of gp27–30 corresponds to the time (postnatal day 4) when massive sprouting occurs and synapses are being established in the cortex (Jacobson, 1978). However, by postnatal day 18, gp27–30 is reduced to a level just above the detection limit of RCA II.

The glycoproteins in the 27–30-kD region are likely to be more than one polypeptide. This conclusion is supported by several facts. First, at least two distinct bands at 27 and 29 kD are observed in growth cone membranes with WGA and the ricins. Second, after neuraminidase treatment, three polypeptides at 29, 27, and 25 kD are detected. The lowest molecular mass species is not likely to result from a molecular

mass shift due to sialic acid removal, because if polypeptides are treated with neuraminidase after they are separated by SDS PAGE and immobilized on nitrocellulose, an RCA II-binding species is clearly observed at 25 kD (data not shown). Third, multiple bands (23–29 kD), all of which are developmentally regulated, are seen in the developmental series (Fig. 7).

Gp27–30 has unusual molecular properties. It has an extremely acidic isoelectric point (pI 4.0), more acidic than any other protein found in growth cone membranes to date. The acidic nature of the molecule is due, at least in part, to its high sialic acid content. The presence of abundant sialic acid is best demonstrated by the significant shift in the isoelectric point of the protein and resultant charge train after neuraminidase treatment. Consistent with this result, WGA binding to the protein is enhanced by oxidative/reductive phenylamination (see Bartles and Hubbard, 1984), a procedure that reportedly alters the sialic acid residue. Other acidic residues, such as phosphate, sulfate, and/or acidic amino acids may also contribute to the acidity of the protein. However, gp27–30 is not likely to be a major phosphoprotein, since phosphate is not incorporated into the protein under conditions that favor protein phosphorylation (Ellis et al., 1985*a*; Katz et al., 1985).

Gp27–30 has somewhat similar properties with molecules of the N-CAM family, which includes the growth-regulated 5B4 antigen (Ellis et al., 1985*b*; Wallis et al., 1985). These proteins are developmentally regulated, are enriched in the growth cone particle fraction, have a high sialic acid content, and are freeze-thaw sensitive (Rothbard et al., 1982; Ellis et al., 1985*b*). Thus, we have considered the possibility that gp27–30 is a fragment of an N-CAM family member. This possibility is unlikely for many reasons. First, growth cone membranes are prepared in the presence of protease inhibitors. Second, antibody 5B4 does not recognize the major isoelectric variant of gp27–30 at a pI of 4.0 (nor does the 5B4 antibody recognize the major neuraminidase-shifted isoelectric forms of gp27–30). Third, RCA II binds to the adult form of N-CAM (175- and 135-kD polypeptides) which are prominent in synaptosomes, but not to their fetal counterpart (180–250 kD) in growth cones. In contrast, gp27–30 is prominent in growth cone particles, even without neuraminidase treatment, but not detected with RCA II in synaptosomes. Finally, if the predicted location of the sialic acid–rich region within N-CAM is correct (Cunningham et al., 1983; cf. Gennarini et al., 1984), the minimum molecular weight of a sialic acid–rich N-CAM fragment that would purify with salt-washed (integral) membrane proteins would be considerably greater than that of gp27–30.

In addition to the major 5B4-binding species described in previous reports (Ellis et al., 1985*b*), we demonstrate here that the 5B4 antibody binds to minor species at 29 and 32 kD, pI 4.7–5.5, and 94 kD, pI 4.7. These newly detected, minor 5B4-positive species have been revealed due to the use of the more sensitive biotin–avidin, immunodetection system (Anderson et al., 1984) than used previously (cf. Ellis et al., 1985*b*).

Gp27–30 also shares many properties with the Thy-1 antigens, a family of surface glycoproteins found predominately on thymocytes and neurons (e.g., Williams and Gagnon, 1982; Letarte, 1984). Thy-1 (24,000–27,000 D) and gp27–30 have similar apparent molecular masses, are both glycoproteins, and are developmentally regulated. However, many differences exist: the adult brain contains at least fifteen times the amount of Thy-1 compared with neonatal brain (Williams et al., 1976); the isoelectric point of Thy-1 (charge train from pI 5 to 9) is higher than that of gp27–30 (e.g., Letarte, 1984); while gp27–30 seems to be sialic acid–rich, brain Thy-1 is not; Con A binds to all forms of Thy-1 (e.g., Letarte, 1984) but does not appear to recognize gp27–30. Thus, gp27–30 is not likely to be a member of the Thy-1 family, but we cannot exclude the possibility that it is an unusual form of Thy-1.

Presently, it is not possible to distinguish if the change in RCA II binding to gp27–30 during development is due to a decrease in the expression of the protein or a modification of its carbohydrate tree. This question may be answered by obtaining antibodies directed against the polypeptide backbone of this glycoprotein.

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