A Neuronal Surface Glycoprotein Associated with the Cytoskeleton

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ABSTRACT A cytoskeleton-associated glycoprotein of 130-kilodalton molecular mass (GP 130) was purified from a nonionic detergent-insoluble fraction of 10–16-d-old chicken embryo brains. GP 130 is tightly associated with other proteins in actin-containing complexes (Moss, D. J., 1983, *Eur. J. Biochem.*, 135:291–297); thus, pure protein preparations were obtained only after the partial dissociation of the complexes with the zwitterionic detergent, dimethyl dodecyl glycine (EMPIGEN BB), followed by ion-exchange chromatography and electrophoresis on preparative SDS polyacrylamide gels. Specific monoclonal and polyclonal antibodies were raised to GP 130 and used to examine its distribution in the developing nervous system. Experiments with these antibodies revealed that GP 130 is confined to nervous tissue and is restricted to the surface of neurons in cultures derived from both the central and peripheral nervous systems. This novel glycoprotein is immunologically unrelated to the neuronal cell adhesion molecule (N-CAM), or to vinculin, a protein of similar molecular mass which has been suggested to link actin filaments to the plasma membrane. In the developing chicken embryo brain, GP 130 is first detectable around day 8 after fertilization and increases to ~50% of its adult level by embryonal day 13. In contrast, no increase is observed over a similar developmental period in sciatic nerve. In the adult chicken, GP 130 is most abundant in brain and has a particularly high content in areas rich in dendrites and synapses.

Membrane components, particularly glycoproteins, that are associated with the cytoskeleton may well play a crucial role in the development of the nervous system. Dynamic processes such as the generation of cell shape, locomotion, the formation of cellular contacts, and redistribution of membrane receptors, require a force-generating cytoskeletal system able to respond to external signals (reviewed in reference 1). This is of particular importance for the numerous events that precede and maintain the establishment of correct connections between neurons. Axonal extension requires the contractile machinery within the growth cone to interact with the substratum via a mechanically robust linkage possibly involving transmembrane glycoproteins. There is a distinct preference for particular pathways, and later-arriving growth cones follow the axons already present by fasciculation into nerve bundles (2, 3), presumably moving along surface receptors that are firmly attached to the axonal cytoskeleton. An association between surface proteins and the cytoskeleton is also likely to play a vital part in synapse formation. Recognition between neurons and their target cells will be mediated by surface molecules, such as glycoproteins, but the segregation and stabilization of the receptors that ultimately leads to the formation of a mature synaptic junction, probably requires them to be coupled to the cytoskeleton. Many cytoskeleton components have been found to occur in synaptic junctions and postsynaptic densities (4, 5), and actin is found enriched in dendritic spines (6) and in the neuromuscular junction (7).

Cytoskeleton-associated glycoproteins have been identified in a number of tissues (8, 9) by virtue of their insolubility in detergent. In a similar approach in this laboratory, a number of concanavalin A (Con A)[1]-binding glycoproteins were iden-

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CNS, central nervous system; Con A, concanavalin A; eMCF, enriched membrane–cytoskeleton fraction; F1, fluorescein; GalC, galactocerebroside; GAM-Ig, goat anti-mouse immunoglobulin; GAR-Ig, goat anti-rabbit immunoglobulin; GP 130, cytoskeleton-associated glycoprotein of 130-kilodalton molecular mass; kD, kilodalton; MCF, membrane–cytoskeleton fraction; N-CAM, neuronal cell adhesion molecule; PBS, phosphate-buffered saline; PNS, peripheral nervous system; RAM-Ig, rabbit anti-mouse immunoglobulin; Rd, rhodamine; rGP 130, rabbit antibodies against GP 130; RIA, radio immune assay.

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tified in high molecular-weight detergent-insoluble, actin-containing complexes that were obtained from pure cultures of chicken sympathetic neurons and whole chicken brain (10). In the present communication, we report the derivation of monoclonal and polyclonal antibodies against the most prominent glycoprotein of this complex, a protein of 130-kilodalton (kD) molecular mass (GP 130). We also describe the use of these reagents to establish the distribution of GP 130 in the developing and adult nervous system.

MATERIALS AND METHODS

Antisera used in this study were kindly provided by Dr. J. Couchman (rabbit anti-chicken smooth muscle vinculin) (11) and Ms. D. Cox (guinea pig anti-bovine fibronectin) (12) (Unilever Research Laboratories, Bedford, U.K.). Rabbit antibodies against the neuronal cell adhesion molecule (N-CAM) (13) were a generous gift from Dr. R. Brackenbury and Dr. G. Edelman (The Rockefeller University, New York City, New York), and Dr. J. Wood (Welcome Research Laboratories, Beckenham, U.K.) kindly supplied the RT 97 monoclonal antibody against the neurofilament 210-kD polypeptide (14). Bovine corneal endothelial cells (15) were generously donated by Dr. C. Kintner (MRC Cell Biophysics Unit, King's College, London, U.K.).

Purification of GP 130: Brains from 10–16-d-old chicken embryos (40–50 g wet wt) were freed of meninges and homogenized at 4°C in 2 mM $MgCl_2$, 0.2 mM dithiothreitol, 10 mM Tris/HCl, pH 7.6 (buffer A), with 1 mM phenylmethylsulfonyl fluoride and Nonidet P-40 (1–5%) to give a 5:1 (wt/wt) detergent/protein ratio. The detergent-insoluble material was pelleted for 30 min at 100,000 *g* and resuspended in buffer A. Fractions were separated for 3 h at 100,000 *g* on a discontinuous sucrose gradient consisting of layers of 50, 30, and 10% sucrose in buffer A. According to a previous report from this laboratory (10), most of the GP 130-containing material collects at the 10–30% interface. This material was sampled, diluted in buffer A, and pelleted at 100,000 *g* as the membrane–cytoskeleton fraction (MCF). The pellet was homogenized in 10 mM Tris-HCl, pH 7.6, and partly dissociated by the addition of a 30% solution of dimethyl dodecyl glycine (EMPIGEN BB, Albright & Wilson Ltd., Whitehaven, Cumbria, UK) to a final concentration of 2% (vol/vol) or 0.67% detergent. This fraction was then applied to a DEAE-cellulose ion-exchange column (DE52, Whatman, Springfield Mill, Maidstone, Kent, U.K.) (3-cm diameter × 30 cm) equilibrated in 10 mM Tris-HCl, pH 7.6, and 0.2% EMPIGEN BB (buffer B). The column was washed with two column volumes of buffer B. Proteins were eluted with a gradient of 0–150 mM NaCl in buffer B and collected in 10-ml fractions. After analysis by SDS PAGE (16), GP 130-containing fractions were pooled as enriched membrane–cytoskeleton fraction (eMCF) and concentrated by vacuum dialysis. Pure GP 130 was obtained by preparative SDS-gel electrophoresis on 7% polyacrylamide gels (17). Separated bands were detected by brief staining of the gels with Coomassie Brilliant Blue (18). The band at 130 kD was excised and eluted electrophoretically. Before their use in immunization procedures, electrophoresis buffer and excess SDS, were removed from GP 130 on a Sephadex G25 column (1-cm diameter × 5 cm; Pharmacia, Uppsala, Sweden). In both gel procedures, ox neurofilaments (mol wt 200,000, 156,000, and 70,000), phosphorylase b (mol wt 95,000), and actin (mol wt 43,000) were used as molecular weight standards.

Protein concentrations were determined after spotting samples onto filter paper and staining with Coomassie Brilliant Blue G 250 (19). Bovine serum albumin (BSA) was used as a standard.

Concanavalin A (ConA)–binding glycoproteins were detected by SDS PAGE with ^{125}I-Con A as described previously (10).

Monoclonal Antibody Production: BALB/c mice were immunized with detergent-insoluble brain fractions of increasing GP 130 content. The first injection was with 200 μg of MCF protein in Freund's complete adjuvant, the second, after 2 wk, was with 90 μg of eMCF protein in Freund's incomplete adjuvant. Thereafter, the mice were boosted four times at intervals of 20, 16, 20, and 22 d with 25–30 μg of pure GP 130 eluted from preparative SDS gels. GP 130 was applied intraperitoneally in all cases. Serum activity against purified GP 130 was determined after each boost in an enzyme-linked immunosorbent assay (20) carried out essentially as described below for a radioimmune assay (RIA). Rabbit anti-mouse immunoglobulin (RAM-Ig) conjugated to alkaline phosphatase as described in reference 21 was used as a secondary antibody. Serum titers were considered satisfactory at 1:10,000. For the final boost, 3 d before sacrifice, pure GP 130 was injected into the tail veins of the mice. Spleen lymphocytes from these mice were fused with hypoxanthine–aminopterine–thymidine-sensitive SP2/0-Agl4 (22) myeloma cells according to published procedures (23, 24). Cells were seeded onto four trays of NUNC 24 (NUNC A/S, Roskilde, Denmark) dishes, which were preconditioned with feeder layers of intraperitoneal macrophages. Hybrids were selected by growth in hypoxanthine–aminopterine–thymidine–containing medium and grew in 80% of the wells. Six hybridoma lines (mGP 130-1 to mGP 130-6) from one fusion were selected on the basis of a RIA and subcloned twice by limiting dilution on macrophage feeder layers. Ascites fluid was produced in pristane-treated BALB/c-exbreeders and, for some experiments, subjected to precipitation with 50% $(NH_4)_2SO_4$ before use.

Screening of Hybridoma Cells for Antibody Production: Clones producing antibodies to GP 130 were detected in a RIA with eMCF in the initial, and with pure GP 130 in the subsequent test. Briefly, 1–2 μg of protein in 50–100 μl of phosphate-buffered saline (PBS) (pH 7.4) were bound to each well of polystyrene plastic (NUNC A/S, Roskilde, Denmark) in an overnight incubation at 4°C. Unbound protein was washed off with PBS, and the remaining free binding sites on the plastic were blocked for 2–3 h with 1% immunoglobulin-free BSA in PBS. Antibodies in undiluted hybridoma culture supernatants were incubated in the protein-coated wells and their binding to the antigen detected after subsequent incubations in presence of 1% BSA with ^{125}I-RAM-Ig $(Fab)_2$-fragments (50,000 cpm/ml, Amersham Bucks, U.K.) or RAM-Ig (Nordic, Maidenhead, Berks, U.K., 10 μg/ml) and ^{125}I-Protein A (50,000 cpm/ml). Each incubation step was carried out for 1–2 h at 37°C and was followed by extensive washing of the wells with PBS. Individual wells were dried and counted in a Packard Gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Protein A (0.5 mg) was iodinated using 1 mCi of Bolton–Hunter reagent (25).

Production of a Rabbit Antiserum against GP 130: The primary immunization of a Dutch rabbit was intramuscular with 400 μg of pure GP 130 in Freund's complete adjuvant. The animal was boosted after 4 and 8 wk with 150 and 100 μg of protein in Freund's incomplete adjuvant. Thereafter, GP 130 (40–100 μg) was applied intravenously in PBS in monthly intervals. Serum activity against GP 130 was first detected on immunoblots after four injections.

Detection of Antibody Binding to Proteins Separated by SDS PAGE: Proteins separated by SDS PAGE on minigels (0.4–0.5-mm thick) (16) were transferred electrophoretically to nitrocellulose paper (Schleicher & Schull, Dassel, Federal Republic of Germany) (26). The transfer was essentially complete after 4 h for proteins of <150 kD. Unspecific binding sites on the nitrocellulose paper were blocked by an overnight incubation with 3% BSA in 10 mM Tris-HCl, pH 8.4, 150 mM NaCl, and 0.1% NaN_3 (buffer C). Monoclonal or rabbit antibodies (1:50–1:100) were incubated with the paper and the monoclonal antibodies were followed by incubations with RAM-Ig (Nordic, Maidenhead, Berks, U.K., 100 μg/ml). Binding of antibodies from both species was detected by ^{125}I-Protein A (500,000 cpm/ml). All incubations were carried out for 1 h at room temperature in buffer C containing 3% BSA and were followed by 1–3-h washes with at least five changes of buffer C. The nitrocellulose paper was dried and exposed for autoradiography with Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) at −70°C with Cronex (DuPont Instruments, Wilmington, DE) intensifying screens (27). In some experiments the antibodies were bound directly to the separated proteins by overlaying the gel with undiluted mGP 130 culture supernatants (28). These gels were processed as described above, stained with Coomassie Brilliant Blue, dried, and exposed for autoradiography. Bands labeled with ^{125}I were identified by matching the labeled bands with the Coomassie Brilliant Blue–stained gel. Relative amounts of GP 130 in different samples were quantitated by densitometry of the labeled bands on immunoblots. This method compared samples within a single autoradiograph by using exposure times so that the increase in intensity was linear with respect to the time of exposure.

Cell Culture: Cultures of sympathetic neurons for biochemical analysis were prepared essentially free of non-neuronal cells as previously described (18). For immunofluorescence, sensory and sympathetic ganglia were dissected from 10–11-d-old chicken embryos in Hanks' balanced salt solution with or without Ca^{++} and Mg^{++}, respectively. The ganglia were dissociated after trypsin treatment (0.25% in Hanks' balanced salt solution without Ca^{++} and Mg^{++} for 30 min at 37°C) and grown in L15 medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum, 0.5% methylcellulose, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 0.6 g/l glucose, and nerve growth factor. The cells were seeded onto the extracellular matrix of bovine corneal endothelial cells (15) on glass coverslips.

Cultures from the central nervous system (CNS) were prepared from the cerebral hemispheres of 6–7-d-old, or the cerebella of 13-d-old chicken embryo brains. The tissues were freed of their meninges and dissociated after incubation with trypsin by gentle trituration through a wide-bore pipette. Aggregates were removed by passage of the cell suspension through a fine nylon mesh. The cells were plated onto extracellular matrix glass coverslips in NUNC 24-well plates (NUNC A/S, Roskilde, Denmark) in Dulbecco's modified Eagle's medium with 10% fetal calf serum at a density of $1–2 \times 10^5$ cells per well and stained

for indirect immunofluorescence after 2–6 d in culture.

Indirect Immunofluorescence: Indirect immunofluorescence staining was carried out on primary cultures from the CNS and peripheral nervous system (PNS). Cultures were incubated sequentially with one of the monoclonal mGP 130 antibodies (undiluted hybridoma culture supernatants or ascites fluid [1:50 or 1:100]) and rhodamine (Rd)-conjugated RAM-Ig (Nordic, 1:50). In some experiments the staining intensity of the monoclonal antibodies was enhanced by incubations with unlabeled RAM-Ig (100 μg/ml, Cappel Laboratories, Inc., Cochranville, PA) and Rd-goat anti-rabbit immunoglobulin (GAR-Ig) (1:100, Cappel Laboratories, Inc.) as secondary antibodies. Binding of rabbit antibodies against GP 130 (rGP 130) (1:50 or 1:100) was detected with Rd-GAR-Ig (1:100, Cappel Laboratories, Inc.). All antibody incubations were in L15-medium with 10% fetal calf serum and 0.02% sodium azide for 30 min at room temperature. After each incubation the coverslip cultures were washed by immersion through five changes of L15-medium. The staining with GP 130 antibodies was always carried out on living cells. The cultures were postfixed with acidic ethanol (ethanol/acetic acid, 95:5, vol/vol) at −20°C after the incubations with GP 130 antibodies and the appropriate fluorochrome conjugates. The coverslip cultures were mounted with gelvatol (29). Staining was examined under a Zeiss fluorescence microscope with appropriate filters for fluorescein and rhodamine.

Suspension staining of living cells was carried out as previously described (30), except that, before examination, the cells were fixed with acidic ethanol to the glass coverslips.

In double-labeling experiments of GP 130 with cell type-specific markers, staining with GP 130 antibodies followed by the appropriate fluorochrome conjugate was always carried out first. Indirect double immunofluorescence was performed sequentially with one of the mGP 130 antibodies, Rd-conjugated goat anti-mouse immunoglobulin (Rd-GAM-Ig), rabbit antibodies against neurofilament 70-kD polypeptide (1:40) (31) or filamin (1:40) (32), and finally fluorescein (Fl)-conjugated GAR-Ig (1:50, Nordic). Alternatively, rGP 130 and Rd-GAR-Ig were used in combination with a monoclonal antibody against neurofilaments (1:50) (14) and Fl-GAR-Ig (1:100, Cappel). Before the application of antibodies against intracellular filaments, the cultures were fixed with acidic ethanol. Double labeling of GP 130 with fibronectin, a marker for fibroblasts and leptomeningeal cells (33) was carried out in an identical way. In this case the cultures were stained with rGP 130 and Rd-GAR-Ig, fixed, and then exposed to guinea pig antibodies against fibronectin (1:50) (12) and Fl-goat anti-guinea pig immunoglobulin (1:50, Nordic). For double labeling of GP 130 and galactocerebroside (GalC), a marker for oligodendrocytes and Schwann cells (34, 35), the labeling was sequentially with rGP 130, Rd-GAR-Ig, monoclonal antibody against GalC (1:200) (30), and Fl-GAM-IgG3 (1:50, Nordic). These cultures were postfixed after having completed all the incubation steps. Secondary antibodies were cross-absorbed against mouse or rabbit immunoglobulin or were affinity-purified on immobilized Ig.

RESULTS

Purification of GP 130

GP 130 was isolated from whole brain homogenates in four steps as shown in Fig. 1 *a*. Components of the cytoskeleton were obtained by extraction with nonionic detergent followed by sedimentation (Fig. 1 *a*, lane *1*). The pellet was enriched for GP 130 by fractionation on a discontinuous sucrose gradient. GP 130 was found to accumulate at the 10–30% interface of the gradient together with about 20 other polypeptides including actin (Fig. 1 *a*, lane *2*). As shown previously by chromatography on Sepharose 4B, these polypeptides are associated in large complexes (10). The first two purification steps depend on the association of GP 130 with actin or other molecules of the cytoskeleton; however, for further purification it was necessary to break up the actin-containing complexes. The zwitterionic detergent EMPIGEN BB proved to dissociate the complexes sufficiently without interfering with subsequent purification steps. By ion-exchange chromatography on DEAE cellulose, a number of proteins were sepa-

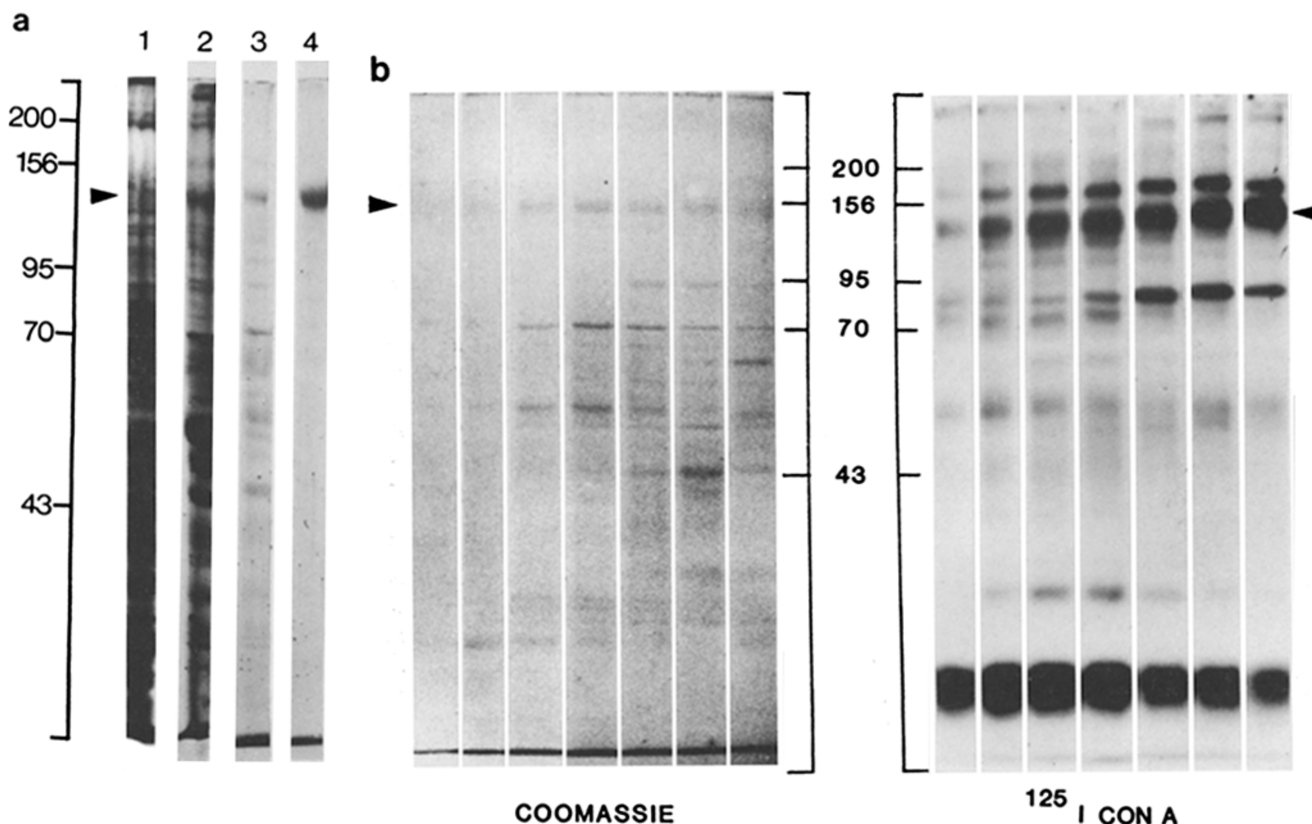


FIGURE 1 Purification of GP 130. (a) Steps in the purification of GP 130 from 10 to 13-d-old chicken embryo brains. (Lane *1*) Detergent-insoluble material. (Lane *2*) MCF from 10–30% interface of the discontinuous sucrose gradient. (Lane *3*) Peak fractions from the DEAE ion-exchange column (eMCF). (Lane *4*) GP 130 isolated from eMCF by 7% preparative SDS PAGE. Numbers in both figures show the molecular mass $\times 10^{-3}$. (*b*) Fractions of MCF were eluted from a DEAE column and analyzed on 9% SDS polyacrylamide gels. Proteins were detected with Coomassie Brilliant Blue and glycoproteins with ^{125}I-Con A.

rated from GP 130, including two or three proteins with similar electrophoretic mobility. The elution profile throughout the column was analyzed by SDS PAGE; the glycoproteins were detected with Coomassie Brilliant Blue or by autoradiography of gels after incubation with ^{125}I-Con A. The GP 130-containing peak fractions are shown in Fig. 1 *b*. Identical elution profiles were obtained with both stains, indicating that GP 130 is the major, if not the only 130-kD protein. Therefore, GP 130-containing column fractions were pooled as eMCF (Fig. 1 *a*, lane *3*) and subjected to preparative gel electrophoresis as the final step in the purification of GP 130 (Fig. 1 *a*, lane *4*). A Con A-binding glycoprotein of slightly lower electrophoretic mobility than 130 kD could be separated from GP 130 by reducing the percentage of polyacrylamide from 9 to 7%, thus increasing the distance between these proteins for preparative SDS PAGE. From 150–200 brains of 10 and 16-d-old chicken embryos, ~0.5 mg of GP 130, was routinely isolated with a percentage yield of 10–15%.

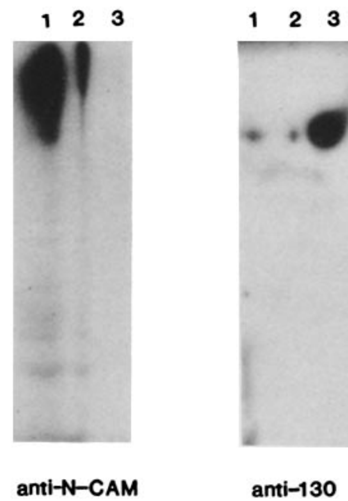


FIGURE 3 Comparison of N-CAM and GP 130. Western blots were incubated with either anti-N-CAM or rGP 130 followed by ^{125}I-Protein A. (Lane *1*) 8.5-μg, 10-d-old embryo brain detergent - soluble fraction. (Lane *2*) 3.5-μg, 10-d-old embryo brain detergent-insoluble fraction. (Lane *3*) eMCF.

Production and Characterization of Monoclonal and Polyclonal Antibodies

For the production of monoclonal antibodies, injection of BALB/c mice with increasingly pure GP 130 proved to elicit good serum titers for this protein. The fusion of spleen lymphocytes from one mouse with SP2/0-Ag14 myeloma cells produced six hybridoma lines (mGP 130-1 to mGP 130-6) reacting with pure GP 130 in a RIA. In addition, nine hybridoma clones secreting antibodies that bound to molecules in the eMCF fraction in the RIA and stained peripheral neurons in indirect immunofluorescence were isolated and kept frozen for later reference.

In Ouchterlony double diffusion (36) with class-specific rabbit or goat anti-mouse immunoglobulins, all mGP 130 antibodies were identified to be of the IgG1 subclass.

The antigen specificity of each of the mGP 130 antibodies was demonstrated in two ways: (*a*) Gels with separated MCF and eMCF were overlaid with mGP 130 antibodies and processed for autoradiography. The gel band labeled by the monoclonal antibodies could be matched up with a Coomassie-stained protein of 130 kD. In Fig. 2 *a* the specificity of mGP 130-2, mGP 130-3, and mGP 130-4 (lanes *2*, *3*, and *4*, respectively) for GP 130 is shown. (*b*) Western blots of whole brain homogenates demonstrated that the monoclonal antibodies recognized only a protein of 130 kD out of a greater variety of polypeptides. In Fig. 2 *b* immunoblots with mGP 130-1, mGP 130-5, and mGP 130-6 (lanes *3*, *4*, and *5*, respectively) are shown. Similarly, the polyclonal antiserum for GP 130 was shown on gel overlays and immunoblots to be monospecific for the 130-kD glycoprotein (Fig 2 *b*, lane *1*).

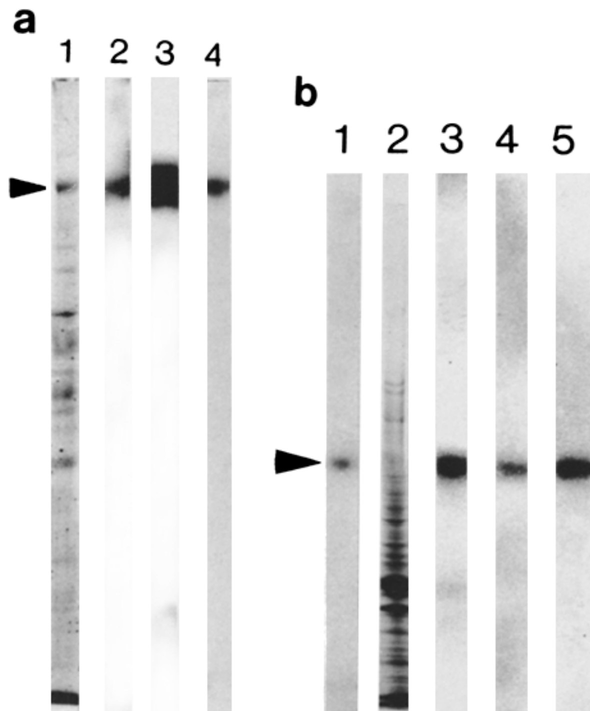


FIGURE 2 Specificity of monoclonal and polyclonal antibodies. (*a*) 9% SDS polyacrylamide gels of eMCF were incubated with culture supernatants and followed by ^{125}I-RAM F(ab)$_2$ fragments before staining with Coomassie Brilliant Blue. The relative intensities of the bands are not related to the binding properties of the antibodies because variable concentrations of the antibodies and different exposure times were used. (Lane *1*) eMCF stained with Coomassie Brilliant Blue. (Lane *2*) mGP 130-2. (Lane *3*) mGP 130-3. (Lane *4*) mGP 130-4. (*b*) 5 μg of whole brain homogenates of 13-d-old embryo brains were separated on 7% polyacrylamide gels and transferred to nitrocellulose. Monoclonal antibodies (ascites fluid [1:100]) were incubated with the paper and followed by RAM and ^{125}I-Protein A. The rabbit antiserum (1:50) was followed by ^{125}I-Protein A only. (Lane *1*) rGP 130. (Lane 2) Brain homogenate stained with Coomassie Brilliant Blue. (Lane *3*) mGP 130-1. (Lane *4*) mGP 130-5. (Lane *5*) mGP 130-6.

GP 130 Is Unique to Nervous Tissue

As a first step in the characterization of GP 130, it was necessary to establish if GP 130 is related to known neuronal or cytoskeleton-associated proteins. In preparations from chicken brain the neuronal cell adhesion molecule N-CAM (13) was not recognized by the polyclonal antiserum against GP 130. Conversely, rabbit antibodies against chicken N-CAM (37) did not bind to GP 130 on immunoblots (Fig. 3). It should be pointed out that N-CAM also has different solubility properties being largely extracted in 1% Nonidet 40, whereas GP 130 is largely resistant to this detergent. When equal amounts of protein from both detergent-soluble and insoluble fractions are examined on gels, GP 130 is usually two- to threefold enriched in the detergent-resistant fraction. Antibodies against chicken smooth muscle vinculin (11), a 130-kD protein that links actin filaments to the plasma membrane (38), and the related 150-kD plasma membrane protein, meta-vinculin (39), did not detect GP 130 in brain tissue on immunoblots (data not shown).

TABLE I
Distribution of GP 130 in Chicken Tissues

	Embryonal day 10	Adult
Breast muscle	ND	–
Heart	–	–
Liver	–	–
Lung	–	–
Gizzard	ND	–
Spleen	ND	–
Skin	–	ND
Brain	+	+

5 μg of each tissue was processed for immunoblots as described in Materials and Methods. On one occasion >10 μg was tested with the same results. A negative result corresponds to <5% of the amount in adult brain or <0.01% of the total protein.

Cytoskeleton-associated glycoproteins have been characterized from erythrocytes (8) and intestinal brush border (40). To examine the relation of GP 130 to these proteins, detergent-resistant fractions were prepared from both sources and tested with the polyclonal GP 130 antiserum on immunoblots. In neither preparation were bands labeled in the 90–140 kD region, although weak staining of low-molecular-weight material was occasionally seen. As the molecular weights of these latter bands varied with the experiments, it is difficult to comment on a possible cross-reactivity. From these experiments, GP 130 appeared to be unrelated to known proteins of similar molecular weight or potentially similar properties.

A variety of tissues from both the embryonic and adult chicken were examined for the presence of GP 130 with the monoclonal and polyclonal antibodies on immunoblots. The result of this survey is summarised in Table I. GP 130 was found only in tissues from the nervous system. Brain or spinal cord from mammals (rat, mouse, calf, and ox) and from an amphibian newt (*Notophtalmus viridescens*, from Lee's Newt Farm, Oak Ridge, TN) did not contain GP 130 when tested in a similar way. Only a reptile, green lizard (*Lacerta viridis*), was found to express GP 130 in nervous tissue.

GP 130 Is Expressed on Neuronal Cell Surfaces

In order to examine the cell type specificity of GP 130 in cells of the central and peripheral nervous system, we stained cultures of brain and peripheral ganglia (sensory and sympathetic) in indirect immunofluorescence with mGP 130 and rGP 130 antibodies. These experiments revealed that GP 130 is expressed on neurons in both the PNS and CNS. Fig. 4, *a* and *b* show the surface labeling of cultured sympathetic neurons with mGP 130-2. Neurons in these cultures are readily distinguished from non-neuronal cells by morphological and immunological criteria. Double immunofluorescence labeling with rGP 130 and a monoclonal antibody against the 210-kD component of neurofilament (14) confirmed the neuronal restriction of GP 130. In Fig. 4, *d–f* an overlapping pattern of staining is seen on most neurites, the only exception being some very thin neurites that appear to be stained only by the rGP 130 antibody. All but one of the monoclonal and the polyclonal antibodies against GP 130 stained neurons in an identical way over their entire cell surface including the growth cones of younger neurites (Fig. 4 *c*). Only mGP 130-4 did not detect an antigen determinant accessible to this antibody on living cells or cells fixed with 1% paraformaldehyde, methanol, or acidic ethanol. Non-neuronal cells in cultures from the PNS were identified by the positive staining with a rabbit antibody against filamin (32), an actin–cross-linking protein, and showed quite distinct morphologies and staining patterns from GP 130-stained cells (data not shown).

In cultures from the cerebral hemispheres of 6–7-d-old chicken embryos, GP 130 was also found on cells with a neuronal morphology (Fig. 5, *a* and *b*). An overlapping staining pattern was seen with neurofilament-positive cells (Fig. 5, *c–e*). In older cerebellar cultures (corresponding to a 17-d-old chicken embryo), no staining of GP 130 antibodies was detected on GalC-positive oligodendrocytes (Fig. 5, *f–h*). GP 130 was always found on process-bearing cells and never on flat cells expressing fibronectin or filamin (putative fibroblasts and protoplasmic astrocytes). There are, at present, however no satisfactory criteria for the identification of astrocytes in chicken brain. Antibodies against human, rat, or fish glial fibrillary acidic proteins, an intermediate filament specific to astrocytes (41), do not cross-react with cultured astrocytes from chicken, and it is not clear whether filamin is expressed on fibrous astrocytes. However, it is unlikely that under the chosen culture conditions fibrous astrocytes will develop in these cultures. From these data GP 130 appears, at present, as a marker molecule for chicken neurons.

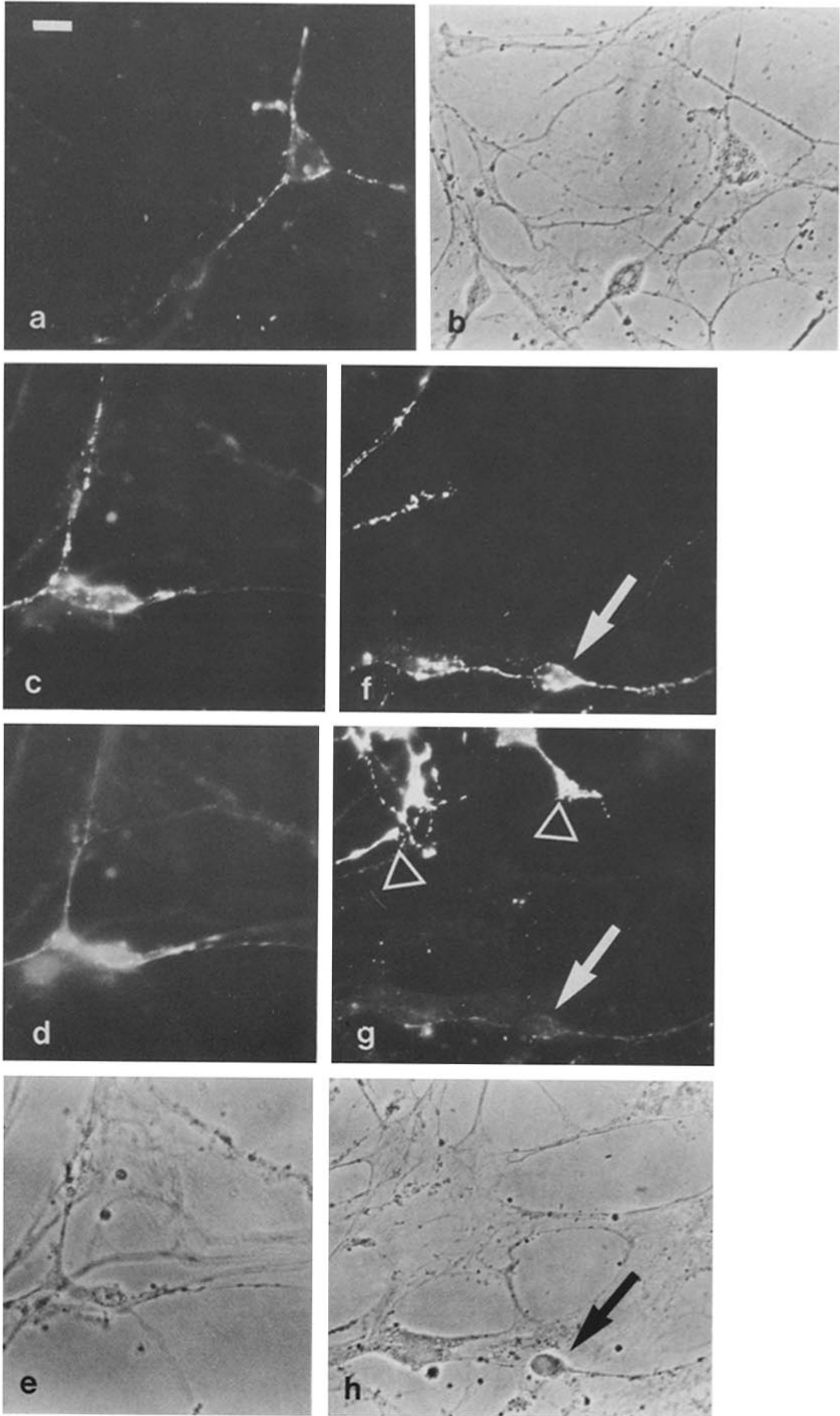
The immunofluorescence staining indicated that GP 130 molecules are exposed on the neuronal plasma membrane. When cells were fixed with methanol or acidic ethanol before the application of GP 130 and fluorochrome-conjugated antibodies, no staining of membrane or intracellular antigens could be detected. Because these fixation methods usually preserve the cytoskeleton, it seems probable that GP 130 was extracted or its antigenic sites were changed by organic solvents. Consequently, we cannot tell from these results, whether GP 130 is present on intracellular membranes. However, biochemical analysis of intact sympathetic neurons in 5-d-old cultures after treatment with trypsin suggested that most of GP 130 is located on the plasma membrane, while internal membranes and vesicles have relatively little. As shown in Fig. 6, after treatment with trypsin for 1 h, 90% of GP 130 was degraded in intact neurons, whereas the neurofilament 70-kD polypeptide—a prominent intracellular component—was unaffected in the same sample.

GP 130 Increases with Time in Neuronal Cultures

To relate the expression of GP 130 to a particular process during neuronal development, sympathetic neurons from 10-d-old chicken embryos were allowed to regenerate in culture. GP 130 was examined on cells without neurites, neurons passing through the early phases of neurite outgrowth, and after an elaborate network of fasciculated axons had formed. The sympathetic ganglia were dissociated with trypsin. This cell suspension was immediately stained in indirect immunofluorescence with rGP 130 antiserum. GP 130 was not detectable on the surfaces of any of these cells. Within 4 h, however, some staining with GP 130 antibodies could be observed on rounded neurons. These cells were prevented from attaching to a solid substratum by agitating them for 30 s at intervals of 5 min on a vibrating platform. Besides indicating the trypsin sensitivity of GP 130, this observation showed that the expression of GP 130 is independent of the formation of neurites. When the same material was analyzed on immunoblots, GP 130 could not be detected, possibly indicating a different level of sensitivity of the applied methods. However, within 24 h, during which the cells had formed growth cones and extended short axons, the content of GP 130 had recovered and exceeded the starting level in the 10-

FIGURE 4 Cell-type specificity of GP 130 in the PNS (*a* and *b*). Living cultures from the sympathetic ganglia of 10-d-old chicken embryos were stained after 3 d in vitro sequentially with mGP 130-2 (1:100), RAM-Ig (1:100), and Rd-GAR-Ig (1:100). Cells with a neuronal morphology are stained. (*a*) GP 130. (*b*) Interference optics. (*c*) Growth cone in a 24-h culture from the sensory ganglia of a 10-d-old chicken embryo stained with rGP 130 (1:50) and Rd-GAR-Ig (1:100) before fixation. (*d*, *e*, and *f*) Double labeling of GP 130 and neurofilaments: 2-d-old cultures from the sensory ganglia of 10-d-old chicken embryos were stained with rGP 130 (1:50) and Rd-GAR-Ig (1:100). After fixation with acid ethanol, neurofilaments were stained with the RT 97 monoclonal anti-neurofilament (1:50) and Fl-GAM-Ig (1:100). An overlapping staining pattern is seen on most neurites. Note: the Fl bleaches more rapidly than the Rd while taking the photograph. (*d*) GP 130. (*e*) Neurofilaments. (*f*) Phase contrast. Bar, 10 μm. × 650.

FIGURE 5 Cell-type specificity of GP 130 in the CNS (*a* and *b*). Cultures from the cerebral hemispheres of 6-d-old chicken embryos were stained after 3 d in vitro with rGP 130 (1:50) and Rd-GAR-Ig (1:100). Only cells with a neuronal morphology are stained. (a) GP 130. (*b*) Phase contrast. (*c*, *d*, and *e*) In the same cultures an overlapping staining pattern is seen with neurofilament-positive cells. After staining of GP 130 as above, neurofilaments were stained with the monoclonal anti-neurofilament (1:50) and Fl-GAM-Ig (1:100). (*c*) GP 130. (*d*) Neurofilaments. (*e*) Phase contrast. (*f*, *g*, and *h*) Cultures from the cerebellum of 13-d-old chicken embryos were sequentially stained after 4 d in vitro with rGP 130 (1:50), Rd-GAR-Ig (1:100), mGalC (1:200), and Fl-GAM-IgG3 (1:50). GP 130 and GalC are expressed on different cells. Arrows point out GP 130-positive cells, empty triangles (in *g*) indicate GalC-positive oligodendrocyte processes. (*f*) GP 130. (*g*) GalC. (*h*) Phase contrast. Bar, 10 μm. × 650.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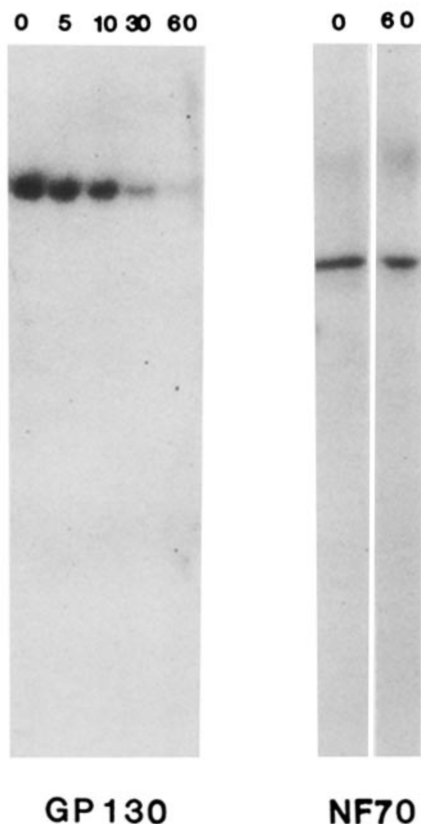


FIGURE 6 Trypsin sensitivity of GP 130 in cultured neurons. Cultures of sympathetic neurons grown for 5 d were treated for various time intervals with 1 mg/ml trypsin at pH 7.4 in 150 mM NaCl, 0.1 M $NaHCO_3$. The reaction was quenched with an equal volume of 1 mg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride. Cells were collected in this buffer centrifuged at 800 *g* and processed for SDS PAGE. GP 130 and the 70,000-mol-wt neurofilament (NF 70) were detected on Western blots using the appropriate antisera and ^{125}I-Protein A. Numbers represent the incubation times with trypsin in minutes. 0 min represents the simultaneous addition of trypsin and trypsin inhibitor.

d-old sympathetic ganglia. As shown in Fig. 7, GP 130 more than doubled in content during the time in culture. The level of GP 130 in 7-d-old pure neuronal cultures corresponded to that in a 13-d-old chicken embryo brain and was considerably higher than that in sympathetic ganglia at any age embryo tested. Immunofluorescence staining with GP 130 antibodies could be observed throughout the entire culture period. In well-developed cultures the staining was more intense than in rounded neurons or 24-h cultures.

GP 130 in Developing and Adult Nervous Tissue

The ontogeny of GP 130 was examined with rabbit and monoclonal GP 130 antibodies on immunoblots with whole brain homogenates from chicken embryos of different ages. The relative amounts of GP 130 in the developing chicken embryo brain as determined from densitometer scans of an immunoblot with mGP 130-3 are depicted graphically in Fig. 8. GP 130 was first detected at day 10 after fertilization and increased to ~50% of its adult level at embryonal day 13. From this point up to hatching, GP 130 was accumulated at considerably lower rates. With all GP 130 antibodies identical results were obtained, although, in some cases, GP 130 was detected as a faint band in brain homogenates 8 d after fertilization. The absolute amount of GP 130 in adult brain was estimated by comparison of densitometer scans of the Coomassie-stained 130-kD gel band in eMCF with known amounts of actin to give an estimate of the concentration of GP 130 in this sample. Densitometer scans of the radiolabeled GP 130 band in the same eMCF sample were then compared with the radiolabeled band from adult brain to give an estimate of the amount of GP 130 in the adult brain. According to these results, GP 130 comprises 0.2–0.3% of the total protein in adult chicken brain. As no correction was made for the relatively weaker staining of glycoproteins with Coo-

FIGURE 7 Expression of GP 130 in cultured sympathetic neurons. 5 μg of total protein from cultures grown for 1, 3, and 7 d and from 13-d-old embryo brain were analyzed on Western blots after incubation with a mixture of the six monoclonal antibodies followed by RAM and ^{125}I-Protein A. After 7 d in culture the sympathetic neurons contained approximately the same amount of GP 130 as a 13-d-old brain. (Lane *1*) 1-d-old culture. (Lane *2*) 3-d-old culture. (Lane *3*) 7-d-old culture. (Lane *4*) 13-d-embryo brain.

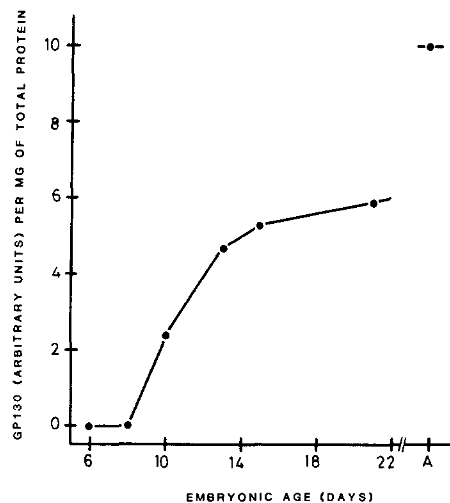
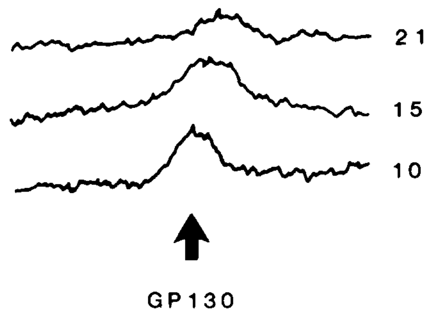


FIGURE 8 Increase of GP 130 content in developing chicken brain. 5 μg of whole brain homogenates from chicken embryos of various ages were analyzed on Western blots using mGP 130-3, RAM, and ^{125}I-Protein A. Densitometer scans of each track were obtained and plotted as shown. Various exposures of the autoradiographs were checked to ensure that all the bands were within the linear range. (*A*) Adult level.



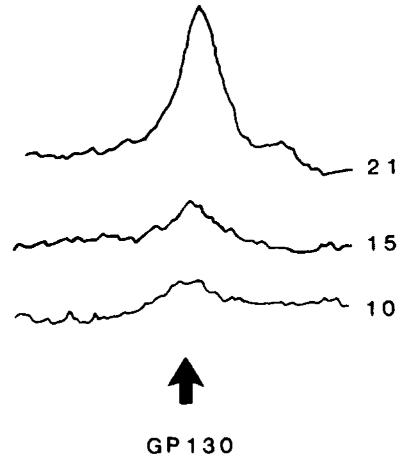


FIGURE 9 GP 130 content in developing sciatic nerve and sympathetic ganglia. 5 μg of total protein was analyzed on Western blots by incubation with the mixture of monoclonal antibodies against GP 130 followed by RAM and ^{125}I-Protein A. Densitometer scans of each track are displayed. Numbers represent days after fertilization.

massie Brilliant Blue, this is a minimum estimate.

Regarding the PNS, sciatic nerves from 10-, 15-, and 21-d-old chicken embryos were examined for their GP 130 content. As shown in Fig. 9, it is hard to detect significant differences in amounts of GP 130 in sciatic nerve taking into account experimental errors. If anything, amounts of GP 130 seem to be lower at day 21 than at day 10 or 15. In another part of the PNS, the sympathetic ganglia, GP 130 increased over the same developmental period, although, at hatching, the relative amount was still considerably lower than in brain tissue of animals of identical age.

Initial experiments were performed, in which brain and sciatic nerves from a 10-d-old chicken embryos were compared. The small differences in GP 130 content observed in these tissues could be accounted for by variation in the percentage neuronal membrane or by experimental error. However, the ontogeny of GP 130 suggested that there may be a different distribution of GP 130 in the adult nervous system. As demonstrated in Fig. 10, amounts of GP 130 varied widely between different regions of the nervous system. GP 130 was most abundant in the CNS, with cerebrum, cerebellum, and brain stem showing the highest amounts. White matter (dissected by inspection from the midbrain) and spinal cord contained less. Particularly striking were the very low levels of GP 130 in nerve bundles projecting to or from peripheral (sciatic nerve, brachial nerve) or central targets (optic nerve). This result was not due to an incomplete dissociation of these nerves, inasmuch as after treatment of nerve bundles with 1% collagenase 1 for 45 min, identically low amounts were detected on immunoblots. The relative amounts of GP 130 could not be quantitated by densitometry of the bands because the variation in intensity was not within the linear range of response of the photographic film. Indeed, a comparison of the three major brain parts with white matter and spinal cord was not possible for this reason.

DISCUSSION

Previous work has shown that GP 130 is a cytoskeleton-associated glycoprotein. It is tightly associated with actin in complexes containing approximately 20 major polypeptides. Actin cannot be extracted or dissociated without disrupting the complexes (10). Two pieces of evidence, presented in this paper, suggest that GP 130 is a single molecular species. First, the elution profile of the MCF after ion-exchange chromatography showed the 130-kD protein in a single peak as identified by staining with both Coomassie and [^{125}I]-Con A. Secondly, all six monoclonal antibodies and the rabbit antiserum that were raised against this 130-kD protein band had identical specificities for a neuronal 130-kD antigen which behaved in the same way on SDS-polyacrylamide gels and had a closely similar, if not identical, course of development. However, heterogeneity of GP 130, for example between different classes of neurons, cannot be ruled out at present.

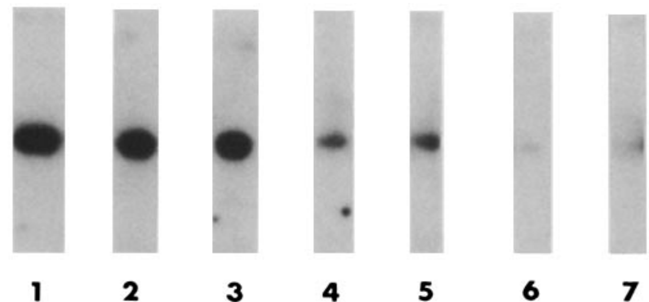


FIGURE 10 Content of GP 130 in different parts of the adult nervous system. 5 μg of total protein was analyzed on Western blots by incubation with the mixture of six monoclonal antibodies against GP 130 followed by RAM and ^{125}I-Protein A. (Lane *1*) Brain stem. (Lane *2*) Cerebellum. (Lane *3*) Cerebrum. (Lane *4*) White matter. (Lane *5*) Spinal cord. (Lane *6*) Optic nerve. (Lane *7*) Sciatic nerve.

The present data suggest that GP 130 is a protein unique to the nervous system. As judged by the coexpression with neurofilaments, GP 130 is located on neurons in cultures from both the CNS and PNS. In older cultures from the CNS, GP 130 is expressed on additional process-bearing cells that do not contain the 210-kD neurofilament polypeptide. This result is consistent with the observation that the 210-kD

component of neurofilaments is not found in all neurons (42) and that GP 130 is expressed on most neurons of the CNS and PNS. Antibodies against GP 130 do not stain other process-bearing cells such as oligodendrocytes and occasional microglia and, from the chosen culture conditions, it seems unlikely that fibrous astrocytes would be present in our CNS cultures. Flat cells expressing filamin or fibronectin are never recognized by GP 130 antibodies. In cultures from the PNS, these cells comprise the entire non-neuronal cell population (32) and, in CNS cultures, putatively fibroblastlike cells and protoplasmic astrocytes. These data strongly suggest that GP 130 is a specific marker for chicken neurons in culture. Comparison of the staining with intact and permeabilized neurons in culture, together with the evidence of trypsin treatment of cultures, show that GP 130 is located predominantly, if not exclusively, on the cell surface. No cross-reactivity of GP 130 antibodies with mammalian proteins was detected, although, in nervous tissue of a phylogenetically lower species, lizard, a protein of identical molecular weight was found to be recognized by GP 130 antibodies. At present it is not known whether different antigen determinants are recognized by the monoclonal and polyclonal antibodies.

Between 1 and 7 d in culture the neurons from sympathetic ganglia form an extensive network of axons including conspicuous axon bundles. GP 130 increases two- to threefold during this time. However, since GP 130 is located almost exclusively on the neuronal surface and the membrane-to-cytoplasmic ratio increases approximately twofold as axons are extended, the change in percentage GP 130 of total protein may be largely accounted for by the increased fraction of plasma membrane. It is difficult to measure a change in the concentration of GP 130 per unit area of membrane, but these results suggest that any change would be quite small.

Three parts of the nervous system were selected for a more detailed examination of the appearance of GP 130 during development. Brain showed a striking increase in GP 130 between days 10 and 13, whereas the content in sciatic nerve remained constant or even decreased during days 10 and 21 of embryonic development. An increase was observed in sympathetic neurons in vivo and in culture. This result indicates a different time course in the ontogeny of sciatic nerve, sympathetic ganglia, and brain. However, the final level of GP 130 reached in the adult chicken is quite distinct in different regions of the nervous system. GP 130 is most abundant in cerebrum, cerebellum, and brain stem, less in spinal cord and white matter, and present in comparatively small amounts in the PNS and optic nerve. As discussed in connection with the cultures, neuronal membrane will increase with respect to total protein during differentiation. This is particularly true in regions of the brain where neurons form extensive dendritic trees. Furthermore, the ratio of neuronal to non-neuronal plasma membrane varies within the different parts of the nervous system. However, because the difference in GP 130 content between, for example, the cerebellum and the sciatic nerve considerably exceeds an order of magnitude, these explanations alone may be insufficient. It is conceivable that GP 130 may be more concentrated per unit area of neuronal membrane in points of interneuronal contact such as dendritic spines and synapses. Unfortunately, there is as yet no specific neuronal membrane marker of uniform distribution with which to compare our results and thus clarify this issue.

Whatever the subcellular concentration and function of GP 130 in the nervous system, the antibodies should be valuable as markers for chicken neurons and as tools to study the development of connections between the neuronal cytoskeleton to molecules from the environment.

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