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Purification and Characterization of p68/70, Regeneration-Associated Proteins from Goldfish Brain

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Abstract: Two acidic proteins (p68/70) previously shown to be associated with regeneration of the goldfish optic nerve were purified 887-fold from brain homogenates of *Carassius auratus*. Purification to homogeneity was achieved by sequential chromatography of a 100,000 *g* brain supernatant fraction on DEAE-Sephacel, Cu^{2+}-charged iminodiacetic acid agarose, and gel filtration. The Stokes radius of the doublet was determined to be 5.8 nm, and the sedimentation coefficient calculated to be 5.2. From these values a molecular mass of 128 kDa and a frictional coefficient ratio of 1.6 were calculated. Chromatofocusing on a high-resolution DEAE column resolved the protein doublet into three dimeric species of p68, p68/70, and p70. These results indicate that the proteins are highly elongated and associate as homodimers or as a heterodimer. Subcellular localization and membrane extraction experiments indicated p68/70 to be a component of the plasma membrane associated primarily through hydrophobic interactions. p68/70 demonstrated biphasic behavior in phase partition experiments using Triton X-114. Analysis of hydrolytic products indicated p68/70 to be a glycoprotein, containing 11% carbohydrate. **Key Words:** Goldfish—Glycoprotein—Plasma membrane—Regeneration—Optic nerve.
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The goldfish visual projection provides a useful model in which to study nerve regeneration in the CNS. When the optic nerve is cut, the severed axons from the retinal ganglion cells (RGCs) grow out, reforming their contacts with the optic tectum (Sperry, 1963). Regeneration of RGC axons can be verified experimentally by morphological (Grafstein and McQuarrie, 1978) and biochemical changes (Agranoff et al., 1980), as well as by recovery of vision. Because optic nerve and other intrinsic CNS fibers of warm-blooded vertebrates do not share this ability to restore function, the details of the recovery process in both warm- and cold-blooded vertebrates have been investigated extensively.

Our laboratory has been studying two proteins, termed p68/70, that were found to be synthesized at elevated levels during regeneration of the goldfish optic nerve (Heacock and Agranoff, 1982). This doublet, purified from goldfish brain, is here termed p68/70. A polyclonal antibody to p68/70 has established immunoreactive material in all goldfish tissues tested, with highest levels found in brain, egg, and ovary (Wilmot et al., 1993). p68/70 is axonally transported with the slow component (Heacock and Agranoff, 1982; Perry et al., 1985). Immunocytochemical studies of retinal explants from postcrush fish (i.e., fish in which the optic nerve had been crushed, and undergoing regeneration) indicate p68/70 to be enriched in axonal varicosities and growth cones (Wilmot et al., 1993). The increased expression of this protein during regeneration and the intense immunolabeling observed in areas known to mediate cellular growth in the goldfish suggest p68/70 plays a role not only in axonal regrowth, but in neural development as well (Wilmot et al., 1993).

The development of a scheme to purify goldfish p68/70 to homogeneity from a soluble brain fraction has allowed us to further biochemically characterize these proteins. The results indicate p68/70 to be a highly elongated protein dimer associated with the plasma membrane, as well as with the cytosolic fraction of goldfish brain homogenate. In addition, p68/70 was found to be a glycoprotein, a finding that likely explains some of the previously observed properties of the doublet (Wilmot et al., 1993).

MATERIALS AND METHODS

Materials

TSK-3000SW and TSK-4000SW columns and HT-hydroxylapatite were obtained from Bio-Rad (Richmond, CA,

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Abbreviations used: IDA, iminodiacetic acid; LDH, lactate dehydrogenase; RGC, retinal ganglion cell; R_s, Stokes radius; $s_{20,w}$, sedimentation coefficient; SDH, succinate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

U.S.A.). The Mono P HR 5/20 column and Polybuffer 74 were the products of Pharmacia (Piscataway, NJ, U.S.A.). Goat anti-rabbit ^{125}I-IgG was purchased from ICN Biomedicals (Irvine, CA, U.S.A.). Goat anti-rabbit IgG horseradish peroxidase conjugate was obtained from GIBCO BRL (Gaithersburg, MD, U.S.A.). S & S NC nitrocellulose was purchased from Schleicher & Schuell (Keene, NH, U.S.A.). The DIG glycan differentiation kit and glycosidases were from Boehringer Mannheim Corp. (Indianapolis, IN, U.S.A.). Trifluoromethanesulfonic acid and anisole were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). DEAE-Sephacel, iminodiacetic acid (IDA) agarose, Sephadex G-50, protein standards, molecular weight markers and all other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Goldfish, 3–4 in. in length, were purchased from Grassy Forks Fisheries, Martinsville, IN, U.S.A. They were maintained at 25°C in 50-gal tanks before use.

Purification of p68/70 from goldfish brains

Buffers used were as follows: A, 20 m*M* HEPES (pH 7.0) containing 0.32 *M* sucrose, 10 m*M* EDTA, and 1 m*M* phenylmethylsulfonyl fluoride; B, 20 m*M* sodium acetate (pH 5.2) containing 1 m*M* EDTA; C, 20 m*M* sodium acetate (pH 4.2) containing 1 m*M* EDTA; D, 20 m*M* sodium acetate (pH 4.2) containing 100 m*M* NaCl and 1 m*M* EDTA; E, 20 m*M* Tris-HCl (pH 8.3) containing 100 m*M* NaCl; and F, 20 m*M* NaH_2PO_4 (pH 6.0) containing 200 m*M* NaCl, 0.1 m*M* EDTA, and 0.01% azide.

Brains were homogenized in 5 volumes (wt/vol) of ice-cold buffer A using 10 strokes of a Teflon-on-glass Dounce homogenizer. The remaining steps were performed at 4°C. The homogenate was centrifuged for 20 min at 10,000 *g*. The pellet was resuspended in 5 volumes of buffer A and recentrifuged. The combined supernatants were centrifuged for 60 min at 100,000 *g*. The high-speed supernatant was dialyzed against 4 L of buffer B, with one change. The sample was then centrifuged for 20 min at 38,000 *g*, and the supernatant was applied to a 2.2 × 10-cm column of DEAE-Sephacel, equilibrated in buffer B, at a flow rate of 2 ml/min. This and subsequent chromatographic procedures were performed using an LKB HPLC system (Pharmacia) equipped with a 2158 Uvicord SD detector and a 2220 recording integrator. For large-scale operations, a peristaltic pump was used to load samples. The column was washed with 2 volumes of buffer B, 2 volumes of buffer C, and then with buffer D until the main protein peak had eluted. p68/70 was detected in this and subsequent chromatographic separations using western analysis quantitated by means of a ^{125}I-labeled goat anti-rabbit antibody (Wilmot et al., 1993). Fractions (10 ml) containing p68/70 were combined and dialyzed against 4 L of buffer E for 2 days, with three changes. This sample was applied to a 2.2 × 15-cm column of IDA agarose charged to 80% capacity with cupric chloride (Cu^{2+}-IDA agarose column), at a flow rate of 0.5 ml/min. Ten-milliliter fractions were collected, and those enriched in p68/70 were combined (typically for a final volume of 100 ml), made 10 m*M* in EDTA by addition of 1/9 volume of 100 m*M* EDTA (pH 7.0), and concentrated to 1 ml using an Amicon concentrator equipped with a PM 10 membrane (Amicon Corp., Danvers, MA, U.S.A.). Aliquots of 100 μl were applied to an 8 × 300-mm TSK-3000SW column equilibrated in buffer F, at a flow rate of 0.5 ml/min. Fractions of five drops were collected. Tubes containing p68/70 were combined and stored at −70°C. Relatively large amounts of p68/70-like proteins could be purified from the eggs of carp (*Cyprinus carpio*) and goldfish by addition of a HT-hydroxylapatite column step after the Cu^{2+}-IDA agarose column.

The method used to purify essentially p68-free p70 was a modification of the above procedure in which the high-speed supernatant was brought to pH 5 with 1 *M* acetic acid, stirred at 4°C for 1.5 h, and then centrifuged at 10,000 *g* for 20 min. The procedure used to purify p68/70 was followed from this point.

Determination of Stokes radius (R_s)

A 7.5 × 300-mm Ultropac TSK-4000SW column was equilibrated in buffer F at 4°C, and 100-μl (200 μg of protein) samples were applied. The column was eluted at a flow rate of 0.5 ml/min. The void and total volume were determined using salmon sperm DNA and potassium dichromate, respectively. Standards used were bovine thyroglobulin (R_s = 8.6 nm), bovine gamma globulin (R_s = 5.1 nm), chicken ovalbumin (R_s = 2.8 nm), and horse myoglobulin (R_s = 1.9 nm). The Stokes radius of p68/70 was determined as described by Laurent and Killander (1964) from the linear plot of the Stokes radius versus $(-\log K_{av})^{1/2}$ (Siegel and Monty, 1966).

Determination of sedimentation coefficients ($s_{20,w}$)

Linear sucrose gradients (2–10%, wt/vol) were prepared in either H_2O or D_2O by means of a two-chamber gradient mixer. After the sample, in buffer F, was layered on top of the gradient, centrifugation was performed in an SW Ti40 rotor at 40,000 rpm for 14 h at 4°C. Fractions (0.5 ml) were collected by pumping buffer from the bottom of the tube, at a flow rate of 0.5 ml/min. Standard proteins were run together with p68/70. Samples of 50 μl were applied to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). The gel was then stained with Coomassie Brilliant Blue (G-250) and the density of the individual bands was determined using a microcomputer-assisted densitometer (Dell Computer Corp., Austin, TX, U.S.A.). The elution position for each protein from the gradient was determined from a plot of the band density versus tube number. For membrane-bound p68/70, plasma membrane proteins were solubilized in 1% β-octyl glucoside and applied to gradients containing 1% of the detergent. For membrane-bound p68/70, detection was by quantitative western analysis. Standards used were cytochrome *c* ($s_{20,w}$ = 1.9), ovalbumin ($s_{20,w}$ = 3.55), bovine serum albumin ($s_{20,w}$ = 4.6), and IgG ($s_{20,w}$ = 7.2). The sedimentation coefficient of p68/70 was determined as described by Martin and Ames (1961).

Calculation of the physical properties of p68/70

From the Stokes radius and sedimentation coefficient, the molecular weight of p68/70 in solution was calculated by the formula $M_r = R_s \times 4{,}240 \times s_{20,w}$ (Siegel and Monty, 1966). The frictional ratio was calculated from $f/f_0 = 1.393$ (R_s/M_r^{-3}), assuming a solvation factor of 0.2 g/g of protein and a partial specific volume of 0.735 cm^3/g (Sherman, 1975). The axial ratio was calculated, assuming the shape of a prolate ellipsoid, using the equation $F = f/f_{sph} = (1 - p^2)^{1/2}/p^{2/3} \ln\{[1 + (1 - p^2)^{1/2}]/p\}$ (Cantor and Schimmel, 1980).

Chromatofocusing

Chromatofocusing was performed by means of a Pharmacia Mono P HR 5/20 column. The column was equili-

brated in 25 m*M* methylpiperazine-HCl buffer (pH 5.7), containing 1 m*M* EDTA and 5 *M* urea. p68/70 in buffer F was made 5 *M* in urea and applied to the column, which was then washed with 5 ml of equilibration buffer, followed by Polybuffer 74 (pH 4.0), containing 1 m*M* EDTA and 5 *M* urea, at a flow rate of 0.5 ml/min. Fractions of five drops were collected and monitored by SDS-PAGE.

Membrane extraction

Synaptic plasma membranes were prepared as described by Sun et al. (1988) and stored at −70°C. Before use the thawed membranes were pelleted at 38,800 *g* for 20 min and resuspended at a concentration of 2 mg/ml of protein in 10 m*M* HEPES-HCl buffer (pH 7.0) containing 1 m*M* EDTA, 2 m*M* phenylmethylsulfonyl fluoride, and 20 m*M* leupeptin (control buffer). For extraction studies, 100 μl of this suspension was mixed with 100 μl of 2× extraction buffer. Final buffer concentrations used for extraction were as follows: 1 *M* NaCl; 1 *M* NH_2OH, pH 8.0; 5 *M* urea; 0.1 *M* Na_2CO_3, pH 11.0; and 1% Triton X-100. With the exception of the 0.1 *M* Na_2CO_3, all were in control buffer. After 30 min at 4°C, the samples were centrifuged at 100,000 *g* for 30 min. The supernatant was removed and made 400 μl with SDS-PAGE gel sample buffer. The pellet was resuspended in 200 μl of control buffer and 200 μl of SDS-PAGE gel sample buffer. p68/70 concentration was determined by quantitative western analysis. Levels of p68/70 were calculated as the percentage of distribution of p68/70 between the supernatant and the pellet, and as the total recovery (supernatant and pellet) for a given treatment versus the control.

Phase partition

The procedure of Bordier (1981) was followed. Both membrane-bound and soluble p68/70 were tested. Synaptic plasma membranes were resuspended in 20 m*M* Tris-HCl (pH 7.4) containing 100 m*M* NaCl, 0.01% azide, 1 m*M* EDTA, and 10 μ*M* leupeptin (TBS with inhibitors) to make 1 mg/ml of protein. A 20 μg/ml sample of p68/70, which had been purified from cytosol, was used to test soluble p68/70. The concentration of p68/70 was determined by quantitative western assay. The effects of phosphatidylethanolamine, phosphatidic acid, phosphatidylcholine, phosphatidylserine, and cholesterol on phase partitioning were tested by making the sample buffer 0.1% in the respective phospholipid or cholesterol. To test the effects of metal ions, the sample buffer was made both 5 m*M* in Mg^{2+} and Ca^{2+}.

Sequential phase partitioning was performed by a modification of the above procedure. Synaptic plasma membranes were resuspended in TBS with inhibitors to make the final protein concentration 1 mg/ml. This sample was made 1% (wt/vol) with respect to Triton X-114 and placed on ice for 5 min. Phase separation was effected by incubation at 30°C for 3 min. The phases were separated by centrifugation for 1 min in a microfuge at 5,000 rpm. A sucrose cushion was not used. The supernatant was removed and placed in an Eppendorf tube, and 55 μl of 10% Triton X-114 added. The pellet was resuspended in 0.5 ml of TBS with protease inhibitors, and 5 μl of 10% Triton X-114 was added. Both samples were placed on ice for 5 min, and the separation was repeated. This procedure was repeated six times, with the supernatant from each tube being transferred to the pellet of the next. The final samples were adjusted to contain identical amounts of detergent and buffer by addition of 55 μl of 10% Triton X-114 to tube 8 (the final supernatant), and 0.5 ml of TBS to tube 1 (the starting tube). Fractions of 50 μl were assayed by western analysis. For this experiment, a goat anti-rabbit IgG horseradish peroxidase conjugate was used in place of the goat anti-rabbit ^{125}I-IgG. Bands were visualized by incubation of the nitrocellulose sheet in 50 ml of reaction buffer, which consisted of 20 m*M* Tris-HCl (pH 7.4) containing 20% (vol/vol) methanol, 100 m*M* NaCl, 0.05% (vol/vol) of 30% hydrogen peroxide solution, and 0.06% (wt/vol) 4-chloro-1-naphthol.

Subcellular localization

The procedure of Sun et al. (1988) was adapted to prepare subcellular fractions from goldfish brain. Approximately 10 g of tissue (125 goldfish brains) was used to prepare the subcellular fractions, which were stored at 4°C and assayed within 24 h. Marker enzymes used were as follows: for microsomes, aryl esterase (Shephard and Hubscher, 1969); for plasma membrane, 5′-nucleotidase (Anner and Moosmayer, 1975); for mitochondria, succinate dehydrogenase (SDH) (Sottocasa et al., 1967); and for cytosol, lactate dehydrogenase (LDH) (Johnson and Whittaker, 1963). For p68/70 determinations, fractions were stored at −70°C, and the doublet was quantitated by western analysis. Results for each marker enzyme are plotted as the relative specific activity found in each subcellular fraction, with p68/70 reported as the relative specific concentration. In each instance, the relative specific activity is defined as the percentage of activity of a marker enzyme recovered in a fraction divided by the percentage of protein recovered in that fraction, whereas the relative specific concentration is the percentage of p68/70 by weight recovered in a fraction divided by the percentage of protein recovered in that fraction. The relative specific activity and concentration of the homogenate is defined as 1.0.

Preparation of membrane skeletons

Detergent-insoluble membrane skeletons were prepared from plasma membrane following the procedure of Moss (1983). Fractions were dialyzed overnight against 1 L of 10 m*M* Tris (pH 7.6), with 2 m*M* EDTA, 0.02% sodium azide, and analyzed by SDS-PAGE and western analysis.

Amino acid and carbohydrate analysis

Vapor hydrolysis (75 min in 6 *M* HCl) of proteins and preparation of the phenylisothiocyanate amino acid derivatives was performed on an ABI model 420H amino acid derivatizer. HPLC analysis of the derivatized amino acids was performed using an ABI model 130A microseparation system. No correction was made for losses due to the hydrolysis. For sialic acid determination, samples were hydrolyzed in 0.1 *M* HCl for 1 h at 80°C. For determination of all other monosaccharides, samples were hydrolyzed in 2 *M* trifluoroacetic acid for 5 h at 100°C. Aliquots were analyzed amperometrically on a Dionex BioLC instrument (Sunnyvale, CA, U.S.A.) using a Carbopac PA1 anion-exchange column. An isocratic gradient of 90% water/10% 200 m*M* sodium hydroxide was used to elute neutral and amino monosaccharides, whereas a gradient from 80%:10%:10% to 75%:10%:15% water/200 m*M* NaOH/1 *M* NaOH was used to elute sialic acid. Neutral sugars were also determined using the method of Hodge and Hofreiter (1962). The ratio of amino to neutral sugars, 0.65, was in reasonable agreement with that obtained by an alternative method, in which amino sugar content estimated from the amino acid analy-

sis (4.8%) was compared with neutral sugar content determined with phenol-sulfuric acid (6.08%), ratio 0.79.

Lectin binding

The affinity of different lectins for p68/70 was tested on Ouchterlony plates and the DIG glycan differentiation kit. For the immunodiffusion experiments, plates of 1% agar in 20 m*M* phosphate buffer (pH 7.0) containing 150 m*M* NaCl, and 10 μM $MgCl_2$ were used. The sample, containing 25 μg of p68/70 in 50 μl, was placed in the center of a seven-well plate, and the lectin solutions (0.5 mg/ml) were added to the peripheral wells. Plates were incubated at room temperature overnight. Lectins used (the gift of Dr. Irwin Goldstein) were from *Amaranthus caudatus*, which recognizes Gal(β1–3)-GalNac; *Galanthus nivalis*, which recognizes terminal mannose; *Maakia amurensis*, which recognizes sialic acid–linked α(2–3) to galactose; *Sambucus nigra*, which recognizes sialic acid–linked α(2–6) to galactose; *Ulex europaeus*, which recognizes α-L-fucose; and wheat germ, which recognizes GlcNac residues in β1–4 linkage. For the DIG glycan differentiation kit, 1 μg of p68/70 was applied to SDS-PAGE and electrotransferred to nitrocellulose. Digoxigenin-labeled lectins used recognize terminal mannose (*G. nivalis*), sialic acid–linked α(2–3) to galactose (*M. amurensis*), sialic acid–linked α(2–6) to galactose (*S. nigra*), Galβ(1–3)-GalNac (*A. caudatus*), and Galβ(1–3)-GlcNac (from *Datura stramonium*). Control proteins were tested for each lectin.

Periodate oxidation/borohydride reduction of p68/70

Lyophilized p68/70 (1 mg) was taken up in 1 ml of 100 m*M* sodium acetate buffer (pH 4.5), containing 25 m*M* sodium metaperiodate, and reacted in the dark for 24 h at room temperature. The sample was dialyzed against 100 m*M* boric acid (pH 8.0) in the dark overnight. Sodium borohydride was then added to make the sample 100 m*M*, and the reaction allowed to proceed for 24 h in the dark at 4°C. The reactivity of this sample and untreated p68/70 toward the affinity-purified anti-p68/70 IgG was determined by spotting 2 μl of a serial dilution of the doublet, made in 20 m*M* Tris-HCl (pH 7.4), containing 150 m*M* NaCl and 50 μg/ml of bovine serum albumin, onto nitrocellulose. Spots were visually compared with that of a progressive dilution series (1:2) of p68/70 samples that were not subjected to the above treatment. The western analysis in this experiment used a goat anti-rabbit IgG horseradish peroxidase conjugate. Blots were visualized by incubation in 50 ml of reaction buffer, which consisted of 20 m*M* Tris-HCl (pH 7.4), containing 20% (vol/vol) methanol, 100 m*M* NaCl, 0.05% (vol/vol) of 30% hydrogen peroxide solution, and 0.06% (wt/vol) 4-chloro-1-naphthol.

Miscellaneous techniques

Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Inorganic phosphate was determined using the method of Bartlett (1959). Trifluoromethanesulfonic acid treatment of p68/70 was performed as described by Edge et al. (1981).

RESULTS

Purification of p68/70

The results of the purification procedure for the doublet from goldfish brain are shown in Table 1. p68/70 was purified 887-fold, from which it can be extrapolated that the doublet constitutes 0.11% of total brain protein. The elution profiles for each of the chromatographic separations are shown in Fig. 1. Purification to homogeneity was achieved, as judged by SDS-PAGE (Fig. 2). The Cu^{2+}-IDA agarose column step resulted in the greatest purification, bringing p68/70 to near homogeneity. Two remaining contaminating proteins of apparent molecular masses of 28 and 26 kDa were then removed by TSK-3000SW chromatography.

Amino acid and carbohydrate analysis

The amino acid composition of p68/70 is shown in Table 2. Glutamate and proline values were high and the arginine value was low, compared with a table of typical amino acid compositions of proteins (Creighton, 1984). The inability of the affinity-purified anti-p68/70 antibody to recognize proteins outside of the Cyprinidae family (Wilmot et al., 1993), or to identify a positive clone in immunoscreening a goldfish retinal 10-day postcrush retinal λgt11 expression library (not shown), suggests that the antibody is directed against a carbohydrate substituent present in the isolated doublet. The ability of the affinity-purified anti-p68/70 IgG to bind to p68/70 was reduced by an average of 75% based on comparison of three independent western dilution series (see Materials and Methods) after periodate oxidation/borohydride reduction, a result that supports the hypothesis that carbohydrates contribute significantly to the antigenicity of the doublet. On the basis of comparison with authentic standards, the relative amounts of the following monosaccharides were identified as follows: fucose, 1.0; glucosamine, 1.0; galactose, 0.9; galactosamine, 0.4; and mannose, 0.3.

Physical properties of p68/70

Although p68/70 migrated with apparent molecular masses of 68–70 kDa during SDS-PAGE, the elution position of the doublet from gel filtration columns suggests that the native protein has a molecular mass of ~200 kDa. We therefore examined further the physical properties of undenatured p68/70. The Stokes radius of p68/70 was determined to be 5.8 nm (Fig. 3A), and the sedimentation coefficient was found to be 5.2 (Fig. 3B). We also determined the sedimentation coefficient to be 5.15 in D_2O (Fig. 3C), indicating that carbohydrate covalently linked to the protein did not significantly affect its density, and that the average partial specific volume for the standard proteins of 0.735 cm^3/g could be used for p68/70. From the Stokes radius and sedimentation coefficient the molecular mass in solution was found to be 128 kDa, indicating p68/70 is associated as a dimer, in agreement with the chromatofocusing results (see below). The frictional coefficient ratio was determined to be 1.6. The axial ratio was calculated assuming the shape of a prolate ellipsoid and found to be 12:1.

TABLE 1. *Purification of p68/70 from goldfish brains*

Sample	Protein (mg)	p68/70 (mg)[a]	Recovery (%)	Purification factor
Homogenate	6,198 ± 890	7.0 ± 1.5	100	1.0
High-speed supernatant	1,763 ± 354	1.4 ± 0.3	20.0	0.7
pH 5 supernatant	870 ± 96	1.2 ± 0.20	17.1	1.2
DEAE-Sephacel	154 ± 19	0.89 ± 0.10	12.7	5.1
Cu^{2+}-IDA agarose	2.6 ± 0.7	0.73 ± 0.12	10.4	248
TSK-3000SW	0.44 ± 0.09	0.44 ± 0.09	6.3	887

[a] Determined by quantitative western analysis, using the purified preparation (TSK-3000SW eluant) as a standard. Results are mean ± SEM values of three preparations of 1,000 goldfish brains.

Chromatofocusing

p68/70 exhibits slightly different isoelectric points for the p68 (5.0) and p70 (4.8) components of the doublet (Heacock and Agranoff, 1982). Therefore, we attempted to separate the two forms on the basis of charge using chromatofocusing. SDS-PAGE of the chromatofocusing column fractions is shown in Fig. 4. Three forms of p68/70 were found, homodimers of 68 and 70, and a heterodimer of 68/70. Elution order was consistent with the isoelectric points of the individual subunits, with the light homodimer eluting first, the heterodimer next, and the heavy homodimer last. Urea was present during the chromatography to prevent aggregation of p68/70. The chromatofocusing column buffer Polybuffer 74 proved difficult to remove and resulted in poor protein recoveries, precluding its use for preparative purposes.

Subcellular localization

During purification, a large amount of p68/70 was lost to the particulate fraction in the preparation of the high-speed supernatant fraction (Table 1). In addition, p68/70 was found to have sequence homology to a synaptic vesicle protein. We therefore further investigated the subcellular distribution of the doublet, as shown in Fig. 5. p68/70 was found to have a broad distribution pattern, and appeared to resemble most closely that of 5′-nucleotidase activity, a marker enzyme for plasma membranes ($r = 0.79$, $p < 0.05$), whereas displaying little correlation with the SDH activity ($r = 0.17$), the aryl esterase activity ($r = 0.76$, $p > 0.3$), or the LDH activity ($r = 0.48$), marker enzymes for mitochondria, microsomes, and cytosol, respectively. The plasma membrane fraction had the highest relative specific concentration for p68/70. Aryl esterase activity, a microsomal marker, was more enriched in the goldfish brain plasma membrane fraction than in the microsomal fraction. This unexpected distribution was confirmed by studies with NADPH-cytochrome *c* reductase (not shown), another marker enzyme for microsomes (Masters et al., 1967). It appears then, that when applied to fish brain, the method used results in a significant contamination of the plasma membrane fraction with microsomal proteins. The recoveries of the marker enzymes used were 96 ± 3% for LDH, 92 ± 2% for aryl esterase, 77 ± 9% for SDH, and 86 ± 5% for 5′-nucleotidase. The recovery of p68/70 was 60 ± 7%. Total protein recovery was 97 ± 5%.

Membrane extraction

Membranes were treated with agents known to disrupt specific types of protein-membrane interactions, to investigate the nature of the membrane association observed for p68/70. Results of these studies are shown in Table 3. Treatment with 1 *M* NaCl did not extract p68/70 from the membrane fraction, a result militating against association via electrostatic interactions. p68/70 was not extracted by 1 *M* neutral hydroxylamine, a treatment that would cleave the thioester bond formed between fatty acids and cysteine residues. p68/70 was partially extracted from the membrane by high pH, a treatment that cleaves fatty acid–protein oxyester bonds, disrupts the lipid bilayer of the membrane, and perturbs protein–protein interactions. Urea (5 *M*) treatment, which also should disrupt protein–protein interactions, was found to only partially extract p68/70 from the membrane. Because the sedimentation coefficient of membrane-bound p68/70 was found to be identical to that of the soluble form of p68/70, a complex between p68/70 and an integral membrane protein did not appear likely to explain the membrane association. We further investigated the nature of the membrane association of p68/70 by analyzing for the presence of the doublet in the plasma membrane skeleton (Moss, 1983). SDS-PAGE and western analysis of the membrane skeleton preparation indicated p68/70 to be present mainly in the soluble fraction, whereas the membrane skeleton is found at the 10–30% interface under these experimental conditions. p68/70 was totally extracted from the membrane by Triton X-100. Thus, whereas a portion of p68/70 can be extracted from the membrane with urea or high pH treatment, the doublet appears to be associated with the membrane primarily through hydrophobic interactions, presumably reflecting a substantial stretch of hydrophobic amino acids detected in its primary sequence (Leski et al., 1991).

Phase partition

Because the membrane extraction experiments indicated p68/70 to be associated with the membrane

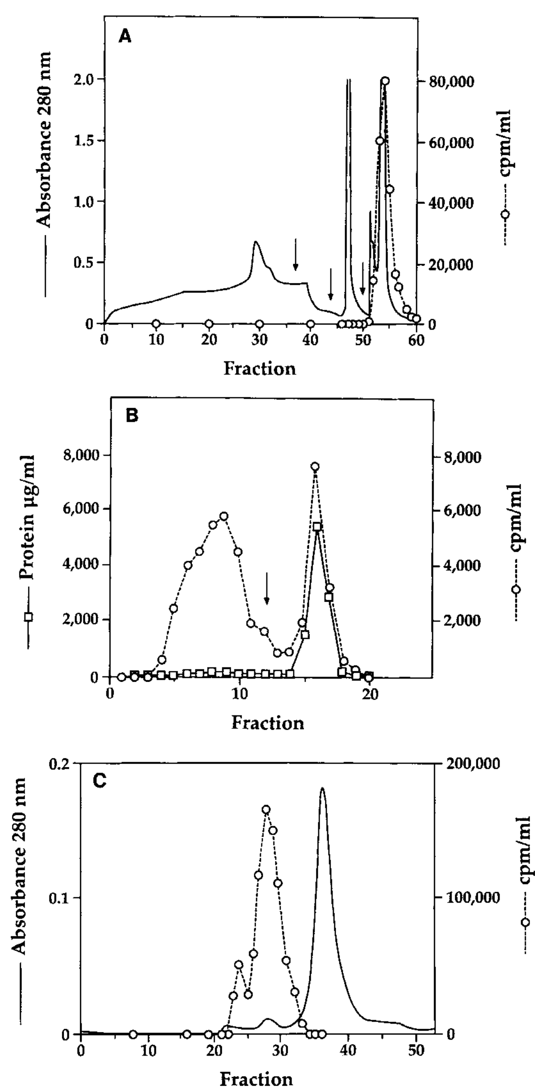

FIG. 1. Purification of p68/70 from goldfish brain. **A:** DEAE-Sephacel chromatography. Arrows (left to right) indicate buffer changes to buffers A, B, and C, respectively. **B:** Copper affinity chromatography. p68/70 was found largely in the flow-through. The arrow indicates a change in buffer to 0.1 *M* EDTA, pH 7.0. **C:** TSK-3000 chromatography. p68/70 eluted primarily in tubes 28–31. The initial minor peak of radioactivity probably contains aggregated p68/70. p68/70 was detected using a western blot assay and plotted as specifically bound cpm of ^{125}I-GAR IgG bound per milliliter.

through hydrophobic interactions, phase partition experiments were performed to further characterize the membrane association. This experiment takes advantage of the low cloud point of Triton X-114, which is soluble at 4°C but forms detergent micelles at 30°C, which can then be separated from the aqueous phase by centrifugation. Hydrophilic proteins partition into the aqueous phase, whereas hydrophobic proteins insert into the micelles and partition with the detergent phase (Bordier, 1981). Soluble p68/70 partitioned almost entirely (>97%) into the aqueous phase. However, membrane-bound p68/70 exhibited biphasic properties, with 23% partitioning into the detergent phase and 77% into the aqueous phase. No difference was observed for either form when phase partitioning was performed in the presence of phospholipids,

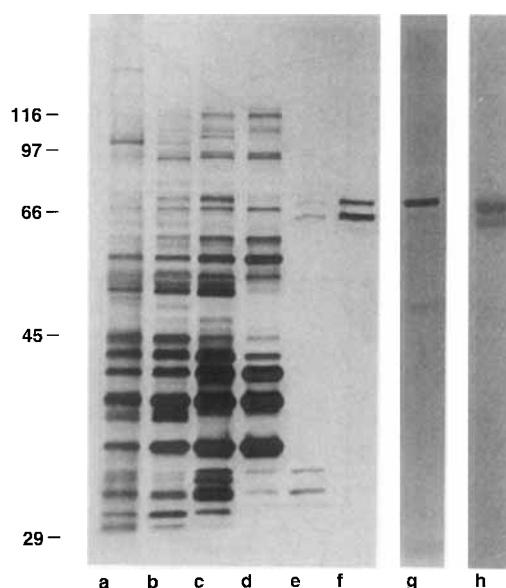

FIG. 2. SDS-PAGE separation of p68/70. Shown are p68/70-containing fractions for the brain preparation (a–f), purified brain p70 (g), and carp egg p68/70-like proteins (h). For (a–d) 10 µg of protein was applied to the gel, for (e–h) 2 µg of protein was applied. Molecular masses (kDa) are indicated on the left. Lane assignments: (a) homogenate; (b) high-speed supernatant; (c) pH 5.2 supernatant; (d) DEAE-Sephacel; (e) Cu^{2+}-IDA agarose column; (f) TSK-3000; (g) brain p70; (h) carp egg p68/70-like proteins. Staining was by silver, except for lane h, which was by Coomassie Brilliant Blue.

TABLE 2. *Amino acid composition of p68/70*

Amino acid	% (mol)
Alanine	8.9
Arginine	2.6
Aspartic acid	8.5
Cysteine	ND
Glutamic acid	20.5
Glycine	6.7
Histidine	0.8
Isoleucine	2.0
Leucine	7.7
Lysine	6.3
Methionine	0.6
Phenylalanine	2.9
Proline	9.4
Serine	5.7
Threonine	7.3
Tryptophan	ND
Tyrosine	2.0
Valine	5.9

ND, not determined.

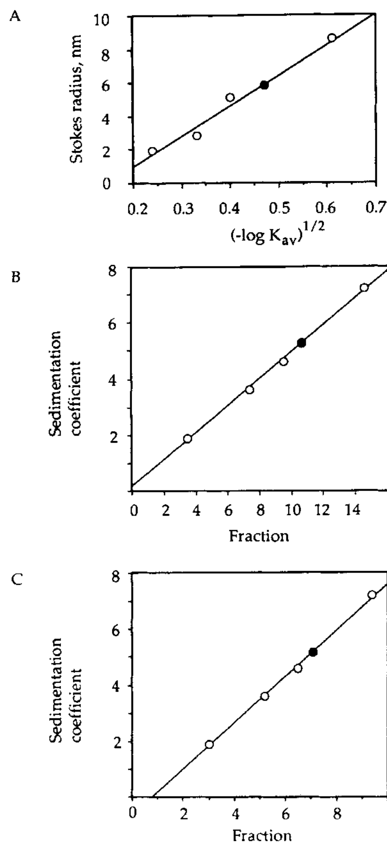

FIG. 3. Determination of the Stokes radius and sedimentation coefficient of p68/70. **A:** The plot used to determine the Stokes radius. A Stokes radius of 5.8 nm was found for p68/70. For sedimentation coefficient determinations, 2–10% gradients of sucrose in buffer F in either H_2O (**B**) or D_2O (**C**) were used. Detergent-solubilized membrane-bound p68/70 was found to have a sedimentation coefficient of 5.2 in H_2O (not shown). Filled circles, p68/70; open circles, standard proteins (see text).

metal ions, or EDTA. Inferences from these experiments of distinct forms of p68/70 were not supported by a sequential phase partitioning experiment. Reduction of micelle rigidity by the addition of linoleic acid (Maher and Singer, 1985) had no effect on the distribution of the doublet.

Lectin binding

The nature of the glycoprotein was further investigated by the use of lectins. Wheat germ agglutinin was found to react very strongly with p68/70 (Fig. 6), which indicated the presence of β-D-GlcNac(1–4)-β-D-GlcNac in the protein. Treatment of p68/70 with trifluoromethanesulfonic acid to remove covalently bound carbohydrates eliminated this reactivity. Lectins that recognize the N-linkage site or the core disaccharide of O-linked glycoprotein, fucose, terminal mannose, or sialic acid were found not to precipitate p68/70. No reactivity for digoxigenin-labeled lectins toward p68/70 was observed.

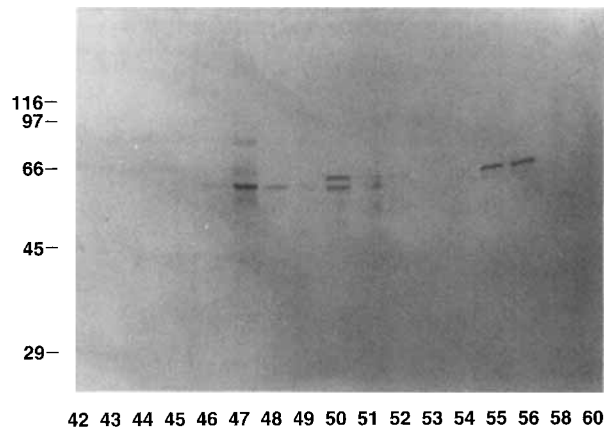

FIG. 4. SDS-PAGE of chromatofocusing fractions. Approximately 100 μg of p68/70 was applied to the column. Samples of 50 μl were analyzed by SDS-PAGE. The doublet was resolved into three dimeric forms (p68, p68/70, and p70), which eluted from the column as predicted from the isoelectric points of the components. p68 homodimer eluted in tubes 47 and 48, p68/70 heterodimer eluted in tubes 50 and 51, and p70 homodimer eluted in tubes 55 and 56. Molecular masses (kDa) are indicated on the left. Fraction numbers are shown at the bottom of the figure. The gel was stained with silver.

DISCUSSION

The nerve regeneration-associated proteins p68/70, initially identified on the basis of their in vivo labeling in the goldfish visual system (Heacock and Agranoff, 1982), were recently further characterized in regard to their tissue distribution via immunohistochemistry (Wilmot et al., 1993), and in the present study have been purified to homogeneity from goldfish brain homogenates. The purification of p68/70 to homogeneity is largely attributable to the removal of contaminating proteins by means of a Cu^{2+}-IDA agarose column step. Separations using metal chelate affinity chroma-

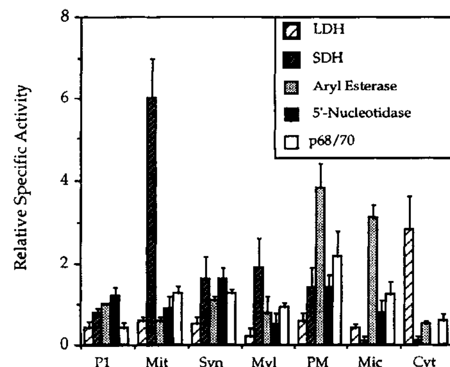

FIG. 5. Subcellular localization of p68/70. The location of p68/70 was determined by comparing the relative specific activities of marker enzymes for specific organelles versus the relative specific concentration of p68/70 in each fraction, prepared as described in the text. The means and standard deviations represent the average of three determinations.

TABLE 3. *Extraction of p68/70 from membranes*

Sample	Supernatant		Pellet		Recovery	
	ng	%	ng	%	ng	%
Control	11.0 ± 3.2	6.2	167 ± 6.8	93.8	178 ± 6	100
1 *M* NaCl	12.9 ± 3.3	6.5	186 ± 6.1	93.5	199 ± 5	111
1 *M* NH_2OH	14.2 ± 5.7	7.5	173 ± 4.9	92.5	187 ± 11	105
5 *M* urea	73.3 ± 34.4	34.3	140 ± 7.1	65.7	213 ± 30	120
0.1 *M* Na_2CO_3	80.0 ± 13.3	51.0	76.7 ± 14	49.0	157 ± 8.4	88.2
1% Triton X-100	149 ± 24.2	100	0	0	149 ± 24	83.7

Results are mean ± SEM values of three experiments.

tography take advantage of the complexes formed between histidine and cysteine side chains with metal ions, particularly copper and zinc, in aqueous solutions (Porath et al., 1975). For example, Cu^{2+}-IDA agarose columns have been used to purify a histidine-free protein, the adipocyte lipid binding protein, on the basis of its inability to bind to the column (Xu et al., 1991). The doublet proteins' inability to bind to the Cu^{2+}-IDA agarose column may reflect the somewhat low histidine content of p68/70, or unavailability of its histidine residues to the column matrix. Other factors such as carbohydrate substituents might interfere with binding of the doublet to the column. The elongated nature of p68/70 may be explained to some degree by its high proline and/or its high glutamic acid content (Table 2, presumed to be mainly glutamate on the basis of preliminary sequence data).

Although previous investigations emphasized the study of cytosolic p68/70, the subcellular distribution experiment, using the western assay, demonstrated a substantial fraction to be particulate. The membrane association studies indicate that p68/70 is bound to membrane via hydrophobic interactions, whereas the phase partition experiments suggest the doublet is hydrophilic. Explanations offered for such anomalous behavior include the incompatibility of the membrane-spanning region of a protein with the rigid detergent micelles formed by Triton X-114 (Maher and Singer, 1985), the existence of distinct forms of proteins (Swanson et al., 1988), and the presence of hydrophilic substituents on proteins such as carbohydrate chains (Volk and Geiger, 1986). Although we were able to rule out the first two, the possibility that the carbohydrate residues of p68/70 prevent the solubilized doublet from associating with the Triton X-114 micelles in phase partitioning experiments could not be tested, as attempts to denude p68/70 of carbohydrate with glycosidases were not successful.

FIG. 6. Ouchterlony analysis of lectin binding. The peripheral wells contained lectins that recognize (1) β-D-GlcNac(1–4)-β-D-GlcNac (wheat germ); (2) sialic acid-Gal(β1–3)-*N*-acetyl GalNac and Gal(β1–3)-GalNac (*A. caudatus*); (3) terminal mannose (*G. nivalis*); (4) sialic acid (α2–3)-Gal (*M. amurensis*); (5) sialic acid (α2–6)-Gal or sialic acid (α2–6)-GalNac (*S. nigra*); and (6) fucose (*U. europaeus*). The center well contained p68/70.

The cellular function of the doublet is unknown. A 13-residue CNBr fragment of p68/70 (Leski et al., 1991) was found to have identity with a sequence (M_{172}–L_{184}) in a membrane-spanning region of VAT-1, a synaptic vesicle protein of *Torpedo* (Linial et al., 1989). Although VAT-1 and p68/70 are both derived from teleost nerve tissue, they do not share similar properties, on the basis of the present and of previous studies (Wilmot et al., 1993). The present finding that p68/70 contains carbohydrate may explain a number of previous observations. Whereas the doublet was named on the basis of its apparent molecular mass on 10% SDS-PAGE, its migration has been found to be anomalous (Wilmot, 1993), probably a reflection of its carbohydrate content (Segrest et al., 1971). The molecular mass of the doublet determined by Ferguson analysis is 53/55 kDa (Wilmot et al., 1993). The predicted molecular masses of the protein portion of the glycoconjugate, based on those molecular masses and an 11% carbohydrate content, would then be 47/49 kDa. A final assignment awaits complete sequencing. p68/70 has not been detected in two-dimensional electrophoretic analysis of in vitro translation products from mRNA isolated from the regenerating goldfish retina (Cauley et al., 1986; Tesser et al., 1986). As these proteins would not be glycosylated, p68/70 apoprotein would migrate at a lower molecular mass. The carbohydrate content may also explain the observed restricted phyletic range of the polyclonal antibody to the Cyprinidae family (Wilmot et al., 1993). Indications that the immunogenicity of the doublet resides largely in the carbohydrate moiety come from the finding that periodate oxidation followed by borohy-

dride reduction of p68/70 greatly reduces the reaction to the anti-p68/70 antibody. The affinity purification step may have selected for a subpopulation of antibodies in the rabbit antisera that recognizes predominantly the glycan portion of p68/70.

Whether the membrane-bound p68/70 is transported axonally at the rate reported for the cytosolic component has not been fully investigated, although evidence indicating the presence of the doublet in the particulate fraction for the slow component has been reported (Heacock and Agranoff, 1982). It has been suggested that an exchange of a larger soluble nonradiolabeled pool with the smaller radiolabeled membrane-bound state could dilute the radiolabeled form during transport, making it appear to be transported in the slow pool (Wilmot et al., 1993). This may be the case, as p68/70 is found in both the soluble and particulate fractions in goldfish brain homogenates. Another possibility would have p68/70 exchanging with a nonradioactive membrane pool. In retinal explants, p68/70 is found enriched in axonal varicosities (Wilmot et al., 1993). The predominant structural inclusion of these varicosities is a tubular smooth endoplasmic reticulum that is organized in an anastomosing, glomerulus-like arrangement (Koenig et al., 1985). These varicosities are also observed in the regenerating optic nerve in vivo (Murray, 1976). If, in transport to the growth cone, p68/70-containing vesicles were to associate with these varicosities, an exchange of the radiolabeled protein with a larger nonradioactive pool could occur.

Because p68/70 appears to be a component of growing cell types rather than a specific indicator of regeneration in the goldfish (Wilmot et al., 1993), evidence presented in this study suggests that the doublet plays a role in membrane-related events in neuronal cell growth and regrowth. Further understanding of its role will be required to clarify whether p68/70 provides a clue to the ability of cold-blooded vertebrates to regenerate their CNSs.

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