IDENTIFICATION AND CHARACTERIZATION OF GLIAL GROWTH FACTOR

Thesis by

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To

my mother

and

my father

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ABSTRACT

A combination of biochemical, cell biological and immunological techniques have been employed to identify a novel and potent polypeptide mitogen of the brain and pituitary. This molecule, named glial growth factor (GGF), stimulates DNA synthesis and cell division in cultured rat Schwann cells, astrocytes, and fibroblasts.

Three independent lines of evidence indicate that GGF activity resides in a basic protein of molecular weight 3.1×10^4. (a) When partially purified preparations are analyzed by native gel electrophoresis at pH 4.5, mitogenic activity migrates with a protein of this molecular weight, as revealed by bioassay coupled with a second dimension of SDS gel electrophoresis. (b) A set of monoclonal antibodies which deplete growth factor activity from heterogeneous solutions specifically recognize a 31,000 dalton protein antigen, as determined by gel immunoautoradiography. (c) GGF activity is recovered at a molecular weight of 3.1×10^4 after denaturing polyacrylamide gel electrophoresis in SDS.

Three large-scale purifications of GGF, employing a combination of column chromatography steps and preparative electrophoreses, are described. The molecule has been purified to apparent homogeneity from anterior lobes of the bovine pituitary.

Through the use of nucleic acid precursor incorporation assays, GGF has been shown to be markedly mitogenic for rat Schwann cells, astrocytes and fibroblasts, but inactive when assayed on oligodendrocytes or microglia. Electrophoretic analyses suggest that all responsive cell types are stimulated by a single (the same) molecular species. GGF is the only defined mitogen to which rat Schwann cells respond.

Glial growth factor from bovine brain has been found to be indistinguishable from bovine pituitary GGF, as determined by biochemical, immunological and bioactivity criteria. GGF is non-uniformly distributed among bovine brain regions. It is present in brain extracts prepared from a wide variety of vertebrate species.

Purified human platelet-derived growth factor (PDGF) shares many important properties with GGF. PDGF has been shown to be unable to significantly stimulate the division of rat Schwann cells, however, and therefore appears to be distinct.

Observations made in vitro suggest several possible biological roles for GGF in vivo. These are discussed.

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INTRODUCTION

The control of cell division is among the most important aspects of cellular and organismal physiology. This control is exercised through the action of both positive and negative agents and is imposed at critical moments during development, in the maintenance and operation of differentiated cells and organ systems, and in response to tissue injury or trauma. The work described in this thesis deals with one kind of positive control element, namely, a soluble protein which specifically signals its target cells to divide.

In contrast to several such molecules described by other investigators, this protein acts principally on a set of highly differentiated, normally quiescent cells: glial cells of the central and peripheral nervous systems. It is a mitogen for only some of these cells, however, being apparently without effect on others. Among its recognized targets are Schwann cells, which arise from the embryonic neural crest and are distributed throughout the vertebrate peripheral nervous system (PNS) (Weston, 1970). These cells serve to produce myelin, a multilayered and compacted wrapping of the extended plasma membrane which allows for rapid impulse conduction along axons (Bunge, 1968). It is also a mitogen for astrocytes of the central nervous system (CNS), which derive from the ventricular zone of the developing brain and spinal cord and which are thought to fulfill several functions, including partial maintenance of the blood-brain barrier (Landis and Reese, 1981) and homeostatic regulation of extracellular potassium levels (Gardner-Medwin, 1981). Non-responsive glia include oligodendrocytes, which appear in the developing neural tube somewhat later than astrocytes and which serve to myelinate axons in the CNS (Webster et al., 1981).

Unlike neurons, many glial cells normally retain the ability to divide. The control of their division is an important component of several differentiation events,

including myelination (Webster et al., 1973), and has been the subject of extensive investigation.

Glial cell proliferation

Studies in vivo. The study of glial cell division in vivo in both the peripheral and central nervous systems has a long history. Anatomical studies of the behavior of vertebrate CNS glia, for example, extend from Virchow's (1859) observation of the "neuroglial" cell division associated with certain degenerative disorders, to recent autoradiographic analyses of the genesis and proliferation of radial glia in the developing monkey telencephalon (Schmechel and Rakic, 1979). At present, our understanding of the mechanisms regulating Schwann cell division in the PNS is more extensive than that for central glial cells. In part this reflects the advantages of analyzing a single cell type in the relatively accessible confines of peripheral nerve (rather than mixed populations of glia present in the CNS). It also reflects the development of culture systems for obtaining purified populations of Schwann cells and neurons.

In the developing PNS, Schwann cells are more obviously associated with axons while they are dividing. A variety of experimental manipulations have demonstrated that this reflects a neural stimulus, in that a full complement of axons is required to generate the normal number of Schwann cells. If, for example, the number of neurons in the superior cervical ganglion is reduced by neonatal administration of antiserum to nerve growth factor, preganglionic axons and Schwann cells are each reduced to nearly one-third of their normal number (Aguayo et al., 1976). This axonal influence is believed to reflect, at least in part, a contact-dependent mitotic signal that is delivered to the Schwann cell. In vitro experiments have provided direct evidence for such a signal associated with the neuritic surface of cultured neurons (see below).

During development, Schwann cells come out of division with kinetics that have been studied in the 2-day-old mouse sciatic nerve (Asbury, 1967). At this age, about

27% of the cells are proliferating and approximately 25% of this population appeared to cease division in the cell cycle under study. In the adult mouse Schwann cells are essentially quiescent, with 0.1% or less of the nuclei being labeled after injection of tritiated thymidine (Bradley and Asbury, 1970). This labeling index increases dramatically ($\geq$ 20-fold) after various lesionings of myelinated fibers, such as transection of the sciatic nerve (Bradley and Asbury, 1970; Abercrombie and Santler, 1957) or crush injury of the cervical sympathetic trunk (Romine et al., 1976). Experiments have shown that this induced proliferation is intense only near the site of the lesion and declines rapidly outside this zone (Romine et al., 1976). The marked division of Schwann cells after injury is clearly an important factor in their ability to remyelinate or re-enfold regenerating axons.

Although there have been extensive studies of glial proliferation after injury in the CNS, the literature is a confusing one. This confusion reflects the difficulty of unequivocally identifying the cell types involved. Although there is general agreement that microglia divide, it remains unclear whether these cells are wholly or in part derived from invasive cells of hematogenous origin (see, for example, Skoff and Vaughn, 1971; Adrian and Williams, 1973; Gall et al., 1979; Ting et al., 1981). There are relatively few reports of oligodendrocyte division after needle or stab wounds. Astrocytes have been unequivocally demonstrated to divide after lesioning (Shultz and Pease, 1959; Cavanagh, 1970; Latov et al., 1979), although hypertrophy is sometimes additionally cited as an important response (Vaughn, Hinds and Skoff, 1970). The rather widespread division that attends injury in the CNS is often suggested as indicating a mitogenic signal diffusing from the region of injury, although we do not have a detailed understanding of this proliferation or of the factors that provoke it.

Studies in vitro. Experiments in cell and tissue culture have provided information about cell-mediated mechanisms controlling proliferation in the periph-

eral nervous system, and offer the best prospect for assaying and hence purifying the molecules that underlie these events. One body of work has provided clear evidence for the existence of a Schwann cell mitogen on the surface of cultured peripheral sensory (Wood and Bunge, 1975; Wood, 1976; Salzer and Bunge, 1980; Salzer et al., 1980a; Salzer et al., 1980b) and sympathetic (McCarthy and Partlow, 1976a,b; Hanson and Partlow, 1980) neurons. These analyses have depended on the ability to obtain purified population of neurons and of non-neuronal (Schwann and satellite) cells through the use of antimitotic agents (Wood, 1976) or selective adhesion (McCarthy and Partlow, 1976a). When the purified populations confront each other in co-culture it is possible to demonstrate, either by autoradiography (Wood and Bunge, 1975) or by thymidine incorporation (McCarthy and Partlow, 1976b), a stimulation of proliferation in the non-neuronal population. A variety of criteria indicate that this stimulation is dependent on cell contact. If, for example, rat sensory neurites and rat Schwann cells are separated by a 6 μm thick collagen diaphragm, Schwann cell proliferation is prevented (Salzer et al., 1980b). Furthermore, an isolated membrane fraction from neurites, but not a soluble fraction, is competent to stimulate the Schwann cells (Salzer et al., 1980a); the addition of membrane preparations from other cell types is not mitogenic. An activity similar to the neurite mitogen has recently been demonstrated in an axolemma preparation from bovine brain (De Vries et al., 1982). (An evaluation of the possible relation of these membrane-associated activities to the soluble factor described in this thesis awaits a physical and chemical characterization of the former.)

Preliminary experiments have also been reported describing mitogenic activities for some CNS glial cells maintained in vitro. Several investigators, for example, have described activities present in high-speed supernatants of brain extracts which initiate DNA synthesis in cultured rat astrocytes (Kato et al., 1981; Pettman et al., 1980; Morrison et al., 1982). For the most part, these activities are not yet well-

characterized biochemically. One example is the glial maturation factor (GMF) described by Lim and his colleagues, which triggers one or two rounds of cell division and promotes the subsequent differentiation (i.e., glial fibrillary acidic protein expression and process extension) of these cells (Lim, 1980). Bovine GMF has been reported to be a relatively acidic, low molecular weight ($=2.0-2.4 \times 10^4$) protein, although similar activities isolated from other species show quite different biochemical properties (Kato et al., 1981; Lim, 1980).

Characterization of growth factors in vitro

The experiments described in this thesis are distinct from those mentioned above and are more closely related to the general study of growth control in cultured cells. It is generally accepted that the growth and division of animal cells in culture require the presence of growth factors and hormones, which are often found in serum. For certain cell types it has been possible to replace the general serum requirement with an appropriate mixture of purified factors (Bottenstein et al., 1979), and it has been suggested that the definition of such factors may lead to the identification of novel molecules of biological significance (Ross and Sato, 1979). In view of their low level in serum, it has generally been easier to seek a tissue source of these components for purification. In one such investigation, tissue extracts were screened for their ability to initiate DNA synthesis and cell division in mouse 3T3 fibroblasts which were arrested by culturing in the presence of low concentrations of serum (Gospodarowicz, 1974). The brain and pituitary were the only tissues which yielded extracts of significant mitogenic activity under these conditions. This effect on 3T3 cells has been adapted as an assay and used to guide the purification of a fibroblast growth factor (FGF) from the pituitary (Gospodarowicz, 1975) and the brain (Gospodarowicz et al., 1978). These molecules have been described as basic polypeptides of molecular weight 13,000, although the structure of brain FGF and, in particular, its possible relation to myelin basic protein are disputed (Westall et al.,

1978; Thomas et al., 1980; Lemmon et al., 1982). It should be noted that the mitogenic effect of nervous tissue extracts on cultured cells has been recognized for quite some time (Trowell, Chin, and Willmer, 1938; Hoffman, 1940), and that recent studies in vitro have demonstrated the presence of a fibroblast mitogen in sonicates and homogenates of cultured neurons (Hanson and Partlow, 1980).

Other investigators have also used the induction of DNA synthesis in quiescent 3T3 cells to identify and purify a potent growth factor from platelet lysates, which is thought to be the principal mitogen in serum (Ross et al., 1974). The platelet-derived growth factor (PDGF), a basic protein of molecular weight 31,000, has been purified to homogeneity (Heldin et al., 1981) and its biochemical properties (Raines and Ross, 1982) and effects on target fibroblasts (Glenn et al., 1982) are now being characterized.

The list of trophic and/or mitogenic molecules whose effects have been analyzed in vitro is by now very long indeed. It includes "classical" agents such as insulin (Bradshaw and Niall, 1978) and epidermal growth factor (Carpenter and Cohen, 1978), as well as more recently described molecules, such as the insulin-like growth factors (Rubin et al., 1982; Hasselbacher and Humbull, 1982) and transforming growth factors (Roberts et al., 1981). The increasingly large number and small range of cellular targets of these identified molecules suggest that cell division is a very carefully regulated phenomenon (see Conclusion). In most instances, however, the role that this growth factor-mediated regulation plays in the development and integrative functioning of higher vertebrates is not known. This is due in large part to the fact that studies of the action of a given agent in vitro are usually far more straightforward than identification of specific physiological effects in vivo. Such effects often point to important aspects of the biology of bioactive molecules, however. The physiological roles that FGF and PDGF play in vivo, for example, remain the subject of speculation, while the critical influence that Nerve Growth

Factor exercises upon the development of vertebrate sympathetic ganglia was first recognized—on the basis of an in vivo experiment—over twenty years ago (Levi-Montalcini and Cohen, 1960).

Glial Growth Factor

This thesis reports work on the identification, purification and characterization of glial growth factor (GGF), a novel and potent polypeptide growth factor/hormone, restricted to neural tissue, which triggers DNA synthesis and cell division in cultured rat Schwann cells, astrocytes and fibroblasts. The definition of this molecule is largely a result of the ability to obtain highly purified cultures of rat Schwann cells and of the exploitation of these cultures in a quantitative proliferation bioassay. As such, this work constitutes a validation of the in vitro approach to the elucidation of new bioactive molecules suggested by Ross and Sato (1979). The experiments described herein establish GGF's biochemical identity, many of its physical and chemical properties, some of its cellular targets, and some of the consequences of its interaction with those targets. They also suggest several possibilities as to roles which GGF might fulfill in vivo. An evaluation of these possibilities is an important avenue of future investigation.

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CHAPTER 1

PURIFICATION AND PRELIMINARY CHARACTERIZATION OF A GLIAL GROWTH FACTOR FROM THE BOVINE PITUITARY

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Purification and Preliminary Characterization of a Glial Growth Factor from the Bovine Pituitary*

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In a normal tissue culture medium containing 10% fetal calf serum, purified rat Schwann cells divide very slowly. We have previously reported that the cells are stimulated to divide by an activity present in extracts of the brain and pituitary (Brockes, J. P., Fields, K. L. and Raff, M. C. (1979) *Brain Res.* 165, 105-118), and this activity appears to be both novel and restricted in its distribution (Raff, M. C., Abney, E. R., Brockes, J. P. and Hornby-Smith, A. (1978) *Cell* 15, 813-822). The pituitary activity has been purified over 4000-fold from a pool of 10 kg of frozen glands and 4000 lyophilized anterior lobes. The activity was assayed by the incorporation of ^{125}I-UdR into DNA of Schwann cells growing in microwells. The most purified (phosphocellulose) fraction was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and displayed a major band of 3×10^4 molecular weight which was approximately 25% of the stained material. When analyzed by native gel electrophoresis at pH 4.5 followed by a second dimension of sodium dodecyl sulfate-gel electrophoresis, the activity was consistently associated with this component.

The effect of the phosphocellulose fraction on proliferation of central glial cells in dissociated cultures of the rat corpus callosum was also investigated by using fluorescent antisera to identify the cells and [^{3}H]thymidine autoradiography to assay proliferation. The oligodendrocytes and "macrophage-like" microglia were not significantly stimulated, but the astrocytes were stimulated over the same range of concentration as Schwann cells. The activity against astrocytes and that against Schwann cells co-migrated in native gel electrophoresis at pH 4.5, providing strong evidence that the same molecule acts on both cell types.

We have recently described the use of immunological methods to both identify and purify rat Schwann cells from dissociated cultures of neonatal sciatic nerve (1, 2). These cultures contain only Schwann cells and fibroblasts as determined by antigenic criteria, and the fibroblasts may be effectively removed by treatment with antiserum to the Thy-1 antigen followed by complement-dependent lysis (2). In a conventional tissue culture medium containing 10% fetal calf serum, the Schwann cells divide very slowly, but can be stimulated by an activity present in extracts of the bovine pituitary (2, 3). This has allowed us routinely to maintain dividing populations of Schwann cells which are greater than 99.5% pure, and whose properties can be studied (4-6).

The nature of the pituitary activity has been investigated in a preliminary study (3). While absent from extracts of non-neural tissues, it was detectable in extracts of bovine pituitary, both anterior and posterior lobes, and of bovine brain. The activity was labile to proteolytic digestion and to boiling. A variety of known anterior and posterior pituitary hormones, as well as fibroblast, epidermal, and nerve growth factors were not mitogenic for the cultured Schwann cells over a wide range of concentrations tested. The activity required the presence of one or more components in fetal calf serum for its action. Furthermore, it did not lead to elevation of the intracellular level of cyclic AMP, an alternative method (4) for stimulating Schwann cell division.

In view of its restricted distribution, apparently novel identity, and the current interest in factors controlling proliferation of glia (7, 8) and other animal cells (9), we have undertaken the purification and characterization of this molecule. A preliminary account of some of this work has appeared (10).

MATERIALS AND METHODS

Wistar-Furth rats were bred in this laboratory. Frozen bovine pituitaries and lyophilized bovine anterior lobes were obtained from Pel-Freez Biologicals and stored at −90°C. [*methyl*-^{3}H]Thymidine (5 Ci/mmol) and ^{125}I-UdR were obtained from New England Nuclear. Whatman carboxymethyl-cellulose CM52 and phosphocellulose P-11 were from Reeve Angel, and Ultrogel AcA44 was from LKB.

DMEM[1] was obtained from Gibco and DMEM without bicarbonate buffer and with 0.02 M Hepes was from Flow Labs. Tissue culture plastics were obtained from Falcon Plastics and Flow Labs.

Cell Culture—Schwann cells were prepared and purified from cultures of neonatal rat sciatic nerves essentially as described previously (2, 3, 5). Fibroblasts were removed by treatment with monoclonal IgM anti-Thy 1.1 (dilution 1:1000) and rabbit complement (1:8) at the first passage, as well as at subsequent passages, if necessary. The cells were generally maintained in medium with the partially purified pituitary factor (CM-cellulose fraction at 10 to 20 μg/ml; see below), but this was removed from the medium for 4 to 6 days before the cells were used to assay the proliferation activity of fractions. Cultures of rat corpus callosum were derived from 4- to 6-day-old rats and grown on coverslips (12- to 15-mm diameter) in multiwell Linbro plates as described (11).

Immunofluorescence and Autoradiography—Procedures for staining cell cultures by indirect immunofluorescence with rabbit antisera to human glial fibrillary acid protein (1:100 dilution) or to galactosylcerebroside (1:25) have been given previously (11). Bound antibody was detected by reaction with rhodamine- or fluorescein-labeled F(ab')$_2$ goat anti rabbit IgG (Cappel Laboratories, 1:25). Macrophage-like microglial cells were stained by incubation with fluorescein-labeled normal sheep IgG (0.1 mg/ml) for 30 min at 37°C (11). Procedures for autoradiography (3), and for autoradiography of fluorescent-labeled cells were as described previously (12).

^{125}I-UdR Incorporation Assay—Schwann cells (generally second or third passage) that had been maintained in the absence of factor for 4 to 6 days were treated with anti-Thy 1.1 and complement and

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[1] The abbreviations used are: DMEM, Dulbecco's Modified Eagle's Medium; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate buffered saline; ^{125}I-UdR, [5-^{125}I]iodo-2'-deoxyuridine.

plated in flat-bottomed microwells in Linbro plates (Catalogue No. 76-003-05, 96 × 0.28 cm^2 wells, 7.5 × 10^3 cells/well) in 0.1 ml of DMEM with 10% fetal calf serum. After 12 to 24 h, fractions (diluted to an appropriate concentration in Hepes-buffered DMEM + 10% fetal calf serum) were added for 48 h and ^{125}I-UdR (2 μCi/ml) was added for the final 16 to 20 h of this period. The medium was removed by aspiration and the cells were treated with 0.05% trypsin (0.1 ml/well) in phosphate-buffered saline (0.9% NaCl solution) without calcium and magnesium for 10 min at 37°C, prior to harvesting onto glass fiber paper discs with a multiple sample device (Titertek cell harvester, Flow Labs). The discs were counted in a Packard well-type gamma counter. All assays were performed on triplicate microwells and averaged. The plateau stimulation by purified fractions varied between 20- to 100-fold depending upon differences in background proliferation between batches of Schwann cells.

Polyacrylamide Gel Electrophoresis—SDS-gel electrophoresis of reduced samples from the purification was performed on small scale 12.5% acrylamide gels (2.6 × 2 × 0.015 cm) using the discontinuous buffer system of Laemmli (13). Nondenaturing electrophoresis of the phosphocellulose fraction was performed in the pH 4.5 buffer system of Reisfeld *et al.* (14), with certain modifications. Urea was eliminated from all solutions, and the gels were cast in small glass tubes (4.5 × 0.3 cm diameter) in order to economize on purified factor. The lower gel (10% polyacrylamide) which contained riboflavin in place of ammonium persulfate, was poured to a height of 2.6 cm and photopolymerized for 16 to 17 h with a fluorescent lamp. After adding the stacking gel (2.8% polyacrylamide) and photopolymerizing for 2 h, the gels were pre-run at 0.8 mA/tube for 30 min at 4°C. The samples of phosphocellulose fraction were mixed with an equal volume of 1% agarose at 42°C and loaded onto the gel. After cooling, the gels were run at 4°C for approximately 2 h, until a marker dye (Pyronin Y) had travelled to near the bottom of the tube. Pyronin Y was not included in sample gels because it tended to complex with some of the components. The gels were extruded and cut into slices (approximately 2 mm) which were shaken for 30 min at 4°C in 0.1 ml of 2× strength PBS (22) to neutralize the gel buffer. After removing the PBS, the slices were eluted by shaking each slice overnight at 4°C in 0.1 ml of Hepes-buffered DMEM with 10% fetal calf serum and 100 μ/ml of penicillin-streptomycin. An aliquot of each eluant (generally 0.01 ml) was added to cultures to assay its effect on proliferation.

For two-dimensional analyses, the pH 4.5 gels were electrophoresed into an SDS slab gel essentially as described by O'Farrell (15). The slab gels (15% polyacrylamide) were, however, scaled down to 2.6 × 2 × 0.015 cm with a 1-cm stacking gel, and the pH 4.5 tube gels were equilibrated for 10 to 12 min at 65–70°C. Each slab was run at 5 mA for approximately 2.5 h and stained with Coomassie blue by conventional procedures.

RESULTS

Purification of Factor—The factor was purified from a pool of 10 kg of frozen pituitaries and approximately 4000 lyophilized anterior lobes. The tissue was processed in four separate batches up to and including fractionation on CM-cellulose. At this point, the CM fractions were combined and processed together through the remaining steps. All steps were performed at 0–4°C. All buffers contained 0.02% NaN_3.

Step 1: Preparation of $(NH_4)_2SO_4$ Fraction—Frozen pituitaries (5 kg) were thawed at 4°C overnight and cut into small fragments before homogenizing in a Waring Blendor in 15 liters 0.15 M $(NH_4)_2SO_4$. Lyophilized anterior lobes (2000) were cut up and swollen in 15 liters of the same buffer for 1 h before homogenization. The tissue was extracted with stirring for approximately 2 h, while the pH was maintained at 4.5 by addition of 1 M HCl. The homogenate was centrifuged for 15 min at 3000 rpm in a Beckman J6 centrifuge to remove large debris and pituitary fragments. The supernatant was filtered through several layers of cheesecloth and passed through a Sorvall RC-5B centrifuge fitted with a continuous flow attachment (flow rate, approximately 120 ml/min, TZ-28 rotor, 18,000 rpm); the pellet in the rotor was discarded. After adjusting the pH to 6.5 with 1 N NaOH, powdered $(NH_4)_2SO_4$ was slowly added to the supernatant to 200 g/liter. The precipitate was removed by continuous flow centrifugation as before, and the resulting supernatant was precipitated by addition of $(NH_4)_2SO_4$ to 250 g/liter. The precipitate was collected by continuous flow centrifugation, dissolved in 300 ml of 0.1 M sodium phosphate pH 6.0 (P buffer), and extensively dialyzed against this buffer.

Step 2: Carboxymethylcellulose Ion Exchange Chromatography—The $(NH_4)_2SO_4$ fraction was applied to a 500-ml column of CM52 carboxymethylcellulose which was equilibrated in P buffer. The column was washed with P buffer until the absorbance at 280 nm (detected on-line with a UA-5 ISCO monitor) was close to baseline, and then eluted with P buffer + 0.05 M NaCl. After washing, the activity was eluted with P buffer + 0.2 M NaCl. The fractions of highest specific activity from the elution with 0.2 M NaCl were pooled and stored at −90°C without detectable loss of activity. The CM-cellulose fraction has been used for routine maintenance of the purified Schwann cells (see "Materials and Methods").

Step 3: AcA44 Ultrogel—A column of AcA44 Ultrogel (5 cm diameter × 90 cm) was poured and equilibrated in P buffer + 0.4 M NaCl. The column was run at a flow rate of 86 ml/h and calibrated with blue dextran, bovine serum albumin, ovalbumin, and myoglobin as molecular weight standards. The four separate CM-cellulose fractions were pooled (volume, 455 ml), concentrated in an Amicon pressure cell with a PM-10 membrane to 194 ml, and then precipitated by addition of 109 g of $(NH_4)_2SO_4$. The precipitate was dissolved in 15 ml of P buffer + 0.4 M NaCl and applied to the AcA44 column. The active fractions (Fig. 1) which eluted at an apparent molecular weight of 5.5 to 6.0 × 10^4, were pooled, concentrated to 13 ml in an Amicon pressure cell, and applied again to the same column. The fractions of highest specific activity were pooled and concentrated to 12 ml in an Amicon cell.

Step 4: Phosphocellulose Ion Exchange Chromatography—A 5-ml column of P-11 phosphocellulose, previously washed according to Burgess (16), was poured and equilibrated in P buffer with 0.2 M NaCl. The conductivity of the concentrated AcA fraction was adjusted to that of P buffer + 0.2 M NaCl by addition of P buffer, and the fraction was then applied to the column. After washing the resin until the absorbance at 280 nm was near baseline, it was eluted with a 150-ml linear gradient from P buffer + 0.2 M NaCl to P buffer + 1 M NaCl. The conductivity of the eluant was continuously monitored with an on-line meter (Radionet CT type CDM 2F). The fractions of highest specific activity, which eluted between 0.65 and 0.75 M NaCl, were pooled, concentrated to

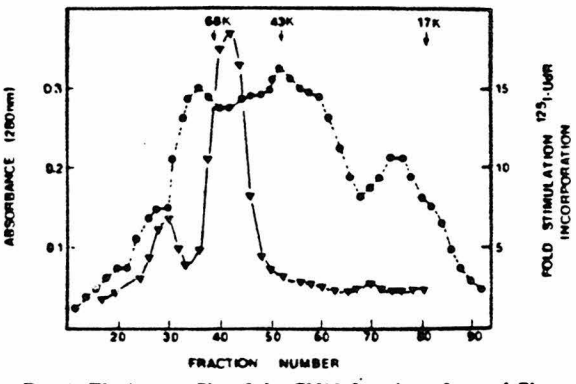

FIG. 1. **Elution profile of the CM52 fraction after gel filtration on AcA44 ultrogel.** The CM52 fraction (405 mg) was chromatographed on a column of AcA44 as described in the text. Fractions of 13 ml were collected and assayed at a final concentration of 1 μg/ml. Fractions 38 to 44 were pooled for further purification. ●, absorbance at 280 nm; ▼, stimulation of ^{125}I-UdR incorporation.

TABLE I

Summary of purification

The activity was purified from a total of 10 kg of pituitaries and 4000 lyophilized anterior lobes as described in the text. The relative specific activity of each fraction was determined from the dose-response curves. The protein yields and units recovered are based on the total quantity of starting material.

Fractions	Quantity	Specific activity	Recovery of activity
	mg	*relative units*	%
1. $(NH_4)_2SO_4$	188,000	1	100
2. Carboxymethylcellulose	409	100	21
3. a. AcA44 No. 1	53	200	6
b. AcA44 No. 2	25	440	6
4. P-11 phosphocellulose	1.1	4400	3

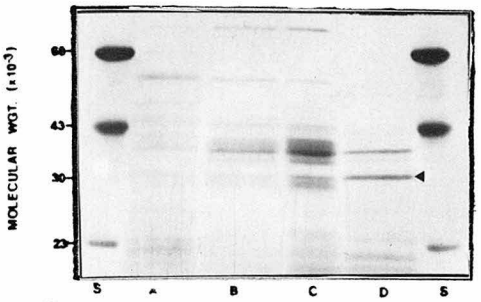

FIG. 2. **SDS-polyacrylamide gel electrophoresis of various fractions from the purification procedure.** An aliquot (7 to 10 μg) of various fractions from the purification procedure was run on SDS-polyacrylamide gels. The gels were stained with Coomassie blue. The standards (*S*) are bovine serum albumin (68,000), ovalbumin (43,000), and trypsin (23,000). *A*, CM52; *B*, AcA-1; *C*, AcA-2; *D*, P-11.

1.8 ml in an Amicon cell, and stored in aliquots of 0.6 ml at −90°C. After an aliquot was thawed out, it was stored at 0°C over a period of 1 to 2 months without detectable loss of activity.

A summary of the purification is given in Table I. Increases in specific activity were estimated from the displacement of dose-response curves. The phosphocellulose fraction is purified over 4000-fold from the $(NH_4)_2SO_4$ fraction with a recovery of approximately 3%.

Electrophoretic Identification of Active Species in the Phosphocellulose Fraction—The heterogeneity of various fractions was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). The phosphocellulose fraction displayed a major band (component b *arrow*), at a position corresponding to a molecular weight of 30,000, accounting for approximately 25% of the Coomassie blue staining material. When the phosphocellulose fraction was analyzed by polyacrylamide gel electrophoresis at pH 4.5 in the absence of detergent (see "Materials and Methods"), the activity migrated as a single peak that was coincident with a major band of staining material. When a parallel pH 4.5 gel was analyzed by a second dimension of SDS-polyacrylamide gel electrophoresis, the activity was associated with component b with an apparent molecular weight of 3×10^4 (Fig. 3). A closely migrating component (*a*) with a molecular weight of 3.5×10^4 (see Fig. 4) was clearly and reproducibly resolved from b. In addition, the peak of component a elutes at a lower ionic strength from phosphocellulose than the peak of activity.[2] The reproducible association of activity with component b on native gel electrophoresis provides strong evidence that the activity is found in a species with a subunit molecular weight of 3×10^4. The major peak of activity eluted on gel filtration at a position corresponding to a molecular weight of 5.5 to 6.0×10^4 (Fig. 1), suggesting that the active species is a dimer.

Activity of the Purified Factor on Central Glial Cells—The activity of the factor on glial cells of the central nervous system was investigated by using dissociated cultures of the rat corpus callosum. Coverslip cultures in DMEM with 10% fetal calf serum were exposed to various concentrations of the phosphocellulose fraction for 48 h. For the last 24 h of this period, the cells were exposed to tritiated thymidine (2 μCi/ml). The cells were reacted with marker antisera and fluorescent anti-immunoglobulin reagents, fixed, dried and coated with emulsion for light microscope autoradiography.

Oligodendrocytes, identified by rabbit antisera to galactosylcerebroside, were essentially quiescent in these cultures (less than 1% nuclei were labeled) and were not significantly stimulated in the presence of phosphocellulose fraction factor (2 μg/ml). Microglia, or "macrophage-like" cells, were identified by their ability to bind fluorescein-labeled normal immunoglobulin and internalize it into cytoplasmic vacuoles (11). Approximately 15% of these cells had labeled nuclei in the absence of factor and this was not significantly increased in medium supplemented with factor. Over 300 nuclei were scored in each case.

The astrocytes in these cultures, which were identified by rabbit antiserum to glial fibrillary acidic protein (17) were, however, stimulated by the factor over the same concentration

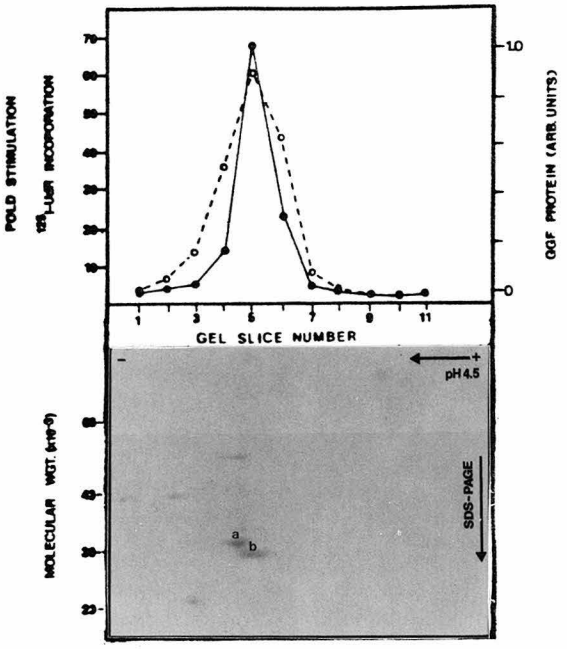

FIG. 3. **Analysis of the activity in the phosphocellulose fraction by native and denaturing gel electrophoresis.** Aliquots (5 μg) of the phosphocellulose fraction were run on parallel small scale pH 4.5 polyacrylamide gels as described under "Materials and Methods." One gel was sliced, eluted, and assayed in the Schwann cell microwell proliferation assay. The other was laid over an SDS-polyacrylamide slab gel and analyzed by a second dimension of electrophoresis, followed by staining with Coomassie blue. ○, stimulation of ^{125}I-UdR incorporation (10-fold stimulation is 1200 cpm); ●, glial growth factor (*GGF*) protein (arbitrary units) derived by correcting -fold stimulation according to the logarithmic dosage relationship. *PAGE*, polyacrylamide gel electrophoresis.

[2] J. P. Brockes, G. E. Lemke, and D. R. Balzer, Jr., unpublished results.

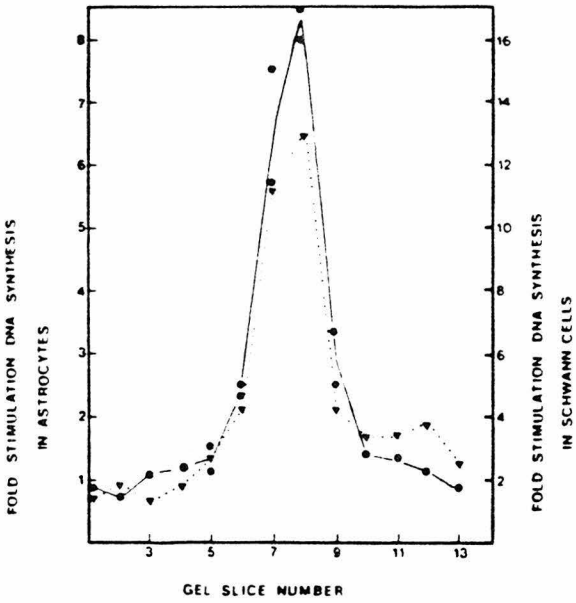

FIG. 4. Activity of the phosphocellulose fraction on astrocytes in cultures of the rat corpus callosum. Co-electrophoresis of activities against Schwann cell and astrocyte. An aliquot (5 μg) of the phosphocellulose fraction was analyzed by native gel electrophoresis at pH 4.5. The gel was sliced and eluted into buffer (see "Materials and Methods") and the eluant was assayed for its effect on ^{125}I-UdR incorporation (10-fold stimulation is 850 cpm) into Schwann cells in microwells, or the proportion of glial fibrillary acidic protein cells (astrocytes) that were thymidine-labeled in cultures of the corpus callosum. A 1-fold stimulation corresponds to 5% labeled nuclei in cultures without added factor. The *solid line* connects data points for the astrocyte (each a single coverslip), the *dotted line* connects points for the Schwann cell (average of three microwells).

range as Schwann cells (0.1 to 1 μg/ml). When the phosphocellulose fraction was analyzed by polyacrylamide gel electrophoresis at pH 4.5, the activity against Schwann cells migrated with the activity against astrocytes (Fig. 4). This provides strong evidence that one molecular species is active on both cell types.

DISCUSSION

Although the displacement of dose-response curves indicated that it had been purified over 4000-fold from the $(NH_4)_2SO_4$ fraction, the phosphocellulose fraction displayed significant heterogeneity when analyzed by SDS-gel electrophoresis. While it clearly will be necessary in the future to purify the factor to homogeneity, the present purification affords an opportunity for initial studies of the activity and its cell specificity. After gel electrophoresis of the phosphocellulose fraction under native conditions at pH 4.5, the activity was reproducibly associated with a component that displayed a molecular weight of 3×10^4 when analyzed in a second dimension by SDS-gel electrophoresis. In view of its behavior on gel filtration, the active species may therefore be a dimer of two identical subunits.

It has been clearly shown that Schwann cells in culture are stimulated to divide by contact with neurites (18, 19). Furthermore, a membrane preparation of rat sensory neurons has also been reported to be mitogenic (20). Although the pituitary and brain activity is extracted as a soluble component, more detailed chemical studies are clearly required before the two activities can be compared. This also applies to the report that crude homogenates or sonicates of chick sympathetic neurons contain mitogenic activity for Schwann cells and other non-neuronal cells (21).

When assayed on central glial cells present in dissociated cultures of the rat corpus callosum, the phosphocellulose fraction had no effect on the initiation of DNA synthesis by oligodendrocytes or macrophage-like microglial cells. It did, however, clearly stimulate astrocyte proliferation in these cultures. An alternative explanation in the mixed cultures of the corpus callosum is that there is a dividing precursor cell which is stimulated to express glial fibrillary acidic protein as a consequence of exposure to the factor. Although this cannot be ruled out, a direct mitogenic effect is more consistent with the action on Schwann cells. The activity against astrocytes co-electrophoresed at pH 4.5 with the activity against Schwann cells, providing strong evidence that the same molecule has both effects.

It would be a great asset for continuing studies on purification and distribution to have a monoclonal antibody to the factor, and we are currently attempting to derive such a reagent by immunizing with purified fractions from this preparation.

Acknowledgments—We thank H. Gordon and D. J. Nishiguchi for their participation in early experiments, and Drs. R. M. Pruss and M. C. Raff for gifts of antisera to glial fibrillary acidic protein and galactosylcerebroside as well as the protocol for corpus callosum cultures.

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ADDENDUM TO CHAPTER 1

This addendum contains additional data relevant to the material presented in Chapter 1. Some of these data have been published previously in the "Journal of Experimental Biology" (1981) 95: 215-230, and in "Development in the Nervous System" (1981) (Garrod and Feldman, eds.), Cambridge University Press, pp. 309-327.

Materials and Methods

Schwann cell materials, culture procedures and assay methods were as for Chapter 1. Primary rat fibroblasts, cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS), were prepared from the adherent population of primary muscle dissociations (Fischbach, 1972) by preplating these dissociations at 37° for 30 minutes. These cells were passaged at least three times (1:10 each passage) prior to use, after which time no remaining myoblasts could be discerned.

Fibroblast proliferation assay. Rat muscle fibroblasts were grown to confluence on 12 mm glass coverslips in DMEM + 10% FCS. The medium was changed and the cells maintained at confluence for 3 days. Test solutions (diluted to appropriate concentration with Hepes-buffered DMEM [HMEM] + 10% FCS) were then added for 30 hr with ^{3}H-thymidine (New England Nuclear) added for the final 24 hr of this period. The cells were washed, fixed and processed for autoradiography exactly as described for the corpus callosum cultures in Chapter 1. Fold stimulation was determined as the number of labeled nuclei per experimental coverslip divided by the number of labeled nuclei per control (HMEM + 10% FCS added). Cells were counterstained with Giemsa (Rhodin, 1974) to more easily visualize unlabeled nuclei.

Results and Discussion

Properties of the ^{125}I-iododeoxyuridine (^{125}I-UdR) incorporation assay. Figure 1 is a dose-response curve generated by testing varying concentrations of a partially purified GGF preparation in the Schwann cell microwell proliferation assay. This curve illustrates several salient features of the assay. Since Schwann cell proliferation in DMEM plus serum alone is very low, signal-to-noise ratios are quite favorable (25 in Fig. 1; typically 20-40). This allows for the reliable detection of minute levels of mitogenic activity. The limits of detection for GGF with this assay can be estimated (from dose-response curves of highly purified fractions in which the amount of GGF protein itself is known—see Chapter 3) to be $\sim 5 \times 10^{-12}$ M. The response varies approximately linearly with the logarithm of protein concentration over most of the range between maximal (plateau) and minimal (background) proliferation.

Dose-response properties of various purification fractions. The data on fold-purification presented in Table I of Chapter 1 were estimated from the displacement of the linear portion of the dose-response curves for each stage in the purification procedure. These curves are illustrated in Fig. 2. Successive steps in the purification procedure after chromatography on CM-cellulose result in a parallel dose-response transposition.

GGF effects on Schwann cell number and morphology. In addition to monitoring the incorporation of ^{125}I-UdR into the DNA of cells growing in microwells, one can also measure cell proliferation by employing ^{3}H-thymidine autoradiography (Wood and Bunge, 1975; see also Materials and Methods of Chapter 1) or by counting cells. Figure 3 illustrates the effect on Schwann cell number obtained when cells are cultured in the presence of the highly purified phosphocellulose fraction of Chapter 1. Schwann cells cultured without GGF (Fig. 3A, lower curve) divide very slowly, taking 7-10 days to double in number. Such cells (Fig. 3C) typically aggregate into small

groups in which individual cells extend only short processes. They do not survive for extended periods in culture. Cells grown in the presence of GGF (upper curve of Fig. 3A and Fig. 3C) double approximately every 30 hr, and are typically observed in large whorls, with cells extending long, parallel-oriented bipolar processes. These cells have been maintained for as long as five months in culture (Brockes et al., 1979) with full retention of expression of the Schwann cell surface antigen, Ran-1 (Brockes et al., 1977, 1979).

Effect of GGF on rat fibroblast proliferation. We have investigated the effect of GGF on the growth of one cell of non-neural origin, namely, rat muscle fibroblasts. These cells, derived as described in Materials and Methods, divide rapidly in serum-containing medium. When assayed at confluence by the ^{3}H-thymidine autoradiographic procedure described in Materials and Methods, cells exhibited proliferative bursts in response to phosphocellulose fraction GGF (Chapter 1) with dose-response properties comparable to those of Schwann cells (data not shown). In order to determine whether or not this activity was the same as that responsible for the stimulation of Schwann cell division, we investigated the migration of each activity after native gel electrophoresis at pH 4.5, as described for the astrocyte experiments of Chapter 1. The results of such an experiment are illustrated in Figure 4. Fibroblast and Schwann cell mitogenic activities show coincident migration under these conditions. Taken with the data of Fig. 4, Chapter 1, this result provides strong evidence that the mitogenic effect of the phosphocellulose fraction on astrocytes, Schwann cells and fibroblasts results from the action of a single molecular species.

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Figure 1. Stimulation of ^{125}I-iododeoxyuridine incorporation into Schwann cells by partially purified extracts of the bovine pituitary. The material was prepared by fractionating a pituitary extract on CM-Sephadex as described by Gospodarowicz (1975). Assay conditions were as described in Materials and Methods, Chapter 1. Plateau stimulation at 25-fold corresponds to an incorporation of 3300 cpm.

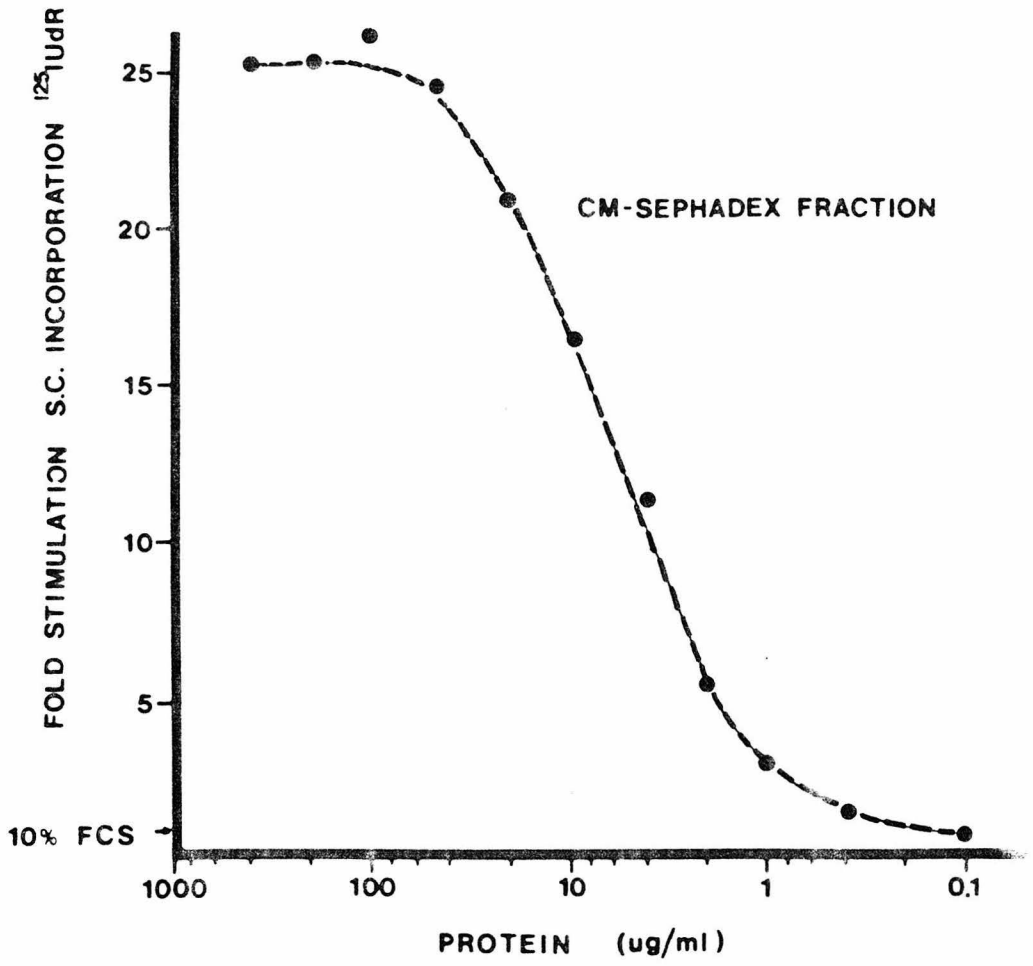

FOLD STIMULATION S.C. INCORPORATION ^{125}IUdR
25
20
15
10
5
10% FCS
1000
100
10
1
0.1
PROTEIN (ug/ml)
CM-SEPHADEX FRACTION

Figure 2. Dose-response curves of ^{125}I-UdR incorporation for the various fractions from the purification procedure of Chapter 1. All fractions were assayed at the same time on purified Schwann cells as described in Materials and Methods, Chapter 1. ● crude extract; ○ ammonium sulfate fraction; ▲ CM 52; △ AcA 1; ■ AcA 2; ● P11.

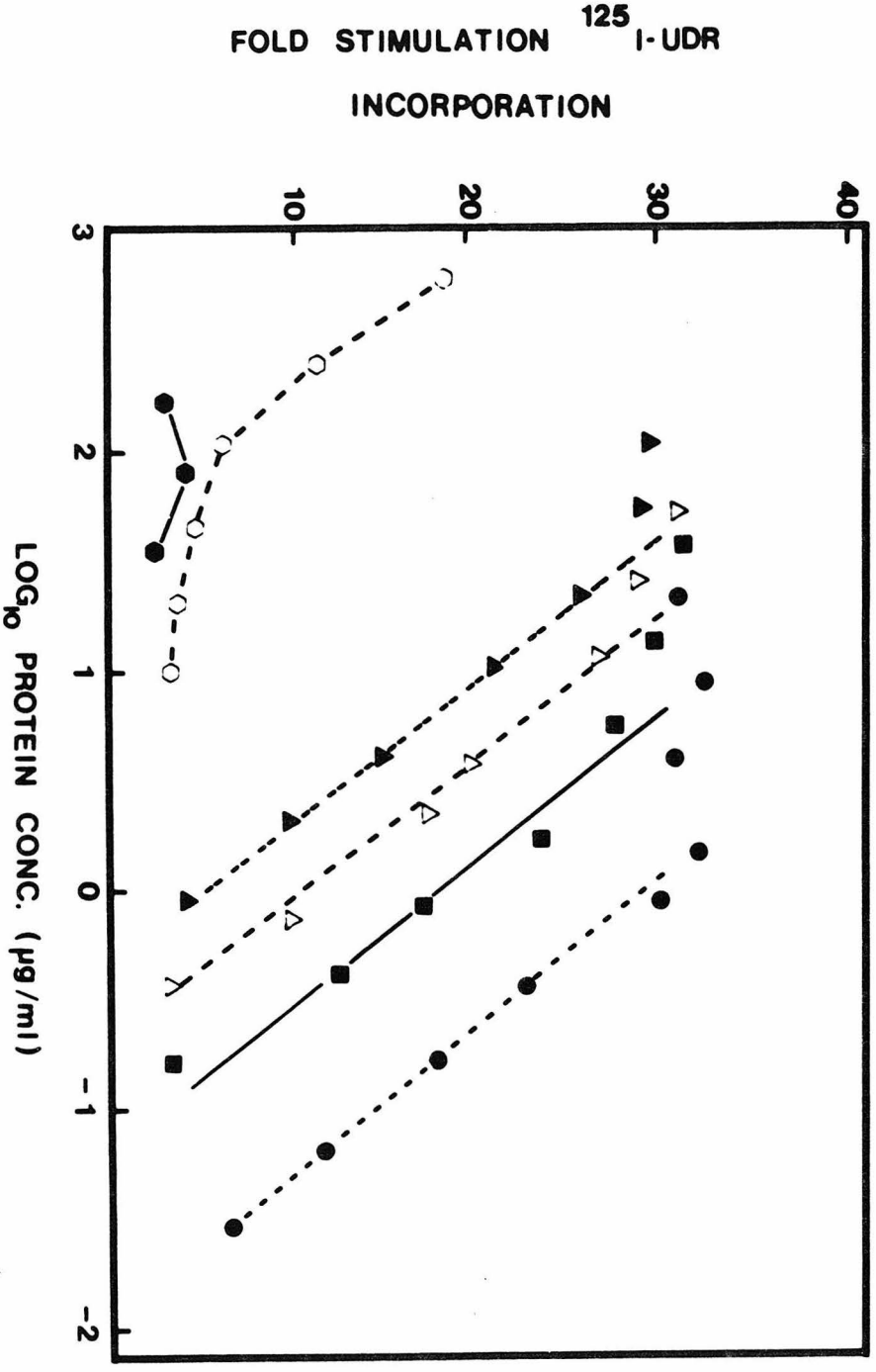

FOLD STIMULATION ^{125}I-UDR
INCORPORATION
10
20
30
40
3
2
1
0
-1
-2
LOG_{10} PROTEIN CONC. (µg/ml)

Figure 3. Effects of GGF on purified Schwann cells. A, Schwann cells were cultured in wells in medium with 10% FCS alone (●) or with 2 μg/ml phosphocellulose fraction GGF (▽, Chapter 1). Cell counts were performed at the indicated times after plating with a hemocytometer. B, Phase-contrast photomicrograph of cells from the experiment of A that had been in factor for 5 days. C, A parallel group of cells from a culture maintained in the absence of GGF.

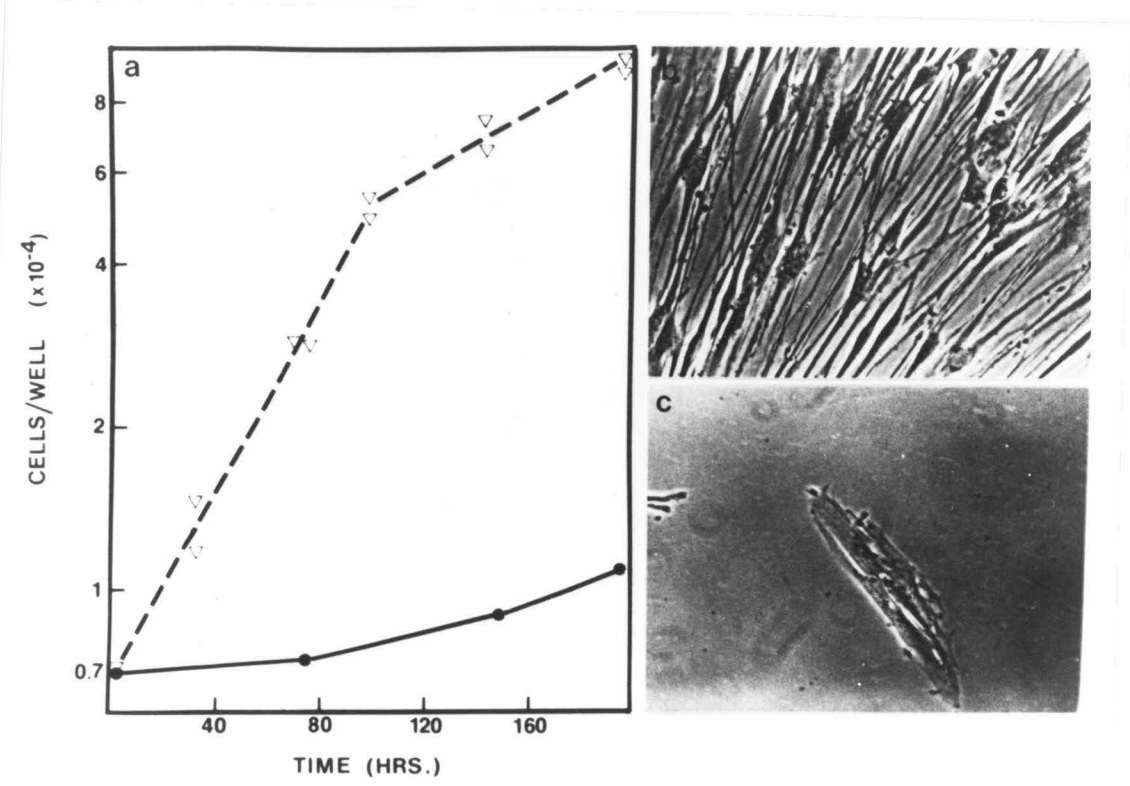
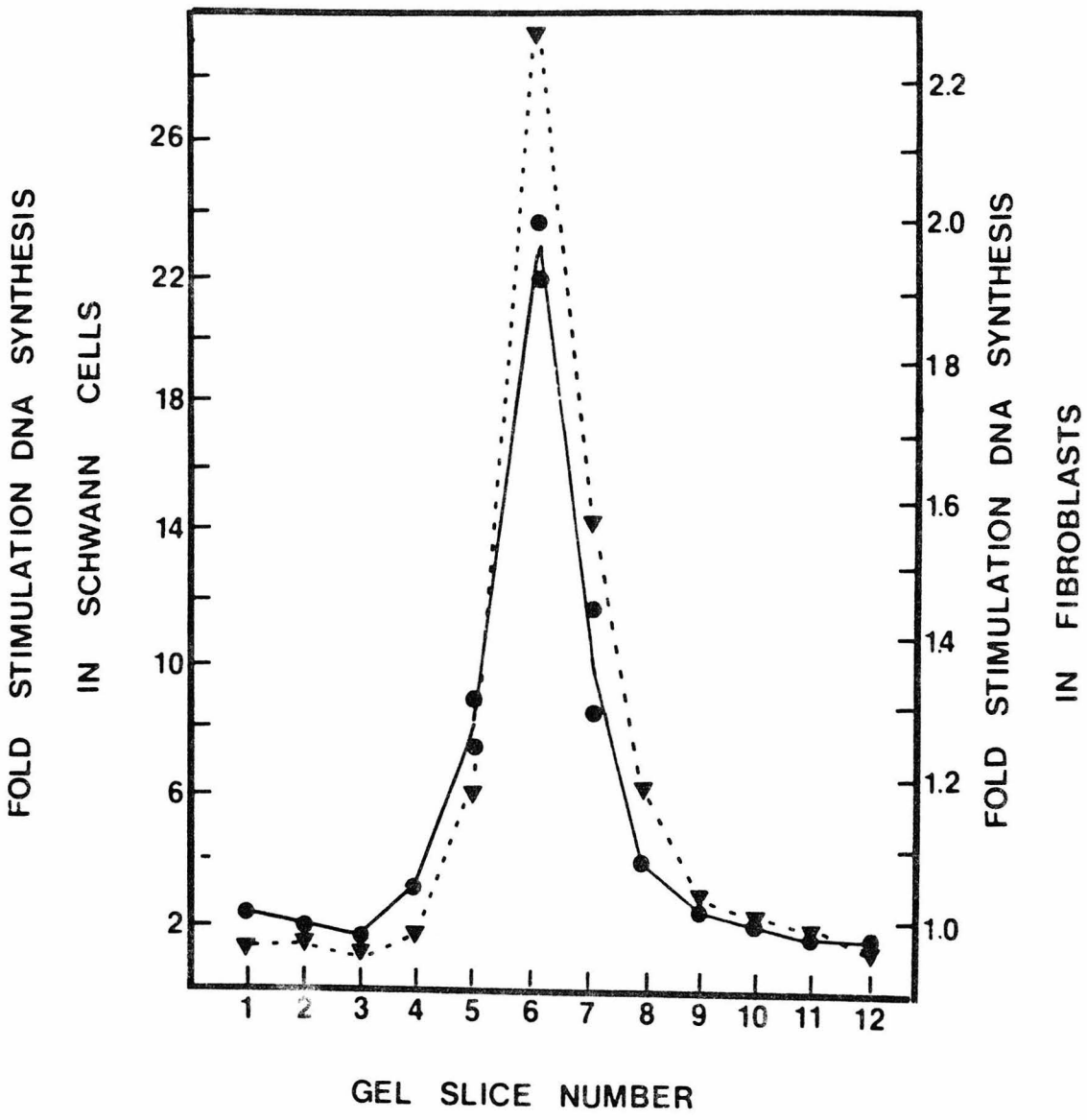

a
b
c
8
6
4
2
1
0.7
CELLS/WELL (x10⁻⁴)
40
80
120
160
TIME (HRS.)

Figure 4. Co-electrophoresis of activities against Schwann cells and rat muscle fibroblasts. An aliquot (5 μg) of phosphocellulose fraction GGF (Chapter 1) was analyzed by native gel electrophoresis. The gel was sliced into segments, the segments eluted with culture medium, and the eluates assayed for their effect on ^{125}I-UdR incorporation into Schwann cells growing in microwells all as described in Chapter 1 and ^{3}H-thymidine incorporation into confluent fibroblasts on coverslips (as described in Materials and Methods). Total fibroblast cell number increased by less than 10% during the 30 hr duration of these experiments, and varied by less than 5% between similarly treated coverslips. Nine to twelve percent of the nuclei in control cultures were labeled. Each point (●) corresponds to a single fibroblast coverslip culture. The solid line connects data points for the fibroblasts and the dotted line connects points for the Schwann cells.


FOLD STIMULATION DNA SYNTHESIS IN SCHWANN CELLS
FOLD STIMULATION DNA SYNTHESIS IN FIBROBLASTS
26
22
18
14
10
6
2
2.2
2.0
1.8
1.6
1.4
1.2
1.0
1 2 3 4 5 6 7 8 9 10 11 12
GEL SLICE NUMBER

CHAPTER 2

CHARACTERIZATION OF GLIAL GROWTH FACTOR ACTIVITY IN VERTEBRATE BRAIN

Some of this material has appeared previously in:

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"Development in the Nervous System" (1981) (Garrod and Feldman, eds.), Cambridge University Press, Cambridge, pp. 309-327.

Abstract

Glial growth factor (GGF) activity is present in vertebrate brain. Three lines of evidence indicate that this activity is very closely related, if not identical, to the pituitary activity described in Chapter 1. (1) The ionic properties of the two activities are indistinguishable, as evidenced by coincident ion-exchange chromatography on phosphocellulose. (2) The activities show co-incident migration when analyzed by native (non-denaturing) gel electrophoresis at pH 4.5. (3) Antibodies which deplete GGF activity from solution specifically recognize a 31,000 dalton protein in partially purified GGF preparations from both pituitary and brain. Growth factor activity is non-uniformly represented in bovine brain. It is widely distributed among vertebrate species.

In view of the phenomena surrounding glial cell division during development and after injury in the central nervous system (Gall et al., 1979) as well as the effects of brain extracts on non-neural cells (Singer et al., 1976), both the activity of pituitary GGF on astrocytes and fibroblasts and the observed presence of Schwann cell mitogenic activity in the brain (Raff et al., 1978) are of considerable interest. I have investigated the distribution of GGF activity in bovine brain, wherein I find a distinct and reproducible variation in the specific activity of crude extracts prepared from different regions. I have also undertaken a series of chromatographic, electrophoretic and immunochemical analyses of the relationship of the brain activity to that of the pituitary. These latter studies suggest that both activities result from the action of the same molecule.

Materials and Methods

Whole frozen bovine brains were obtained from Pel-Freez Biologicals (Rogers, Ark) and were dissected at room temperature by Dr. Hermes Bravo. All other materials were as for Chapter 1. Schwann cell culture, proliferation assays, phosphocellulose ion-exchange chromatography and native gel electrophoresis at pH 4.5 were performed exactly as described in Chapter 1. Crude extracts from dissected regions of bovine brain were prepared by twice homogenizing tissue with three volumes of 0.15 M $(NH_4)_2SO_4$ at 4° for 30 seconds. The pH of each homogenate was lowered to 4.5 with 1 M HCl, and extraction conducted for 2 hours with stirring. This material was centrifuged at 20,000 x g for 30 minutes and then extensively dialyzed against distilled water. Extract dialysands were centrifuged as before and the supernatants assayed for protein with the Bio-Rad dye binding reagent (Richmond, CA). (Those extracts measuring less than 2 mg/ml were lyophilized and then resolubilized with distilled water to this concentration.) Reaction of monoclonal

anti-GGF antibodies (Lemke and Brockes, 1981) with 12.5% SDS gels was performed according to the method of Moore et al. (1982), modified as described in Chapter 3.

Results and Discussion

Distribution of GGF activity in bovine brain. The preliminary studies of Raff et al. (1978) included a description of Schwann cell growth factor activity in bovine brain. Their data indicated that the specific activity of crude extracts of whole brain was approximately 1/3 that of extracts similarly prepared from the pituitary. In order to investigate the regional representation of this activity, crude ammonium sulfate extracts were prepared as described in Materials and Methods from various regions of bovine brain. These extracts were then assayed for GGF activity by titration in the Schwann cell microwell proliferation assay. Figure 1 gives the specific activity of each of these extracts as compared to an extract similarly prepared from the bovine pituitary. A non-uniform distribution of GGF activity is evident, with considerable variation between regions. The composite brain activity, summed over this distribution, is consistent with the relative levels of pituitary vs. brain mitogenicity observed by Raff et al. (1978), although some areas are particularly active. One area, the caudate nucleus, yields extracts of an even higher specific activity than those of the pituitary. The significance of this singularity, and of the regional variation in specific activity is unknown, but these findings are consistent with GGF being localized in neurons rather than glia or other cell types.

Phosphocellulose chromatography. One distinctive feature of pituitary GGF is its extreme charge: it behaves as a very basic protein when chromatographed on the ion-exchange resin phosphocellulose P11. (Greater than 99.5% of the proteins in crude pituitary extracts are less basic than GGF by this criterion—see Fig. 4, Chapter 3.) Figure 2 demonstrates that brain GGF shares this property. Crude extracts of bovine pituitary and caudate nucleus were fractionally precipitated with

ammonium sulfate as described in Chapter 1. These fractions were each extensively dialyzed against 0.1 M sodium phosphate, 0.2 M NaCl, pH 6.0, applied to a 10 ml column of phosphocellulose P11 previously equilibrated with this buffer, and then chromatographed by developing each column with a 100 ml linear gradient from 0.2 M-1.0 M NaCl in 0.1 M sodium phosphate, pH 6.0. Fractions from each column run were assayed for stimulation of ^{125}I-iododeoxyuridine (^{125}I-UdR) incorporation into Schwann cells. Both brain and pituitary growth factor activity are observed to migrate as a single peak at ~0.65 M NaCl.

Native gel electrophoresis. Active fractions from the caudate and pituitary phosphocellulose elutions were pooled, concentrated by ultrafiltration in an Amicon cell to 1.6 mg/ml and further analyzed by native gel electrophoresis using the discontinuous buffer system of Reisfeld et al. (1962), modified as described in Chapter 1. This gel system separates proteins on the basis of both charge and molecular weight. Parallel gels of resolved pituitary and caudate phosphocellulose fractions were sliced, eluted into tissue culture medium and the eluates assayed for GGF activity as described in Chapter 1. Figure 3 illustrates the results of such an experiment: the caudate and pituitary activities show coincident migration as a single peak. Taken with the data of Figure 2, this result argues strongly that brain and pituitary growth factor activity result from the action of a biochemically similar, if not identical molecule.

Reactivity of brain GGF with monoclonal antibodies. We have derived a panel of monoclonal antibodies to pituitary GGF based on their ability to specifically precipitate GGF activity from heterogenous solutions (see Lemke and Brockes, 1981, and Chapter 3 for details as to derivation and characterization). These reagents have been used to identify their target antigens as resolved on SDS gels (Burridge, 1978). Studies with partially purified preparations of pituitary GGF indicate a specific binding of these antibodies to a protein of molecular weight 3.1×10^4 (see Chapter 3).

We have conducted similar investigations with partially purified GGF preparations from whole brain. The result obtained from an experiment in which a CM-cellulose fraction from chick brain was analyzed is illustrated in Fig. 4. Monoclonal antibody E binds specifically to a 31,000 dalton species, with some staining at the dye front. This staining pattern is also observed with material from bovine brain (data not shown). These results are consistent with those obtained from pituitary material (see Chapter 3) and indicate that the same mitogen-related antigen is present in both preparations.

Cross-species occurrence of GGF activity. GGF-like activity has been detected in partially purified (CM-cellulose or phosphocellulose) preparations from chick, frog, newt and axolotl brains in addition to bovine brain. These activities resemble GGF in their high-salt elution from cation exchange resins and in their ability to stimulate DNA synthesis in rat Schwann cells when tested in the ^{125}I-UdR incorporation assay. Figure 5 illustrates one such test—a titration of a CM-cellulose fraction of GGF from 4-day chick brain. The dose-response properties and specific activity of this fraction resemble those of similar fractions prepared from the bovine pituitary.

The above data indicate that, by several independent criteria, GGF is present in the brain as well as the pituitary. The mitogenic activities purified from these sources cannot be distinguished by biochemical behavior, immunological reactivity, or bioactivity properties, and thus appear to derive from the same molecule. This is in contradistinction to the observed differences between pituitary FGF and some preparations of brain FGF (Lemmon et al., 1982). An analysis of the activity of brain GGF, purified from the caudate nucleus as described above, has demonstrated that this material is capable of stimulating the division of cultured rat astrocytes in a manner comparable to that of pituitary GGF (data not shown). Experiments described in Chapter 3 further show that both brain and pituitary GGF activity are recovered at a molecular weight of 3.1×10^4 after denaturing polyacrylamide electrophoresis in

SDS, providing additional evidence for identity between these two molecules.

The presence of an unusually high level of GGF activity in the caudate nucleus is intriguing, although the role that it may play in either the development or subsequent functional organization of this center is not immediately obvious. The observed regional variation in GGF activity is consistent (by extrapolation, for example, from observations of neurotransmitter occurrence [Matsuda et al., 1973] or opiate receptor binding [Snyder and Matthysse, 1975]) with a neuronal site of synthesis, although evidence on this point will of course require direct investigation.

The functional role that glial growth factor in the brain plays in vivo is open to question. It should be noted, however, that the molecule is certainly a viable candidate for the hypothesized diffusible mitogen responsible for the proliferative response of CNS glia to injury (Cavanagh, 1970).

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Figure 1. Regional distribution of GGF activity in bovine brain. The activity of extracts of various areas was determined from dose-response curves (four points each) as generated in the ^{125}I-UdR incorporation assay with the same set of Schwann cells. Dose-response curves for different areas were approximately parallel. Activity is expressed relative to that in the pituitary at an extract concentration of 0.2 mg/ml, i.e.

$$\frac{\text{fold stimulation of extract at 0.2 mg/ml}}{\text{fold stimulation of pituitary extract at 0.2 mg/ml}}$$

Values given are the mean $\pm$ S.D. for analyses performed on four separate brains. CN = caudate nucleus; CCX = cingulate cortex; PUT = putamen; HTA = hypothalamus; HPC = hippocampus; P = pons; COR = cerebral cortex; TH = thalamus; GP = globus pallidus; CBM = cerebellum; MES = mesencephalon; MED = medulla; and PTY = pituitary.

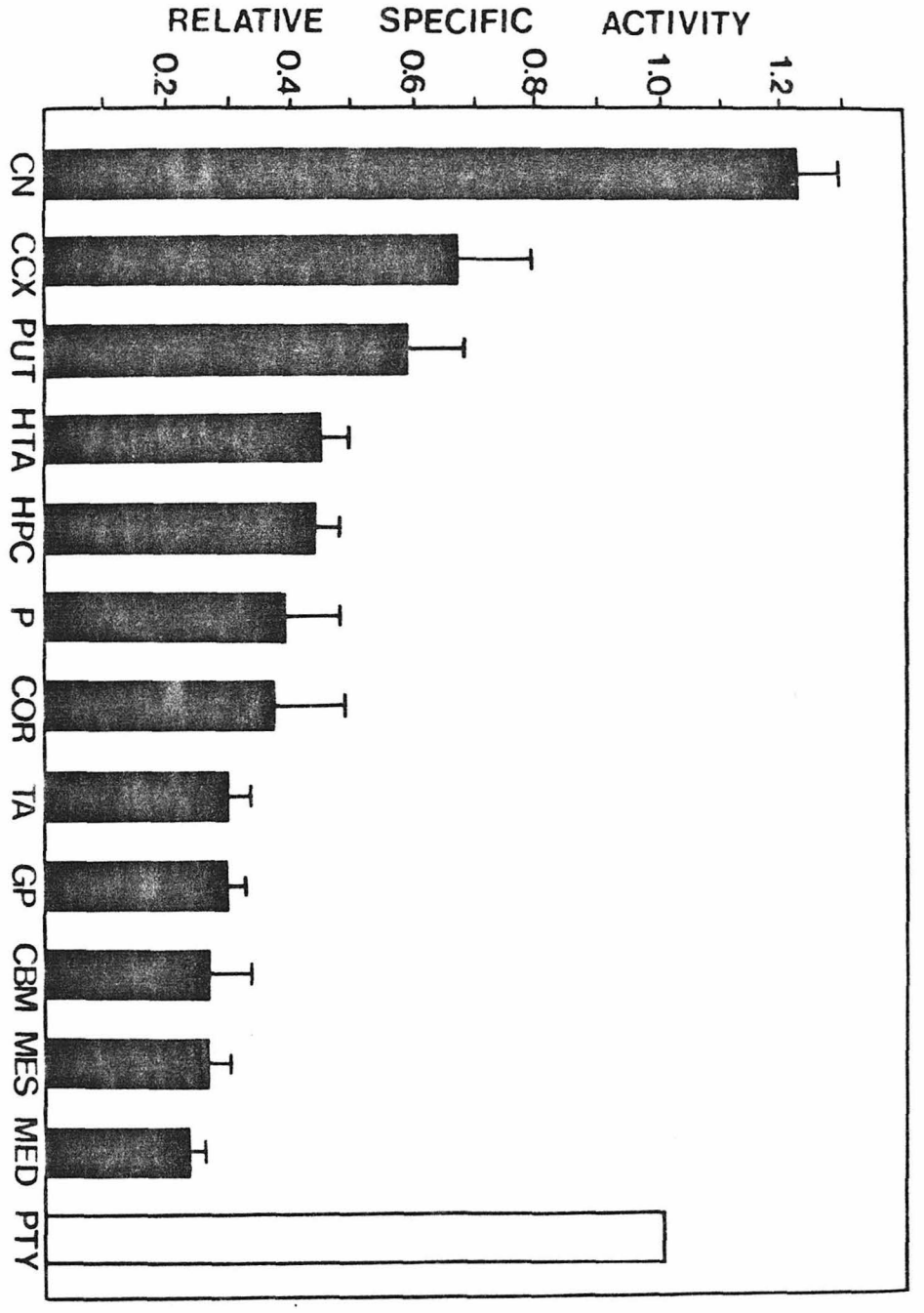

RELATIVE SPECIFIC ACTIVITY
0.2
0.4
0.6
0.8
1.0
1.2
CN
CCX
PUT
HTA
HPC
P
COR
TA
GP
CBM
MES
MED
PTY

Figure 2. Comparative chromatography on phosphocellulose P11. Crude extracts of caudate nucleus and pituitary were prepared and fractionally precipitated with ammonium sulfate as described in the text. Protein (0.25 g of each extract) was applied to the column in 0.1 M sodium phosphate, pH 6.0 and chromatographed as described in Chapter 1. Fractions were assayed (at a constant protein concentration of 10 μg/ml) for their effect on Schwann cell proliferation. (Assaying column fractions at constant volume gave similar profiles.) Less than 5% of the activity applied to the column was recovered in the flow-through and wash. Fractions from 0.6-0.72 M NaCl were pooled for further analysis. o - - - o caudate; ●——● pituitary.

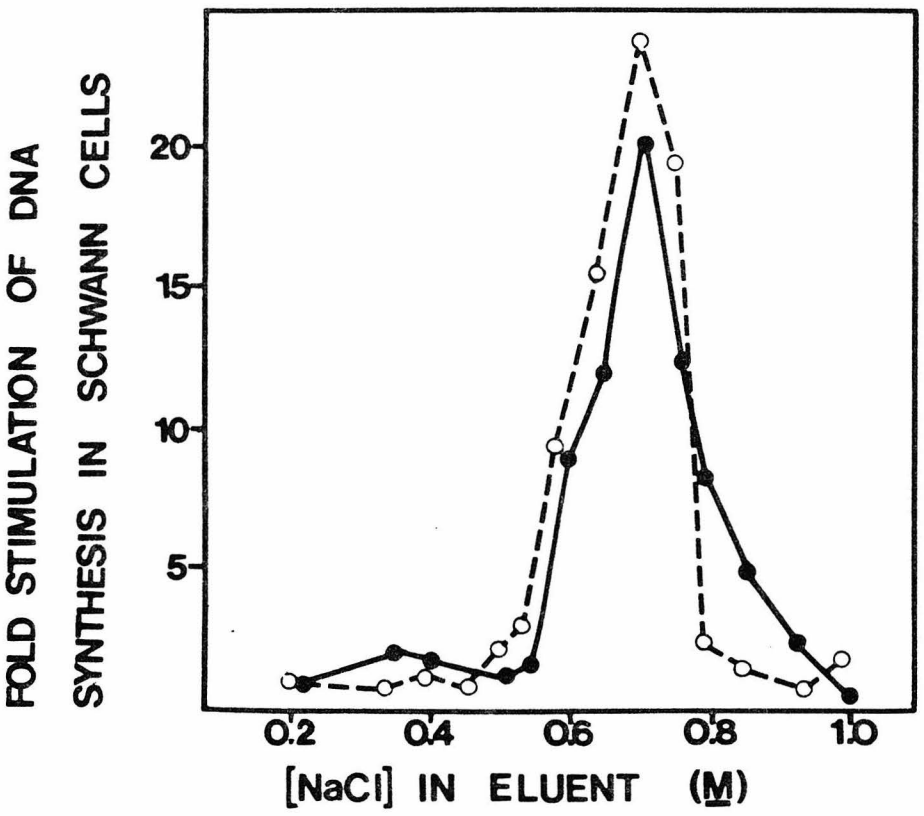

FOLD STIMULATION OF DNA
SYNTHESIS IN SCHWANN CELLS
20
15
10
5
0.2
0.4
0.6
0.8
1.0
[NaCl] IN ELUENT (M)

Figure 3. Comparative native gel electrophoresis. Pooled and concentrated fractions from the caudate and pituitary phosphocellulose elutions (20 µl) were analyzed by native gel electrophoresis at pH 4.5 as described in Chapter 1. The gels were processed as described in the text and Chapter 1. Eluates (15 µl) were assayed for their effect on Schwann cell proliferation. o - - - o caudate; ●——● pituitary.

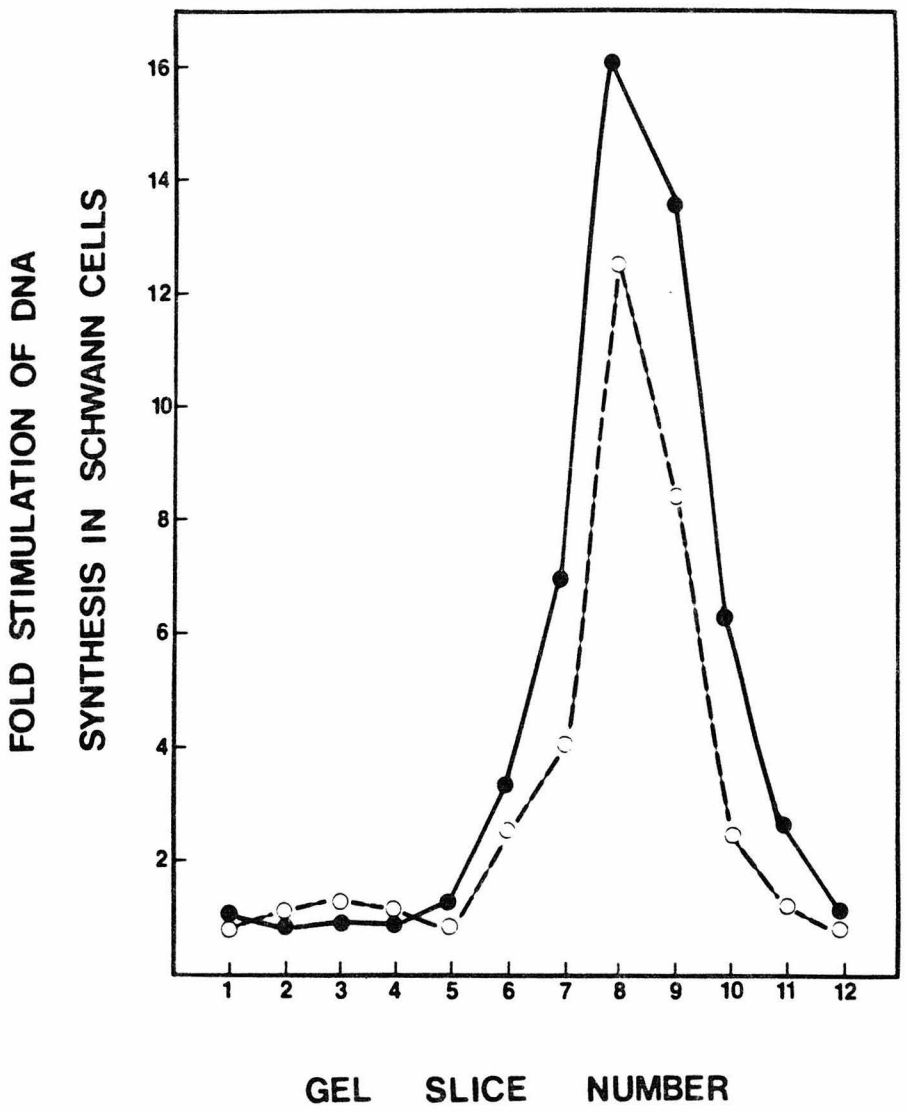

FOLD STIMULATION OF DNA
SYNTHESIS IN SCHWANN CELLS
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
11
12
GEL SLICE NUMBER

Figure 4. Monoclonal antibody reactivity. Whole brains from 4-day chicks were extracted, fractionally precipitated with ammonium sulfate and chromatographed on CM-cellulose 52 in 0.1 M sodium phosphate at pH 6.0 as described for the pituitary CM-cellulose fraction in Chapter 1. An aliquot of this fraction (15 µl containing 45 µg total protein) was analyzed by SDS gel immunoautoradiography with monoclonal anti-GGF E and ^{125}I-protein A at pH 8.5 as described in Chapter 3. Shown is the autoradiogram of the treated gel. Exposure was for 28 hr at -70° with an intensifying screen.

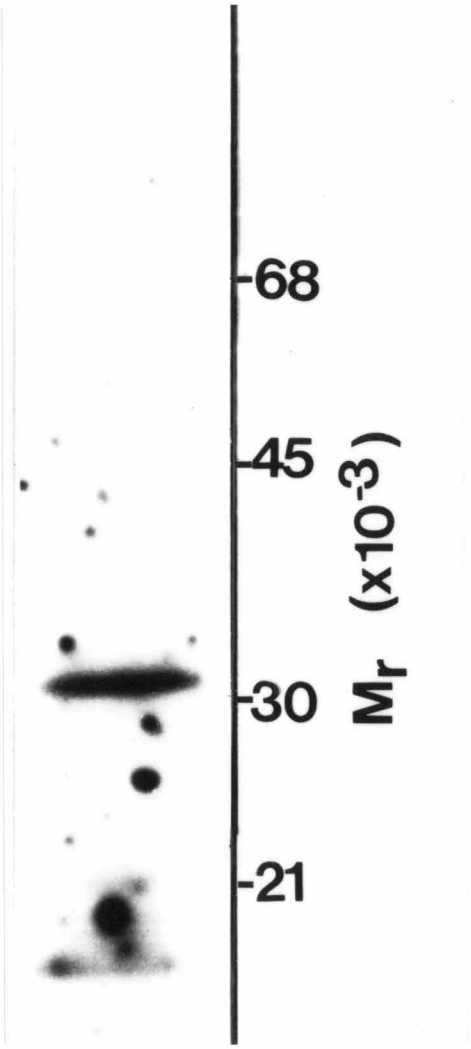
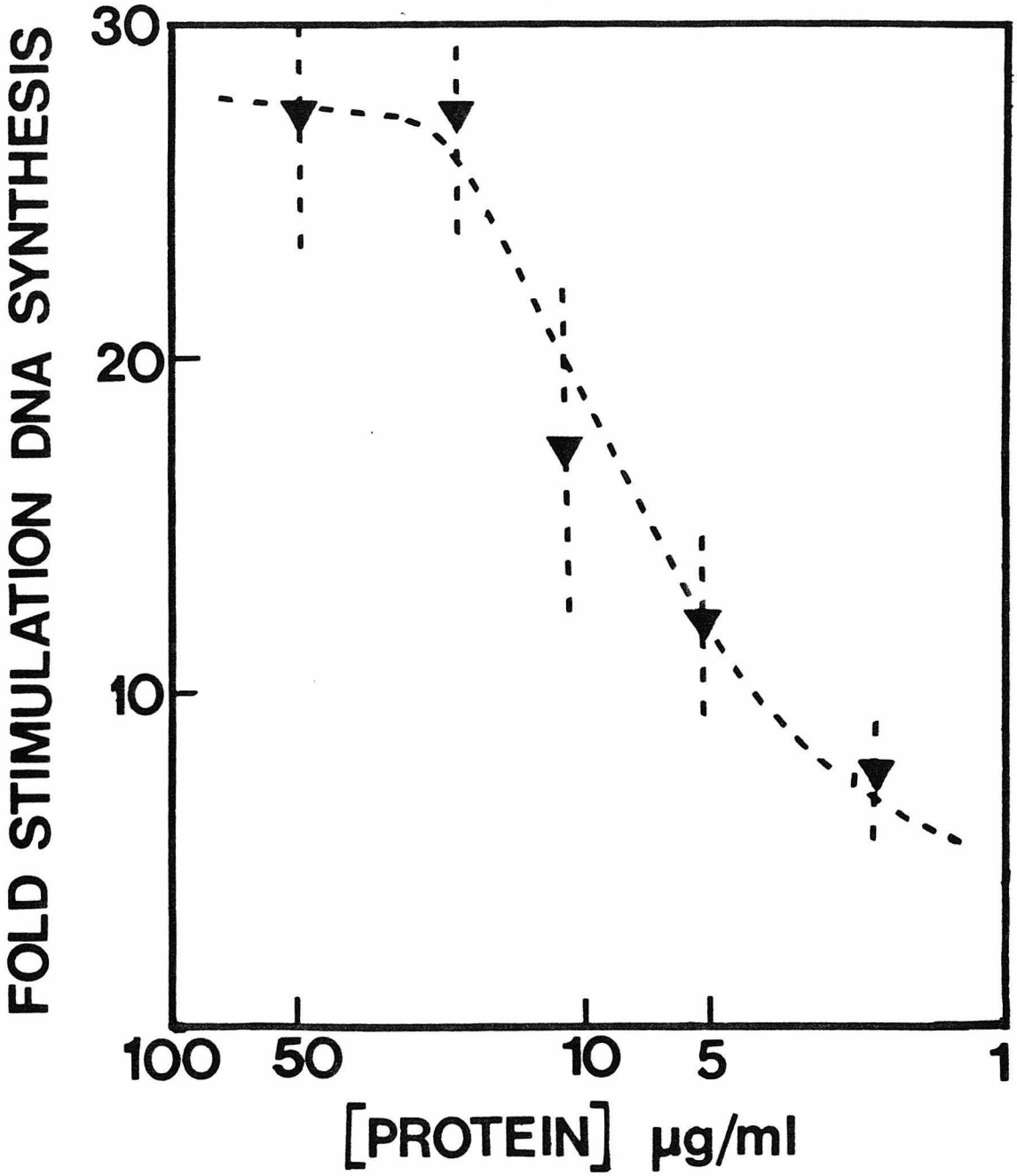

68
45
30
21
M_r ($\times 10^{-3}$)

Figure 5. Titration of chick brain GGF. Varying concentrations of a CM-cellulose fraction of chick brain GGF (as for Fig. 4) were assayed for their effect on Schwann cell proliferation. Dilutions were made into Hepes-buffered Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Twentyeight-fold stimulation at plateau is equivalent to 4,000 cpm incorporated.


FOLD STIMULATION DNA SYNTHESIS
30
20
10
100
50
10
5
1
[PROTEIN] µg/ml

CHAPTER 3

IDENTIFICATION AND PURIFICATION OF GLIAL GROWTH FACTOR

This material has been submitted for publication in the Journal of Neuroscience.

Abstract

Cultured rat Schwann cells are stimulated to divide by a protein growth factor, present in extracts of bovine brain and pituitary, which we have named Glial Growth Factor (GGF). Two lines of evidence indicate that GGF activity in both brain and pituitary resides in a protein of $M_r = 3.1 \times 10^4$. (1) Four independently isolated monoclonal antibodies that immunoprecipitate the activity react with an antigen of this molecular weight in SDS polyacrylamide gels. (2) After SDS polyacrylamide gel electrophoresis of partially purified preparations, mitogenic activity on Schwann cells is recovered at this molecular weight. GGF has been purified approximately 10^5-fold to apparent homogeneity from bovine pituitary anterior lobes by a combination of column chromatography steps and preparative SDS gel electrophoresis. Purified human platelet-derived growth factor, a molecule with properties similar to those of GGF, is inactive on Schwann cells and therefore appears to be distinct.

The growth, division and survival of animal cells in culture is dependent on the presence of growth factors and hormones. These molecules are often present in animal sera, tissue extracts and other undefined medium supplements. For some cell types it has been possible to replace the general serum requirement with an appropriate mixture of purified growth factors (see for example, Bottenstein et al., 1979). It has been suggested that the development of new cell culture systems will lead to the identification of new growth factors of biological significance (Ross and Sato, 1979).

We have described the use of immunological methods to obtain pure populations of rat Schwann cells from the neonatal sciatic nerve (Brockes et al., 1977; Brockes et al., 1979). These cells divide very slowly in a conventional tissue culture medium containing 10% fetal calf serum but they are strongly stimulated by an activity present in extracts of bovine brain and pituitary (Raff et al., 1978; Brockes et al., 1979). This activity is not detectable in extracts of non-neural tissue and is not mediated by a variety of purified growth factors and pituitary hormones (Raff et al., 1978). In a previous report (Brockes et al., 1980), the activity was partially purified from extracts of the bovine pituitary by a combination of cation exchange chromatography and gel filtration. The most purified fraction still displayed significant heterogeneity, but further electrophoretic analyses by native and SDS gels indicated that the activity was associated with a basic protein of molecular weight 3×10^4 which readily dimerized. In addition to its action on Schwann cells, this molecule was active in stimulating division of cultured astrocytes of the rat corpus callosum and of fibroblasts, but not of oligodendrocytes and macrophage-like microglia (Brockes et al., 1980). We have also reported on the activity in bovine brain (Brockes and Lemke, 1981; Brockes et al., 1981) which exhibits a reproducible variation in specific activity between regions and which is indistinguishable from that of the pituitary by electrophoretic and chromatographic criteria. In view of its

localization to the nervous sytem, and its action on Schwann cells and astrocytes, the molecule has been named Glial Growth Factor (GGF).

In this paper we describe two new lines of evidence which substantiate our earlier identification of the active species. In addition, we describe a method which permits purification of GGF to apparent homogeneity, and demonstrate its distinction from a previously characterized growth factor which is related in structure and function.

Materials and Methods

Materials. Lyophilized bovine anterior lobes, rats, Schwann cell tissue culture media and plastics, and radioisotopes were all purchased as described previously (Brockes et al., 1980). Protein A was from Pharmacia and was iodinated according to the method of Moore et al. (1982). Hybridoma materials were prepared or purchased as described by Moore et al. (1982). NIH 3T3 cells were a gift of Dr. Rex Risser, University of Wisconsin, Madison. Purified human platelet-derived growth factor was a gift of Drs. Elaine Raines and Russell Ross, University of Washington, Seattle.

Cell culture. Rat Schwann cells were dissociated from neonatal sciatic nerve, purified by immunoselective methods, and maintained as described previously (Brockes et al., 1977, 1979; Brockes et al., 1980). NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS).

^{125}I-UdR incorporation assay. The incorporation of ^{125}I-iododeoxyuridine (^{125}I-UdR) into Schwann cells growing in microwells was determined as described previously (Brockes et al., 1980; Brockes and Lemke, 1981). For constructing dose response curves, the stimulation of ^{125}I-UdR incorporation was plotted against the logarithm of protein concentration (see for example Figs. 4D and 5). Stimulation of DNA synthesis in NIH 3T3 was determined as follows: cells were grown to confluence in microwells in DMEM + 10% FCS. The medium was then changed and the cells were

maintained at confluence for an additional 4-5 d. Test solutions (diluted to an appropriate concentration in Hepes-buffered DMEM (HMEM) + 0.5 mg/ml bovine serum albumin (BSA)) were added for 48 hr and ^{125}I-UdR (2 μCi/ml) was added for the final 16-20 hr of this period. The cells were then harvested with a multiple sample device and counted as described previously for the Schwann cell microwell assay (Brockes et al., 1980).

Monoclonal antibody methods. Female Balb/c mice were immunized with partially purified fractions of GGF complexed with poly(I):poly(C) as an adjuvant as described previously (Lemke and Brockes, 1981). Spleen cells from responding animals were fused to SP-2/0 myeloma cells as described by Moore et al. (1982). Culture supernatants from the resulting hybridomas were screened for GGF binding by assaying their ability to precipitate growth factor activity from solution as follows: 40 μl of culture medium (or an appropriate dilution) was incubated with 10 μl of CM-cellulose fraction GGF (Brockes et al., 1980) at 400 μg/ml for 4 hr at 37°. Ten microliters of a solution of normal mouse IgG (60 μg/ml in HMEM + 10% FCS) and 10 μl of a solution of affinity purified rabbit anti-mouse IgG (400 μg/ml) were then added. The mixture was incubated at 37° for 30 min and then overnight at 4°. After centrifugation at 20,000 x g for 4 min at 4°, 20 μl of the resulting supernatant was tested in the microwell proliferation assay (see Lemke and Brockes, 1981).

Antibody reactions with partially purified GGF fractions resolved on SDS gels were performed essentially as described by Moore et al. (1982), except that anti-mouse IgG was not used and all reactions were carried out at pH 8.5 to permit ^{125}I-protein A binding to monoclonal IgG_1 antibody (Ey et al., 1978); Anti-GGF antibody E IgG_1 (purified to near homogeneity from ascites fluid by DEAE-cellulose chromatography) was used at 0.9 μg/ml. Autoradiography was performed using Kodak XAR-5

x-ray film with an intensifying screen. Exposures were carried out at -70° for 18-28 hr.

Analytical SDS gel electrophoresis. Small-scale (5.5 x 7.0 x 0.1 cm, 1.5 cm stack) SDS gel electrophoresis was conducted according to the methods of Laemmli (1974), but with modified concentrations of BIS-acrylamide (0.11% for 12.5% acrylamide gels and 0.09% for 15% acrylamide gels). Gels were stained either with 0.2% Coomassie Blue (in 50% methanol, 10% acetic acid) or according to the silver staining procedure described by Morrissey (1981). Samples to be analyzed for migration of growth factor activity were solubilized in 0.5 volumes sample buffer (80 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS) by heating at 37° for 2-3 min in the absence of disulfide reducing agent. After electrophoresis, the sample lane was excised from the gel, sliced into 2.5 mm segments, and the segments placed into sterile snap-cap tubes and washed with 1 ml of sterile phosphate-buffered saline (PBS) for 30 min at room temperature by vigorous agitation. The PBS was replaced with 1 ml of fresh buffer and a second wash performed for an additional 10 min. The PBS was then removed and replaced with 150 µl of HMEM containing 10% FCS. Proteins were eluted from the segments by shaking overnight at 4°. Fifteen to twenty microliters of each eluate was assayed in the standard microwell proliferation assay.

Large scale purification of GGF. CM-cellulose fractions of glial growth factor were prepared from 20,000 and 10,000 lyophilized anterior lobes as described previously (Brockes et al., 1980). In brief, tissue was extracted at pH 4.5, fractionally precipitated with ammonium sulfate, and chromatographed through batch elution from CM-cellulose. This material was then processed in two alternative ways. In procedure I (20,000 lobes, starting material), the CM-cellulose fraction was re-applied to a 200 ml CM-cellulose column equilibrated with 0.1 M sodium phosphate, pH 6.0 (P buffer) + 0.025 M NaCl, and then eluted with a 2 l linear gradient from 0.025 M-0.210 M NaCl in P buffer. Active fractions (see Fig. 4A) were pooled (total

volume = 550 ml), precipitated with 309 g ammonium sulfate, resuspended in P buffer + 0.4 M NaCl and chromatographed on an AcA 44 Ultrogel column exactly as described previously (Brockes et al., 1980). Active fractions (see Fig. 4B) were pooled, applied to a 5.5 ml column of phosphocellulose P11 equilibrated with P buffer + 0.4 M NaCl and then eluted with a 60 ml linear gradient from 0.4 M to 0.85 M NaCl in P buffer. GGF was purified from active fractions of the phosphocellulose elution by small-scale SDS gel electrophoresis and elution by the procedure of Mendel-Hartvig (1981). In procedure II (10,000 lobes, starting material), the CM-cellulose fraction (in P buffer + 0.35 M NaCl) was applied directly to a 25 ml phosphocellulose column, equilibrated with P buffer + 0.35 M NaCl and then eluted with a 200 ml linear gradient from 0.35 M-0.85 M NaCl in P buffer. Active fractions from this run were pooled and concentrated to 3.5 mg/ml in an Amicon cell (total protein recovered = 35 mg). Six-milligram portions of this material were then purified by successive preparative gel electrophoreses under native and denaturing conditions. Native gel electrophoresis was performed in slab gels (10 cm x 12.5 cm x 0.7 cm, 2.5 cm stack) using the buffer system of Reisfeld et al. (1962), modified as described previously (Brockes et al., 1980). Samples were prepared by mixing them with 1/2 volume of 1.5% agarose at 40° for 1 min, applied to a preformed well in the native stacking gel and allowed to solidify. Electrophoresis was conducted at 50 mA at 4° for approximately 7 hr, after which time a gel slice from 0.38 to 0.5 mobility relative to cytochrome C was excised and cut into fine segments. The segments were placed in a 15 ml tube with 5 ml of 2 x PBS and shaken 15 min. The PBS was removed and 5 ml of elution buffer (20 mM Tris/HCl pH 6.8, 0.5% SDS) added. Proteins were then eluted from the segments by shaking overnight at room temperature. The elution buffer was removed, an additional 5 ml of buffer added, and a second overnight elution was performed. The two eluates were pooled, lyophilized and adjusted to approximately 2 ml with distilled water. Glycerol was added to 10% final

concentration and the sample was heated to 37° for 2-4 min before application to second dimension SDS gels. Large-scale (10 cm x 12.5 cm x 0.5 cm, 12.5 cm stack) SDS gel electrophoresis was carried out at room temperature at 25 mA for approximately 18 hr. Resolved proteins were visualized by staining the gel with Coomassie blue (2% in 20 mM Tris/HCl pH 6.8) for 1 hr, followed by a 2 hr destain (in 20 mM Tris/HCl pH 6.8) with several changes. The GGF band was identified by its migration relative to carbonic anhydrase and excised with a scalpel. The gel slice was incubated in SDS running buffer with 0.05% bromophenol blue for approximately 30 sec and then positioned on top of a second SDS slab (4 cm x 12.5 cm x 0.6 cm) for electroelution exactly as described by Mendel-Hartvig (1982). Eluted proteins were collected in 80 mM Tris/HCl pH 8.7, 30% glycerol, 0.5% SDS.

Results

Molecular identification of GGF by monoclonal antibodies. Two new lines of evidence support our earlier tentative identification of GGF as a protein with an apparent molecular weight of 31,000 when analyzed by SDS gel electrophoresis (Brockes et al., 1980).

After immunizing with partially purified fractions from a large scale purification of bovine pituitary GGF, we derived four mouse monoclonal antibodies apparently directed against this activity (Lemke and Brockes, 1981). An assay of one of these reagents (termed antibody ER) is shown in Fig. 1. A partially purified fraction of GGF was incubated with culture medium from cells secreting ER, and immune complexes were then precipitated after addition of an appropriate amount of anti-mouse IgG followed by centrifugation. The resulting supernatant was assayed for GGF activity in the standard Schwann cell proliferation assay. Immunoglobulin from clone ER but not clone 4-F7 produced a dose dependent depletion of activity (Fig. 1). This depletion of activity was only demonstrable after indirect immunoprecipitation

of immune complexes (Lemke and Brockes, 1981). The four independent clonal isolates that showed this reactivity all secreted immunoglobulin of the IgG_1 subclass as determined by Ouchterlony double diffusion analysis with subclass specific antisera.

The antigen reactive with these reagents was investigated by running partially purified fractions of GGF on SDS gel electrophoresis and incubating with each of the monoclonal antibodies after the method of Burridge (1978). Bound IgG_1 was detected by reaction with ^{125}I-labeled protein A at pH 8.5 (Ey et al., 1978) followed by autoradiography. The results of such an experiment with antibody E are shown in Figs. 2A and B. Although many protein species were detectable on the Coomassie blue stained gel (Fig. 2A), the antibody reacted strongly and specifically with a single minor band of M_r = 31,000 (31 K) (Fig. 2B). Each of the four independent monoclonal isolates reacted in the same way. There was evidence of very minor diffuse reactivity with protein species in the 15-30 K range and at the dye front in some but not all preparations (see Fig. 2B), which may be a consequence of degradation. These antibodies also recognize a 31 K antigen in partially purified GGF preparations from bovine caudate nucleus and from whole chick brain (data not shown).

Molecular identification of GGF by SDS gel electrophoresis. We have found that GGF activity can be recovered from SDS gels run in the absence of disulfide reducing agents. Figure 3A shows an experiment in which a partially purified preparation of GGF (phosphocellulose fraction, see Fig. 4C) was analyzed on a 12.5% SDS gel, using the standard Laemmli discontinuous buffer system. One lane was silver stained to detect proteins, and the other was sliced into segments which were washed in buffer and then eluted into tissue culture medium (see Materials and Methods). GGF activity was detected as a single peak of $M_r = 3.1 \times 10^4$. No activity was recovered if the gels were run in the presence of disulfide reducing agents such as 2% β-mercaptoethanol.

GGF activity is detectable in bovine brain (Raff et al., 1978) and shows a regional variation in specific activity with a particularly high level in the caudate nucleus (Brockes and Lemke, 1981; Brockes et al., 1981). The activity in the caudate is indistinguishable from that in the pituitary by the criteria of native gel electrophoresis at pH 4.5 and ion exchange chromatography on phosphocellulose (Brockes and Lemke, 1981). When partially purified preparations of caudate GGF were analyzed by SDS gel electrophoresis, a major peak of activity was detected at 31 K (Fig. 3B). In addition, a minor peak of activity was detectable at 56 K in this experiment. This species, which constitutes less than 10% of the activity in the 31 K peak when corrected for the logarithmic dosage relationship (Lemke and Brockes, 1981; see Fig. 4D), has also been detected at low and variable levels in some less purified preparations of the pituitary activity. The existence of aggregates of the 31 K species is considered further in the discussion.

Large scale purification of bovine pituitary GGF. We have purified the 31 K species of GGF to apparent homogeneity by combining the previously described procedures of column chromatography and native gel electrophoresis (Brockes et al., 1980) together with a final step of preparative SDS gel electrophoresis. In the procedure outlined in Table I, the CM cellulose fraction (see Brockes et al., 1980) from 20,000 lyophilized anterior lobes was further purified by gradient elution from CM cellulose (Fig. 4A), gel filtration on AcA 44 Ultrogel (Fig. 4B), and elution from phosphocellulose (Fig. 4C). Peak fractions from the phosphocellulose column gave plateau stimulation of Schwann cells at a concentration of 200 ng/ml (Fig. 4D) indicating a purification factor of approximately 10,000-fold with a yield of 3% (Table 1). After SDS gel electrophoresis, approximately 10% of the protein (Fig. 4D) and all of the activity (Fig. 3A) resided in a 31 K band. This species could be further purified by preparative SDS gel electrophoresis followed by the elution procedure of Mendel-Hartvig (Fig. 4D), yielding approximately 4 μg per thousand anterior lobes.

In an alternative procedure which employs fewer steps, the CM cellulose fraction (Brockes et al., 1980) from 10,000 anterior lobes was chromatographed on phosphocellulose essentially as in Fig. 4C, and then purified by preparative native gel electrophoresis at pH 4.5 (Fig. 4E) using the buffer system of Reisfeld et al. (1962) as described earlier (Brockes et al., 1980). The pH 4.5 fractions were passively eluted from the gel slices and further purified by SDS gel electrophoresis to yield the 31 K species (Fig. 4F). The material resulting from this procedure (Fig. 4F) showed significant activity in the Schwann cell proliferation assay (after dilution into medium with 1 mg/ml BSA and extensive dialysis to remove SDS) but at a concentration that was consistent with the loss of 95% of the activity due to the multiple electrophoretic steps.

Comparison with the platelet-derived growth factor (PDGF). PDGF has been purified from platelet lysates by virtue of its mitogenic effect on fibroblasts or 3T3 cells (Ross et al., 1979; Heldin et al., 1981a; Deuel et al., 1981). It shares several properties with GGF as defined in this paper and previously (Brockes and Lemke, 1981). Both are basic proteins of $M_r = 3.1 \times 10^4$ that are functionally inactivated by disulfide reducing agents, are relatively heat stable, and act on fibroblasts. To further investigate their relationship, we assayed a preparation of purified human PDGF (Raines and Ross, 1982) for its ability to stimulate DNA synthesis in cultured rat Schwann cells. The preparation was active (Fig. 5) on mouse NIH 3T3 cells in the range of 1-20 ng/ml as previously reported (Raines and Ross, 1982), but gave no significant stimulation of Schwann cells at these concentrations. A small but reproducible stimulation was observed at 100 ng/ml. The Schwann cells used in these experiments were tested in parallel with the CM cellulose fraction (Brockes et al., 1980) of GGF and were strongly stimulated with a normal dose response curve (Fig. 5).

Discussion

The two lines of evidence presented in this paper provide strong support for the identification of GGF activity with a 31 K protein. Our earlier evidence for this was based on native gel electrophoresis of a partially purified preparation and was suggestive but not conclusive. The ability of a set of four monoclonal antibodies to both specifically precipitate GGF activity from solution and bind to the 31 K species is independent evidence for the association of mitogenic activity with this molecule. It might still be argued, however, that GGF activity derives from a smaller component that is adventitiously associated with the 31 K protein. This possibility is made very unlikely by the demonstration that growth factor activity is recovered at 31 K after denaturing electrophoresis in SDS.

Although we now consider this association to be established, the presence of oligomers is not fully understood. In previously reported gel filtration experiments (Brockes et al., 1980), the major peak of growth factor activity was observed to migrate with an apparent molecular weight of 5.6×10^4 (GGF dimer), while in procedure I, activity was observed predominantly at a molecular weight of 3.1×10^4 (GGF monomer). We have found that the GGF dimer is largely removed as a result of the gradient elution from CM-cellulose of this latter procedure: if subjected to SDS gel electrophoresis under non-reducing conditions, material from pool a (Fig. 4A) gives a major peak of activity at $M_r = 5.6 \times 10^4$ (data not shown). Significant dimerization of GGF has not been detected in the more highly enriched fractions (AcA 44 [Fig. 4B] and phosphocellulose p11 [Fig. 3A]) subsequently prepared from pool b (Fig. 4A).

The two purification procedures that are reported above result in apparently homogeneous preparations of GGF, but ones whose bioactivity is significantly reduced after the preparative SDS electrophoresis steps. The second procedure, which employs a combination of native gel electrophoresis at pH 4.5 and SDS gel

electrophoresis, is similar in design to that employed by Barde et al. (1982) in the isolation of a neurotrophic factor from pig brain and is a modification of the two dimensional analytical procedure that we used previously (Brockes et al., 1980). Attempts to purify GGF by using the monoclonal antibodies as immobilized affinity reagents have thus far been largely unsuccessful. Although growth factor activity is purified approximately 10^5-fold and the estimated potency of highly purified GGF is comparable to that of other purified mitogenic growth factors, it might still be formally argued that the final preparation of the 31 K band is not homogeneous and contains one or more components of identical molecular weight which co-purify with GGF through the multiple steps of the purification procedures. For this reason, the homogeneity is termed "apparent". Amino acid sequence analysis of the purified preparation followed by the derivation of antisera to synthetic peptides, or the cloning and subsequent manipulation of the gene coding for this protein are clearly important elements of a definitive identification of the GGF molecule.

While we have not undertaken an investigation of the mechanism of action of GGF at the cellular level, some of our data bear on this question. The equilibrium dissociation constant of the putative Schwann cell receptor for GGF can, for example, be estimated from the midpoint of dose response curves of highly purified fractions (such as the 10% pure preparation of Fig. 4D) to be approximately 10^{-10} M. This value is comparable to that exhibited by high affinity cell receptors for epidermal growth factor (Carpenter et al., 1975) and PDGF (Bowen-Pope and Ross, 1982; Heldin et al., 1981b) and indicates that GGF acts in a "hormonal" concentration range.

Although PDGF and GGF are strikingly similar, our results (Fig. 5) clearly demonstrate a functional disparity with respect to their action on Schwann cells. Additionally, GGF, unlike PDGF, appears to be a single polypeptide, in that treatment with disulfide reducing agents does not alter the observed molecular weight of the

purified protein on SDS gels or the binding of anti-GGF antibodies to the 31 K antigen (data not shown). It seems possible, nevertheless, that these molecules are members of a family of growth factors, some of whose target cells (e.g. fibroblasts) overlap. The lack of effect of PDGF on Schwann cells is consistent with the relatively weak mitogenicity of 10% serum on these cells. It points to the importance of Schwann cell proliferation as a distinctive test for natural growth factors—in our studies we have found no defined mitogen other than GGF which stimulates their division. The relation of GGF to the cell surface mitogen of cultured neurites and CNS axolemma (Salzer and Bunge, 1980; De Vries et al., 1982) awaits chemical and immunochemical characterization of the latter.

Although there is currently considerable interest in the mechanism of action of mitogenic growth factors at a molecular level, almost nothing is known about their significance in vivo. There is little information about their "endocrinology" in the sense of where they are made and released, or of the identity of their targets under circumstances of cell division in development and regeneration. The location of GGF in the nervous system and pituitary raises some possibilities as to its function which we have discussed elsewhere (Brockes and Lemke, 1981). It might be released from the pituitary as a circulating hormone. In addition, it may play a role in the control of glial cell division in development and after injury to the CNS and PNS. Finally, it might be important in that subset of neurotrophic phenomena which appear to reflect a mitogenic effect of nerves. As well as bovine brain and pituitary and rat pituitary (Raff et al., 1978), we have detected GGF activity on rat Schwann cells in chick, frog, newt and axolotl brain (unpublished results). It has also been detected in the regeneration blastema of the axolotol, a context where nerves appear to stimulate division of the progenitor cells of the regenerate (Singer, 1952). The chemical and immunochemical characterization reported in this paper is clearly a prerequisite for a critical evaluation of all these possibilities.

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Figure 1. Titration of monoclonal anti-GGF. 4-F7 (■—■) is a hybridoma secreting IgG that does not react with GGF, whereas AbER (●—●) does. Varying amounts of culture supernatants were incubated with the CM-cellulose fraction of GGF, followed by carrier mouse IgG and affinity purified rabbit anti-mouse IgG as described in materials and methods. After centrifugation, the incubation mixtures were assayed for their ability to stimulate ^{125}I-UdR incorporation into Schwann cells in the microwell assay. The depletion of GGF activity by AbER is corrected by the logarithmic dose-response relationship (see Fig. 4D) to give the estimated amount of GGF protein remaining in solution (▼—▼). One unit of IgG is that present in 40 µl of 4-F7 culture supernatant as determined by a quantitative ELISA procedure. The values for AbER are normalized to this. Each point represents the mean ± S.D. of three microwell assays.

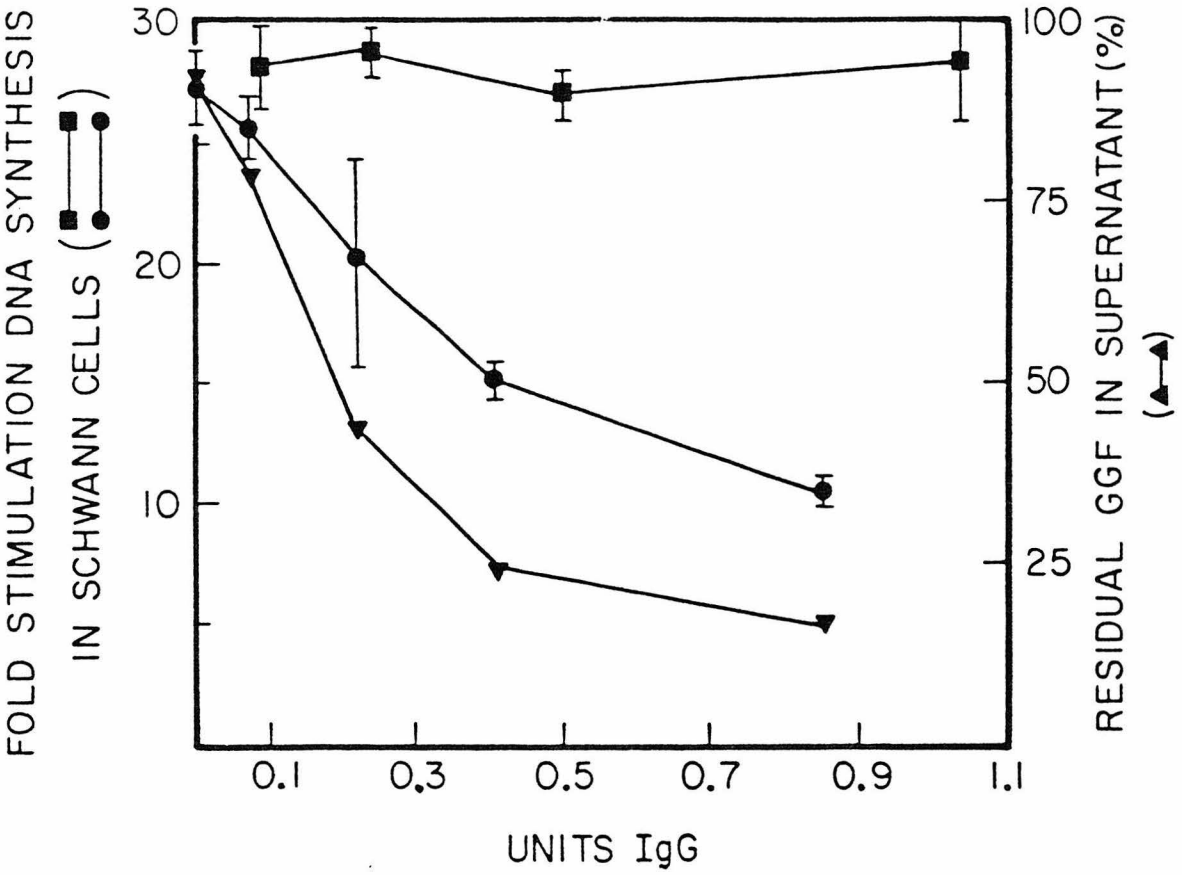

FOLD STIMULATION DNA SYNTHESIS
IN SCHWANN CELLS (■—●)
30
20
10
0.1
0.3
0.5
0.7
0.9
1.1
UNITS IgG
100
75
50
25
RESIDUAL GGF IN SUPERNATANT (%)
(▲—▲)

Figure 2. Analysis of the target antigen of monoclonal anti-GGF by SDS gel electrophoresis. Forty-five micrograms of the CM-cellulose fraction of GGF (Brockes et al., 1980) was electrophoresed through a 15% SDS-polyacrylamide gel (see Materials and Methods) in the presence of 2% β-mercaptoethanol. The gel was reacted with AbE and ^{125}I-protein A, stained with Coomassie blue and then autoradiographed (overnight at -70°) as described in Materials and Methods. Figure 2A shows the proteins as visualized with Coomassie blue. Figure 2B is an autoradiogram of the same gel, showing the binding of antibody/^{125}I-protein A at M_r = 31 K.

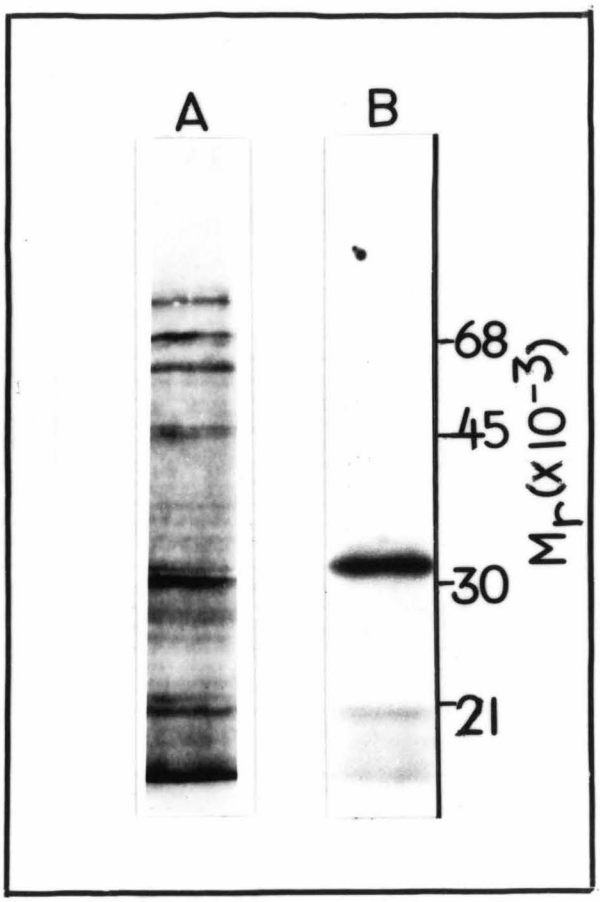

A
B
68
45
30
21
Mr(x10-3)

Figure 3. Identification of GGF activity after SDS-polyacrylamide gel electrophoresis. A, Electrophoresis in a 12.5% gel of approximately 1 μg of a phosphocellulose fraction of GGF from the bovine pituitary (∿10% pure; see below). Samples were solubilized (without disulfide reduction) and electrophoresed as described in Materials and Methods. The sample lane was excised, sliced into 2.5 mm segments, and the proteins were eluted from each segment into medium (see Materials and Methods). The GGF activity eluted from the gel slices was measured in the Schwann cell proliferation assay. The silver-stained profile of unreduced proteins and the migration of reduced molecular weight markers are from parallel lanes of the same gel. B, Electrophoresis in a 12.5% gel of 30 μg of a phosphocellulose fraction of GGF prepared from the caudate nucleus of the bovine brain (0.3% pure). Electrophoresis, elution of proteins and analysis were as for A, except that the proteins were visualized with Coomassie blue.

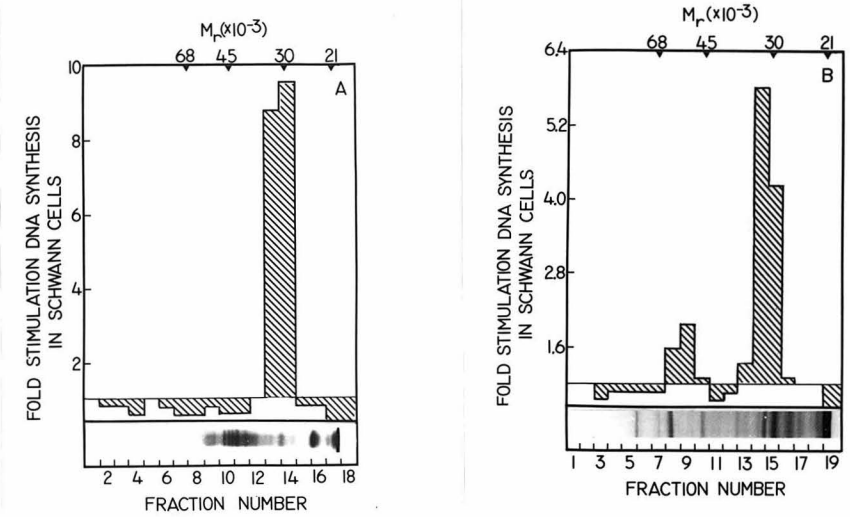
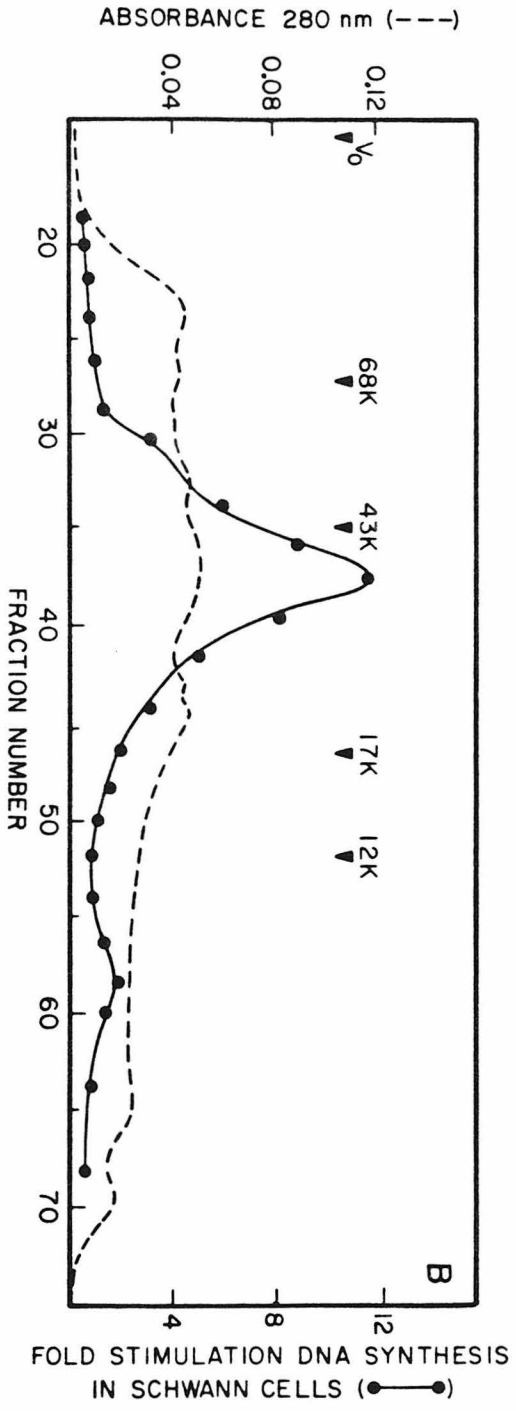

M_r(x10^{-3})
68 45 30 21
A
FOLD STIMULATION DNA SYNTHESIS IN SCHWANN CELLS
10
8
6
4
2
2 4 6 8 10 12 14 16 18
FRACTION NUMBER
M_r(x10^{-3})
68 45 30 21
B
6.4
5.2
4.0
2.8
1.6
1 3 5 7 9 11 13 15 17 19
FRACTION NUMBER

Figure 4. Steps in the large scale purification of GGF. A, Gradient elution of the CM-cellulose fraction from CM cellulose. The batch eluted CM cellulose fraction (1090 mg) was applied to a CM cellulose column and eluted with a linear NaCl gradient as described in Materials and Methods. Fractions were collected and assayed at 3 μg/ml in the Schwann cell proliferation assay. Two pools were made and pool b was used for further purification (the properties of pool a are considered in the Discussion). B, Elution profile of the (gradient eluted) CM-cellulose fraction after gel filtration on AcA 44 Ultrogel. The CM cellulose fraction (210 mg) was chromatographed on a calibrated column of AcA 44 as described in Materials and Methods section. Fractions were collected and assayed at 0.5 μg/ml. Fractions 36 to 40 were pooled for further purification.


ABSORBANCE 280 nm (---)
0.04
0.08
0.12
V_0
68K
43K
17K
12K
20
30
40
50
60
70
FRACTION NUMBER
B
4
8
12
FOLD STIMULATION DNA SYNTHESIS
IN SCHWANN CELLS (●—●)

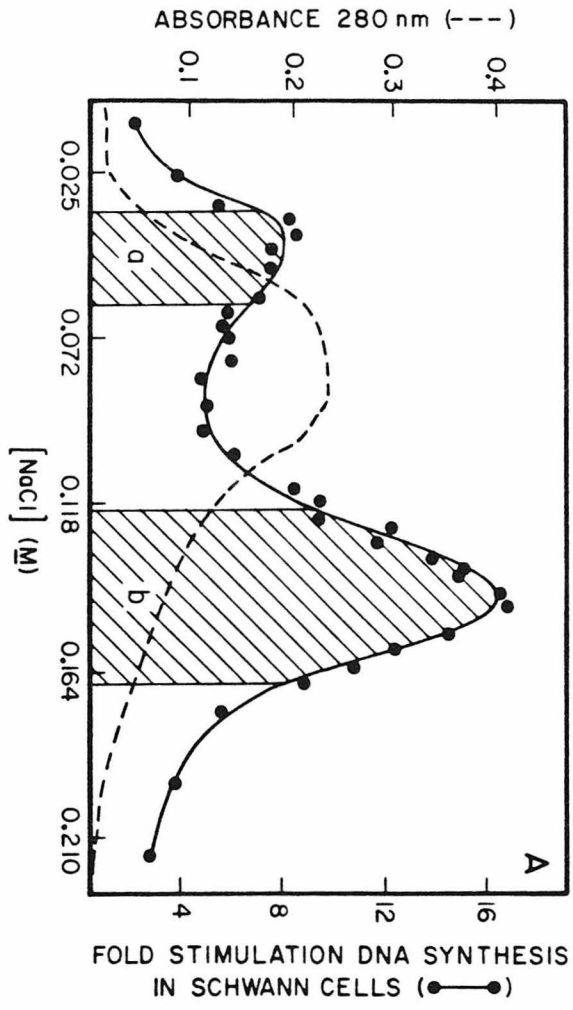

ABSORBANCE 280 nm (---)
0.1
0.2
0.3
0.4
0.025
0.072
0.118
0.164
0.210
[NaCl] (M)
a
b
A
4
8
12
16
FOLD STIMULATION DNA SYNTHESIS
IN SCHWANN CELLS (●—●)

Figure 4C. Elution profile of the AcA 44 fraction from phosphocellulose. The AcA 44 fraction (19 mg) was applied to a phosphocellulose column and eluted with a linear NaCl gradient as described in Materials and Methods section. Fractions were collected and assayed at 0.2 μg/ml; no activity was detectable in the flow through.

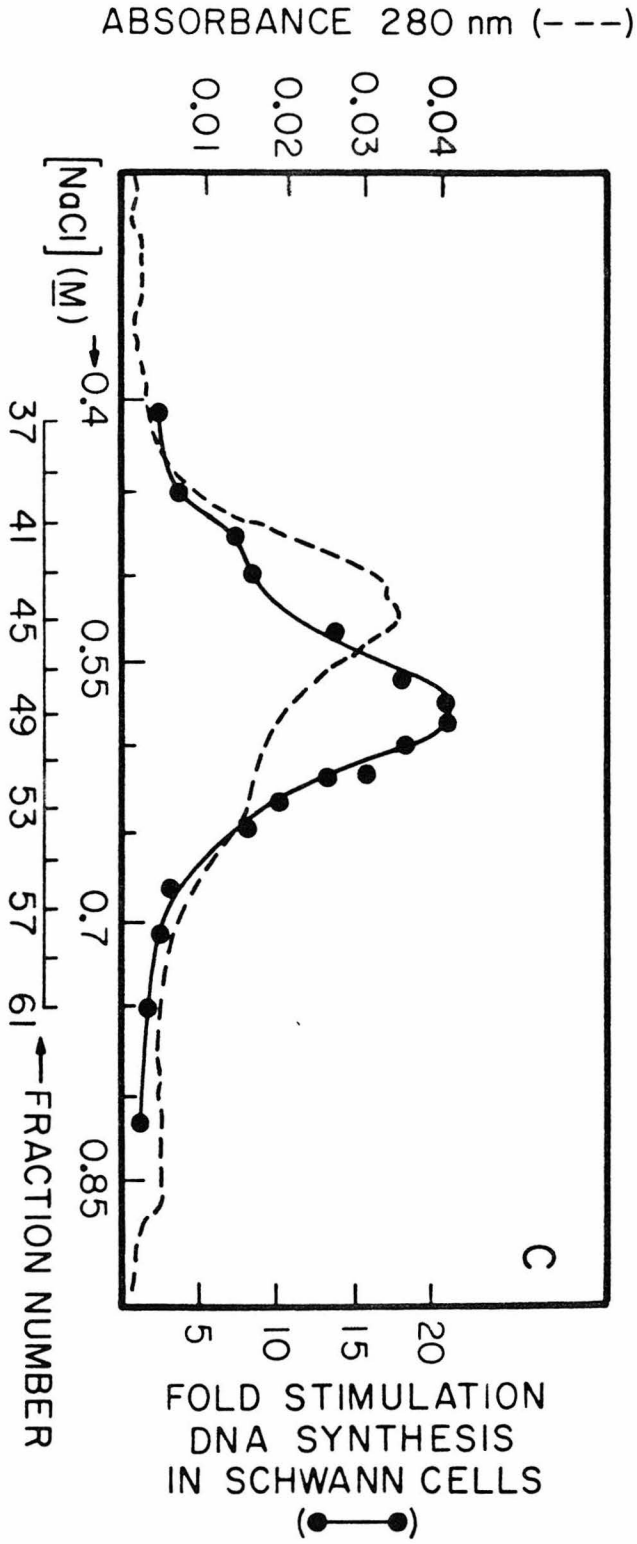

ABSORBANCE 280 nm (- - -)
0.01
0.02
0.03
0.04
[NaCl] (M)
0.4
0.55
0.7
0.85
37
41
45
49
53
57
61
FRACTION NUMBER
C
5
10
15
20
FOLD STIMULATION
DNA SYNTHESIS
IN SCHWANN CELLS
(●—●)

Figure 4D. Dose response curve and further purification of the phosphocellulose fraction. Phosphocellulose fraction (fraction 49 of Fig. 4C) was diluted in medium and assayed in the Schwann cell proliferation assay. Inset (i) Proteins of this fraction (1 μg) were analyzed on a 12.5% SDS gel followed by silver staining. The 31 K GGF band accounts for approximately 10% of the protein as determined densitometrically, and can be purified to apparent homogeneity by excision from the gel and subsequent elution (see Materials and Methods). 0.1 μg of eluted material is shown (ii) after SDS gel electrophoresis followed by silver staining.

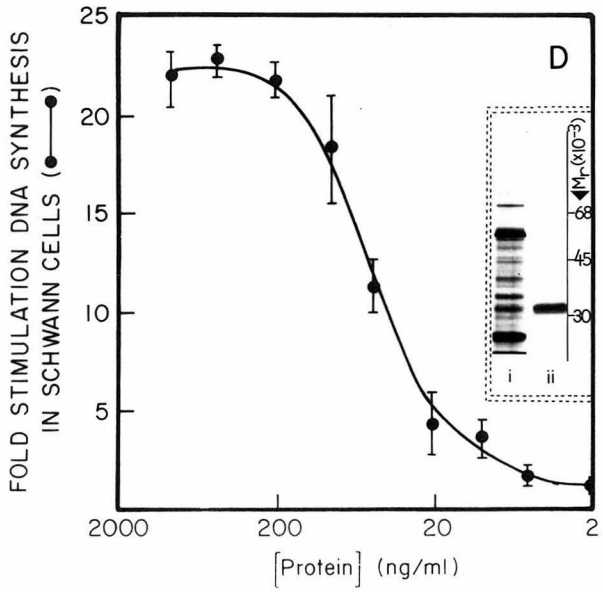

FOLD STIMULATION DNA SYNTHESIS
IN SCHWANN CELLS (●—●)
25
20
15
10
5
2000
200
20
2
[Protein] (ng/ml)
D
M_r (x10^{-3})
68
45
30
i
ii

Figure 4E. Profile of phosphocellulose fractions after native gel electrophoresis at pH 4.5. Phosphocellulose fraction (6 mg), derived as described in the text, was subjected to polyacrylamide gel electrophoresis at pH 4.5 as described in Materials and Methods. The migration of GGF activity relative to cytochrome C was determined previously on pilot scale gels. A slice of $R_{\text{cytochrome C}} = 0.38$ to 0.5 was excised and eluted as described in Materials and Methods. A parallel lane of the pH 4.5 gel was sliced into 3 mm segments which were eluted into medium and assayed in the Schwann cell proliferation assay. The activity profile in the parallel lane is shown together with the region (shaded) excised from the sample lane.

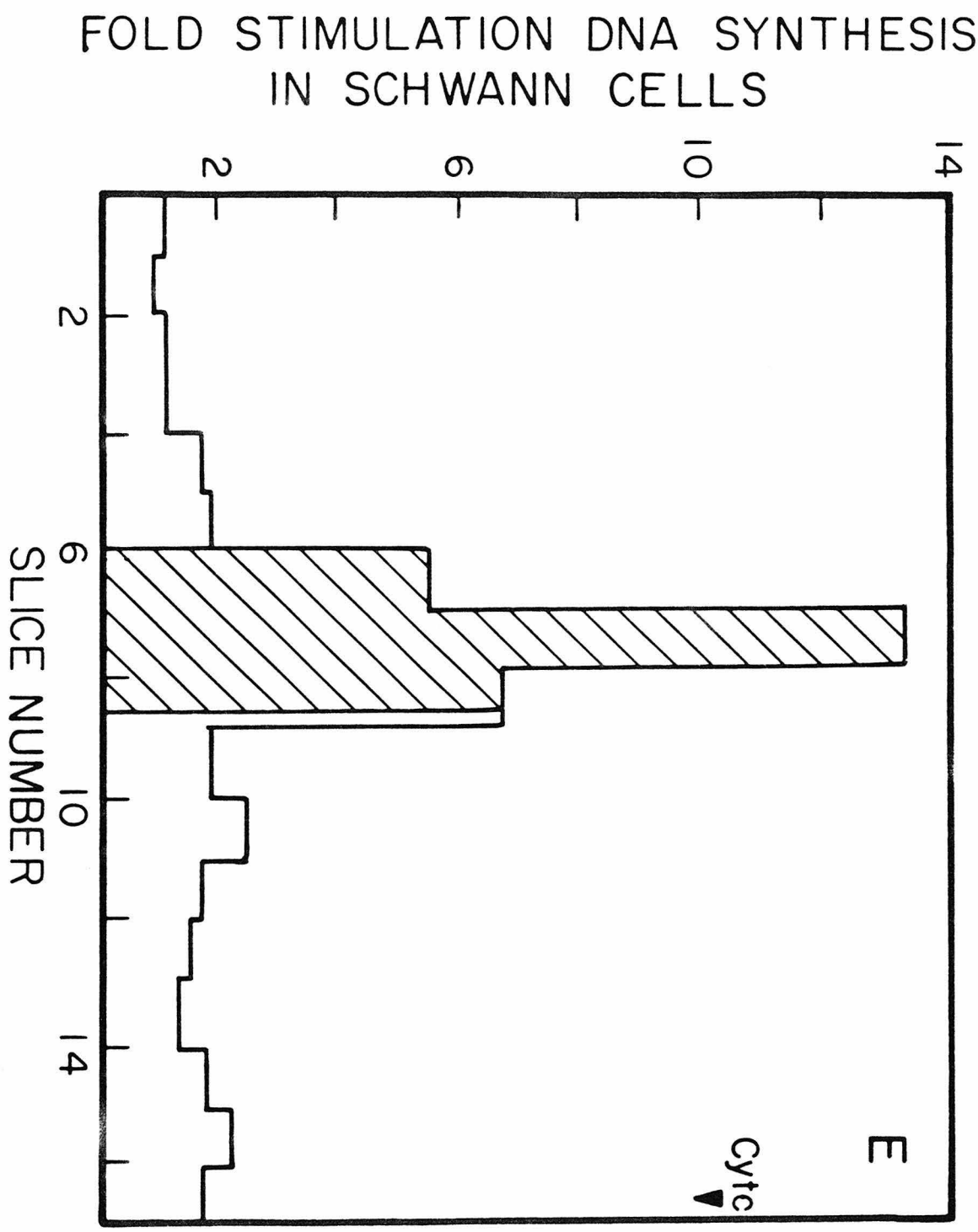

FOLD STIMULATION DNA SYNTHESIS
IN SCHWANN CELLS
2
6
10
14
SLICE NUMBER
2
6
10
14
E
Cytc

Figure 4F. SDS gel electrophoresis. (a) Shown is the native gel eluate as resolved by electrophoresis through a preparative scale SDS polyacrylamide slab gel (see Materials and Methods). The GGF band (arrowed) was identified after staining with Coomassie blue by its migration to a position just above that for carbonic anhydrase. A gel slice containing this band was excised from the slab and GGF was eluted according to the procedure of Mendel-Hartvig (1982). An analytical SDS gel of the eluted material (0.2 μg) is shown in (b).

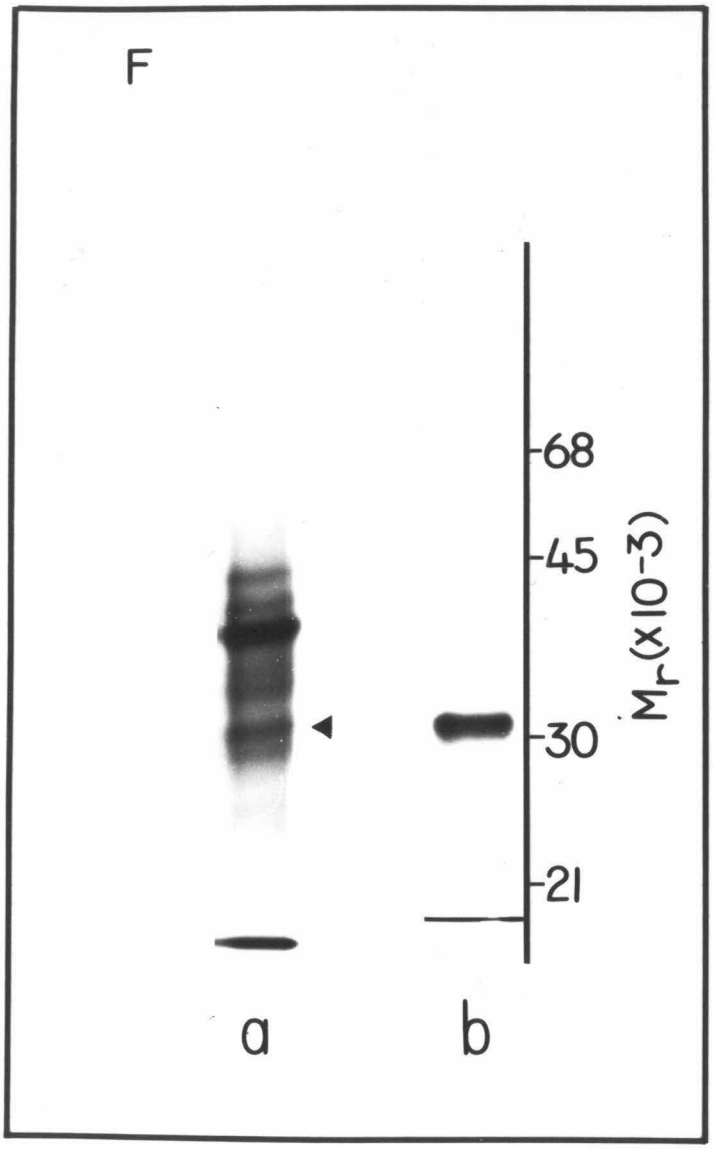

F
68
45
30
21
M_r(x10-3)
a
b

Figure 5. Comparison with PDGF. Purified human PDGF was diluted to varying concentrations with HMEM + 0.5 mg/ml BSA and assayed for mitogenic activity simultaneously on both Schwann cells and NIH 3T3 fibroblasts. Proliferation assays were performed in microwells with ^{125}I-UdR as label as described in Materials and Methods. As a control, a CM-cellulose fraction of GGF was titered against the same batch of Schwann cells tested for PDGF response. ■—■ GGF: Schwann cells; ●—●, PDGF: Schwann cells; ▲- -▲ PDGF: 3T3 cells.

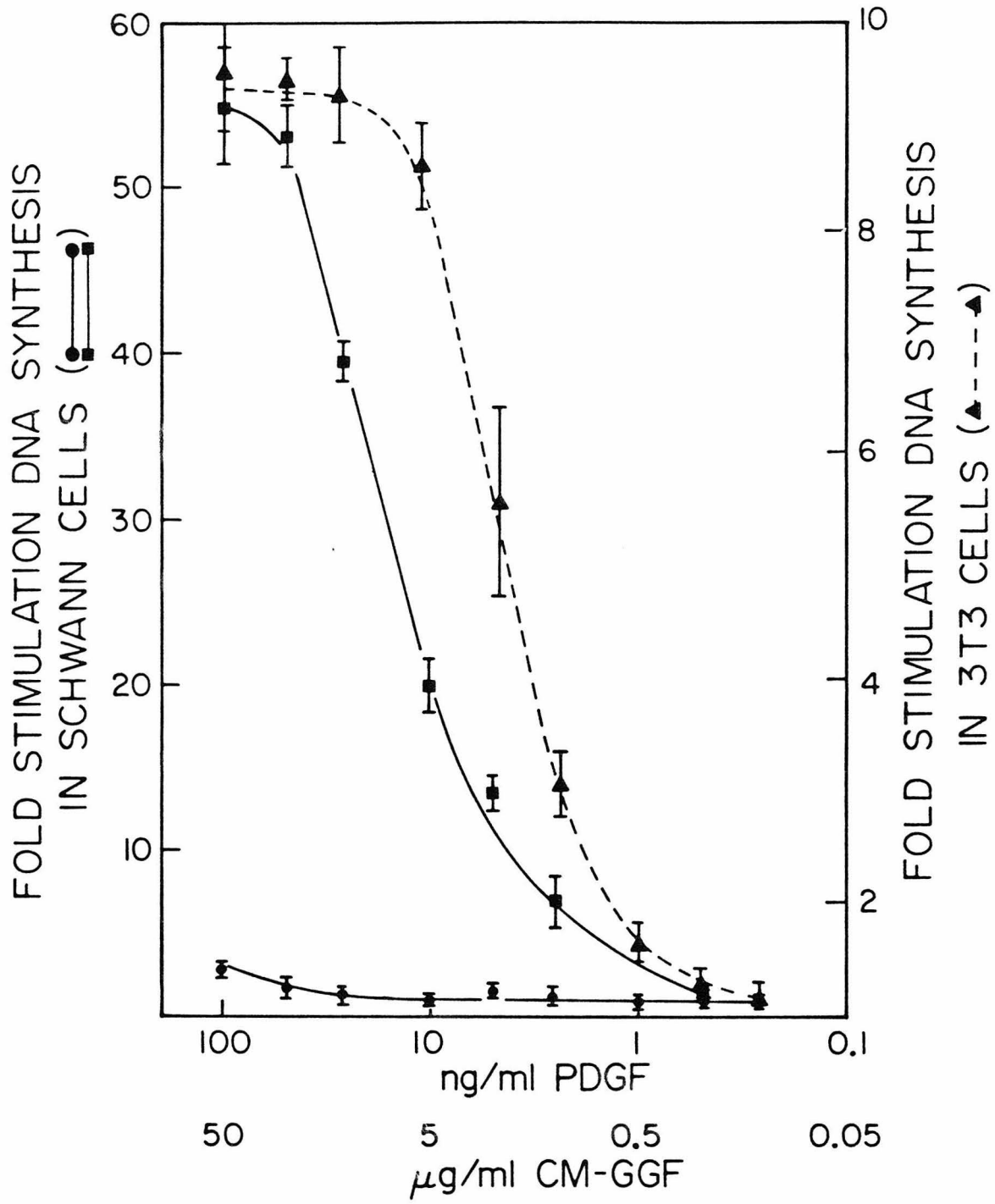

FOLD STIMULATION DNA SYNTHESIS
IN SCHWANN CELLS (●—■)
FOLD STIMULATION DNA SYNTHESIS
IN 3T3 CELLS (▲- - -▲)
60
50
40
30
20
10
10
8
6
4
2
100
10
1
0.1
ng/ml PDGF
50
5
0.5
0.05
μg/ml CM-GGF

Table 1. Summary of Purification

step	total protein (mg)	recovery of activity	fold purification
crude extract	400,000	----	----
ammonium sulfate fraction	202,000	100 %	2
CM-cellulose (batch elution)	1,200 (110 set aside)	30 %	100
CM-cellulose (gradient elution)	210	15 %	250
AcA 44 Ultrogel gel filtration	19	6.8 %	1,250
phosphocellulose	1.1	3.1 %	10,000
SDS gel electrophoresis	0.08*	0.45%**	100,000***

The activity was purified from 20,000 lyophilized anterior lobes as described in the text. The fold purification through the phosphocellulose step was determined from the displacement of dose-response curves (see Lemke and Brockes, 1981).

*This is an estimate based on protein recoveries (as determined by calibrated silver-stained gels) in small-scale elutions by the procedure of Mendel-Hartvig (1981).

**This is an estimate based on activity recoveries in small-scale elutions by the procedure described in Materials and Methods and illustrated in Figure 3.

***This figure is based on the observation that GGF accounts for 10% of the protein in the phosphocellulose fraction.

CONCLUSION

The biochemical identity of glial growth factor has been established through an in vitro convention—the maintenance of purified rat Schwann cells in tissue culture. These are the only cells known to exhibit a unique responsiveness to GGF. As such, this mitogen is to be distinguished from FGF and PDGF, which have been characterized by virtue of their action on "dedifferentiated" mouse 3T3 cell lines and human fibroblasts. Unlike rat Schwann cells, these cells respond to a host of purified polypeptide growth factors (Herschman et al., 1978; Gospodarowicz et al., 1978).

The discovery of GGF is best appreciated in the context of similar efforts to define the specific growth requirements of other functionally distinctive, highly differentiated cells maintained in vitro. T cell lymphocytes, for example, have been demonstrated to respond to a particular polypeptide mitogen—Interleukin-2 (formerly known as T-cell growth factor) (Morgan et al., 1976). Interleukin-2 has been shown to stimulate both the long-term in vitro growth of activated T cells (Gillis and Smith, 1977) and the induction of cytotoxic T cell reactivity (Gillis et al., 1979). These experiments have yielded important insights as to the functional interactions within mixed lymphocyte populations, and thus to key features of the regulation of the cellular immune response. Among other examples of in vitro studies of the growth requirements of highly differentiated cells are those of Farley and Baylink (1982), whose work has identified a polypeptide growth factor for cultured bone-forming cells, and those of Kasper et al. (1982), who have identified a polypeptide mitogen for cultured chondrocytes. For each of these cell types, an important consequence of their differentiated phenotype would appear to be the requirement of a unique signal to regulate their division. Given the diversity of functionally distinct cell types present in higher organisms, we can assume that many more such signals will be identified in the future.

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