Purification of Multiple Forms of Glial Growth Factor*

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Glial growth factors (GGFs) were purified from bovine pituitaries using an in vitro rat Schwann cell mitogenesis assay. In addition to an ~34-kDa species termed GGF-I, similar in molecular mass to a previously identified molecule (Lemke, G. E., and Brockes, J. P. (1984) J. Neuroscience 4, 75–83), two species named GGF-II and GGF-III were characterized with apparent molecular masses of ~59 and ~45 kDa, respectively. Highly purified preparations of all species share a similar dose-dependent stimulation of Schwann cell DNA synthesis at nanomolar concentrations. Forskolin synergizes with all three GGFs, shifting their dose dependence 3–8-fold into the sub-nanomolar range. The GGFs, which contain N-linked carbohydrate groups not essential for their in vitro mitogenic effects, are three distinct members of a novel family of glial cell mitogens.

Glia are fundamentally important cells of the vertebrate nervous system. Major classes include the astrocytes and oligodendrocytes of the central nervous system and the Schwann cells of the peripheral nervous system. Although the precise physiological roles of glial cells are still being defined, it is well established that oligodendrocytes and Schwann cells form myelin sheaths, which are essential for fast impulse conduction along larger diameter axons (Peters et al., 1991). Injury to the myelin sheath through mechanical trauma, neurotoxins, or demyelinating diseases has profoundly damaging functional consequences, which, in the central nervous system, are substantially irreversible (Ludwin, 1988). In contrast, damaged or severed axons in the peripheral nervous system can often regenerate through poorly understood repair processes, which are known to crucially involve the proliferation of Schwann cells (Fawcett and Keynes, 1990). In addition, post-developmental growth of Schwann cells has been implicated in the formation of schwannomas, which occur throughout the nervous system in clinical conditions such as type 2 neurofibromatosis and schwannomatosis and sporadically in a wide range of neural and non-neural sites (Riccardi, 1992).

Attempts to define the regulators of Schwann cell proliferation have been greatly aided by the development of purified cell populations cultured in vitro (Brockes et al., 1977, 1979). Early studies demonstrated that while purified rat sciatric nerve Schwann cells can survive in culture in the presence of fetal calf serum for long periods, they proliferate very slowly (Brockes et al., 1977; Raff et al., 1978a). However, addition of extracts from bovine pituitaries or brain tissue from a variety of vertebrate species considerably stimulates the proliferation of these cells (Brockes et al., 1979; Raff et al., 1978a). Subsequent purification studies employing a rat Schwann cell mitogenesis assay (Brockes et al., 1980; Lemke and Brockes, 1984) have demonstrated that an active component in such extracts is a 31-kDa basic protein named glial growth factor (GGF).1 This factor, which was purified to homogeneity, also stimulates the division of astrocytes and fibroblasts (Brockes, 1987).

The GGF activity defined by these studies has been implicated in two situations of particular biological interest. First, a Schwann cell mitogen with almost identical chromatographic properties to GGF has been observed in extracts of human acoustic neuromas (Brockes et al., 1986). This suggests a role for GGF in the abnormal proliferation of Schwann cells, which are the predominant cell type in these tumors. Second, a GGF-like activity is thought to be involved in newt limb regeneration (Brockes and Kintner, 1986), since a Schwann cell mitogen chromatographically indistinguishable from GGF has been observed in extracts of newt limb blastema (Brockes, 1984). Furthermore, denervation of the limb, which arrests blastemal proliferation, causes the mitogenic activity to fall to background levels suggesting that this factor could mediate the nerve-dependent stimulation of blastemal cell division (Brockes and Kintner, 1986).

Despite these striking observations, studies of the role of GGF in the biology of Schwann cells and other mesenchymal cells have been hampered by the absence of specific molecular probes, due to the low abundance of the protein and the absence of any sequence information. In addition, it is now known that rat sciatric nerve Schwann cells respond mitogenically to a surprisingly small group of growth factors, and this

1 The abbreviations used are: GGF, glial growth factor; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; [125I]dUdR, 5'-[125I]iodo-2'-deoxyuridine.
has stimulated additional interest in the identity of GGF (Raff et al., 1978a, 1978b; Porter et al., 1987; Ridley et al., 1988; Eccleston et al., 1988; Weinmaster and Lemke, 1990; Davis and Stroobant, 1990; Schubert, 1992). We have therefore developed high resolution chromatographic techniques to purify quantities of bovine pituitary GGF sufficient to enable initial studies of structure and cell biology to be performed on this apparently novel factor.

As a result of the work described here, a basic ~34-kDa Schwann cell mitogen with similar molecular properties to those reported for GGF has been purified and named GGF-I. In addition, two novel molecules with GGF activity have also been characterized and named GGF-II (molecular mass, ~59 kDa) and GGF-III (molecular mass, ~45 kDa). We describe procedures that yield highly purified preparations of all three molecules as judged by silver-stained SDS-PAGE and document the preliminary characterization of these Schwann cell mitogens.

MATERIALS AND METHODS

Chemicals

Poly-d-lysine hydrobromide (molecular mass, 30–70 kDa) and rabbit complement were obtained from Sigma; DMEM and 0.05% trypan blue, 0.02% EDTA in modified Puck’s saline were from Life Technologies, Inc.; batch-tested FCS and batch-tested fetal calf plasma were from Imperial Laboratories Ltd. (Andover, England); forskolin was from Calbiochem; anti Thy 1.1 was from Serotec Ltd. (Oxford, England); [3H]UdR was from Amersham International (Amersham, England); rabbit anti-rat S-100 was a gift from Dr. R. Mirsky (University College, London); HPLC solvents were from Rathburn Chemicals Ltd. (Walkerburn, Scotland); electrophoresis reagents were from Bio-Rad; bovine pituitaries were from Biogenesis Ltd. (Bournemouth, England); all other chemicals were reagent grade or better (BDH Laboratory Supplies, Dorset, England).

Purification of Glial Growth Factors

Preparation of GGF-carboxymethylcellulose Fraction—4,000 frozen whole bovine pituitaries (9–12 kg) were thawed over night, washed for 1 min with an equal volume of water, and homogenized for 3 × 45 s in an equal volume of 0.15 M ammonium sulphate in batches of 1,000 in a Waring Blender (4 liters). The homogenate was taken to pH 4.5 by adding 4,900 × g for 10 min. The precipitate was washed twice with 1.0 M HCl and centrifuged at 4,900 × g for 80 min. Patey material was removed by filtering the supernatant through glass wool. After taking the pH value of the supernatant to 6.5 using 1.0 M NaOH, solid ammonium sulfate was added to give a 36% saturated solution. After several hours of stirring, the suspension was centrifuged at 4,900 × g for 80 min and the precipitate discarded. After filtering the supernatant through glass wool, solid ammonium sulfate was added to give a 75% saturated solution, which was centrifuged at 4,900 × g for 80 min after several hours of stirring. The pellet was resuspended in 2 liters of 0.1 M sodium phosphate, pH 6.0, and dialysed against 3 × 40 liters of the same buffer (Spectrapor 6-8 K dialysis tubing, Spectrum). When the conductivity of the dialysate was below 20.0 mSiemens, it was loaded onto a Bioproces column (120 × 113 mm, Pharmacia LKB Biotechnology Inc.) packed with carboxymethylcellulose (CM-52, Whatman) at 2.0 ml/min. The column was washed sequentially with 2 volumes of 0.1 M sodium phosphate, pH 6.0, 2 volumes of 50 mM NaCl, and 2 volumes of 0.2 M NaCl both in the same buffer. During this final step, 10-ml fractions were collected. Fractions containing GGF activity were pooled, dialysed against 10 mM sodium phosphate, pH 6.0, and clarified by centrifugation at 100,000 × g for 60 min.

Hydroxylapatite High Performance Liquid Chromatography—The active pool from carboxymethylcellulose chromatography was filtered (0.2 μm, Nalgene), divided into two aliquots, and each was run on a preparative hydroxyapatite HPLC column (50 × 25 mm, guard column 16 × 25 mm; Bio-Rad) equilibrated at room temperature with 10 mM potassium phosphate, pH 6.0. Elution was carried out at 2.0 ml/min using a step of 0.2 M potassium phosphate, pH 6.0, followed by a 0.2 M to 1.0 M linear potassium phosphate, pH 6.0, gradient. Fractions (60 ml) were collected during the gradient elution. Active fractions were pooled and dialyzed against 50 mM sodium phosphate, pH 6.0.

Movo S Fast Protein Liquid Chromatography—Partial purification of Movo S eluted from Mono S was carried out by a clariying spin at 100,000 × g for 60 min. The supernatant was split into two aliquots, each of which was run on a preparative HR10/10 Mono S cation exchange FPLC column (180 × 10 mm, Pharmacia), which was then re-equilibrated to 50 mM sodium phosphate, pH 6.0, at room temperature. At 1.0 ml/min. After washing with 0.36 M NaCl for 70 min, bound protein was eluted by a 0.36 M to 1.20 M NaCl gradient over 170 min, and 1.0-ml fractions were collected.

Size Exclusion Fast Protein Liquid Chromatography—The active pool of Mono S eluted material was applied to a preparative Superose 12 FPLC column (510 × 16 mm, Pharmacia) in 50 mM sodium phosphate, 0.75 M NaCl, pH 6.0 (previously passed through a C18 reversed phase column (Sep-Pak, Millipore) to remove any UV-absorbing material) at 1.0 ml/min. During the elution of successive aliquots, 0.5-ml fractions were collected into the same tubes, giving a single set of fractions. In the most recent preparation, a Superdex 75-column (600 × 16 mm, Pharmacia) was substituted for the Superose 12 column and run using the same parameters.

Reversed Phase High Performance Liquid Chromatography—(i) Peak active fractions from the Superose 12 column corresponding to an apparent molecular mass of 60 kDa were mixed and divided into three aliquots. Each aliquot was loaded onto a C8 reversed phase column (Aquapore RP-300, 5 μm, C8, 220 × 4.6 mm; guard cartridge RP-3, 10 μm, 220 × 3.2 mm; Applied Biosystems) and equilibrated to 40°C at 0.5 ml/min. After equilibration in 0.1% trifluoroacetic acid, a linear gradient to 60% acetonitrile, 0.1% trifluoroacetic acid over 60 min was used. Fractions (200 μl) were collected into the same siliconized tubes (Multilube tubes, Multitechnology, Salt Lake City, Utah) during the elution of successive aliquots. Peak active fractions from the Superose 12 column corresponding to an apparent molecular mass of 60 kDa were run on reversed phase HPLC under identical conditions. (ii) Active fractions from the first reversed phase run were pooled and divided into eight aliquots. Each aliquot was diluted 3-fold with 0.1% trifluoroacetic acid and loaded onto a second C8 reversed phase column (Aquapore RP-300, 7 μm, C8, 100 × 21 mm; Applied Biosystems) and equilibrated to 40°C at 200 μl/min in 0.1% trifluoroacetic acid (Buffer A). Bound protein was eluted using a programmed gradient to 90% acetonitrile, 0.1% trifluoroacetic acid (Buffer B) incorporating a linear gradient from 16.5 to 49.5% B. Fractions (100 μl) were collected in siliconized tubes.

Schwann Cell Culture and Assay

Rat sciatic nerve Schwann cells were isolated and purified using a modified (Davis and Stroobant, 1990) method of Ironer (1987). The cultures were >99% pure and contained <1% fibroblast contamination as judged by morphology and S-100 staining. Cells were grown on poly-D-lysine-coated plates in DMEM, 10% FCS, 5 μM forskolin, and 0.5 μg/ml GGF-carboxymethylcellulose, fed every second day, and passed every 3–4 days. Cultures passed more than six times were not used in the DNA synthesis assay because of declining sensitivity to GGF. The DNA synthesis assay was performed essentially as described (Davis and Stroobant, 1990) in 96-well plates with 3,300 cells/well in 100 μl/well DMEM, 10% FCS.

Measurement of Cell Proliferation

Quiescent Schwann cells (see above) were seeded into poly-d-lysine-coated 24-well plates at 20,000 cells/well in DMEM, 10% FCS. After 3–4 h, when the cells had adhered, purified GGF samples were added in quadruplicate at 5 nM, a concentration that gave maximal stimulation in the DNA synthesis assay. After 4 days, cells were trypsinized and counted by hemocytometer.

N-Glycanase Digests

GGF samples in 0.1% trifluoroacetic acid and 20% (GGF-I and -III) or 35% (GGF-II) acetonitrile were dried under vacuum (Speedvac, Savant), resuspended in 10 μl 0.25% SDS, 0.1 M β-mercaptoethanol, and boiled for 10 min. β-Mercaptoethanol was omitted from samples for activity elution after SDS-PAGE. The samples were adjusted to 0.1 M sodium phosphate, 10 mM EDTA, 1.25% Nonidet P-40, 80 g/ml, to a volume of 28.5 μl. After mixing, 1.2 μl (0.3 units) of N-glycanase was added to samples and incubated...
overnight at 37 °C. Water was substituted for the N-glycanase in control samples. Reactions were terminated by boiling in SDS-PAGE sample buffer.

Electrophoretic Methods

SDS gels were run as described (Laemmli, 1970) and stained using a silver staining kit (Amersham). Where measurement of GGF activity after electrophoresis was required, reducing agents were omitted from the sample buffer. Immediately after electrophoresis, the gel lanes (7 mm wide) were cut from the gel and cut across the lane at 2.5-mm intervals. These slices (~13 µl in volume) were agitated (Luckham model 804) at room temperature with 0.5 ml of sterile 1% bovine serum albumin in phosphate-buffered saline for 1 h to remove the SDS. Each slice was washed with 0.5 ml of the bovine serum albumin solution and then agitated in a further 50 µl of the same solution overnight. 10 µl of the supernatant was assayed in duplicate in the DNA synthesis assay. A molecular mass marker lane in an unsliced portion of the gel was stained with Coomassie Blue R-250 (Bio-Rad). Molecular mass markers (Bio-Rad) were: hen egg white lysozyme (14,400 kDa), soybean trypsin inhibitor (21,500 kDa), bovine carbonic anhydrase (31,000 kDa), hen egg white ovalbumin (43,000 kDa), bovine serum albumin (66,300 kDa), and rabbit muscle phosphorylase b (97,400 kDa).

Protein Determination

Protein concentrations were measured with the Bradford assay (Bradford, 1976) using bovine γ globulin as a standard (Bio-Rad). Amino acid analysis (model 420A, Applied Biosystems) was used for high purity samples as indicated.

RESULTS

Early Purification of GGF Species—The purification scheme described is routinely carried out with 4,000 whole bovine pituitaries. Each chromatographic step has been carried out at least 20 times and the entire protocol replicated 5 times. The Schwann cell proliferation assay was used to monitor all column fractions, and the examples of activity and protein profiles shown are typical for the preparation.

Homogenization, extraction, selective ammonium sulfate precipitation, and carboxymethylcellulose chromatography were carried out using methods modified from previous studies (Brockes, 1987; see “Materials and Methods”). The pool of GGF activity eluted by a salt step from the carboxymethylcellulose column was divided in half, and each aliquot was applied separately to an hydroxylapatite HPLC column and eluted using a programmed potassium phosphate gradient as indicated in Fig. 1A. All GGF activity bound to the column and eluted as a single peak corresponding to 0.40 M potassium phosphate, representing high affinity binding to the hydroxylapatite matrix. Occasionally a small peak (representing less than 10% of the total activity) eluting at a lower potassium phosphate concentration was also observed. The fractions corresponding to the major GGF peak (hatched bar, Fig. 1A) from the two runs were pooled.

After dialysis into equilibration buffer, the hydroxylapatite pool was divided in half, and each aliquot was applied separately to a preparative Mono S cation exchange FPLC column and eluted with a programmed salt gradient as indicated in Fig. 1B. All the GGF activity bound and eluted as a single broad peak spread across a salt concentration of 0.40-0.60 M. Dose responses of each active fraction were assayed, and the most active fractions from the two parallel runs were pooled (indicated by the hatched bar, Fig. 1B).

Resolution of Multiple GGF Proteins—The Mono S pool was passed over a preparative Superose 12 size exclusion column in 12 separate aliquots. The elution profile of one aliquot is shown in Fig. 1C. Two distinct peaks of activity were observed whose most active fractions were centered at relative apparent molecular masses of approximately 30 and 60 kDa. As this column was run under non-denaturing conditions, it was unclear whether these two peaks represented distinct species or simply different order aggregates of the same molecule. Separate pools of these two peaks, labeled pools 1 and 2, respectively, were carried forward for further purification.

Each size exclusion pool was split into three aliquots, each of which was separately applied to a C8 reversed phase HPLC column in 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile. The elution profile of pool 1 is shown in Fig. 2. The major peak of activity elutes at ~19-21% acetonitrile and is followed by a second minor peak repre-
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**Fig. 2.** Reversed phase HPLC of GGF pools after size exclusion chromatography. The main part of the figure shows programmed gradient elution of size exclusion pool 1 from a C8 reversed phase column. Buffer A was 0.1% trifluoroacetic acid, pH 2.1, and Buffer B was 90% acetonitrile, 0.1% trifluoroacetic acid, pH 2.1. The inset shows the activity profile of size exclusion pool 2 eluted under identical conditions. For explanation of symbols used, see legend to Fig. 1.

The activity profile of size exclusion pool 2 run under identical reversed phase HPLC conditions is shown in Fig. 2 (inset). Two GGF activity peaks are observed, precisely corresponding in gradient elution position to those observed with pool 1. Most of the GGF activity from this pool is found in the second peak, eluting at 30–35% acetonitrile. SDS-PAGE analysis of the fractions including this second peak showed that in the lane corresponding to the two most active fractions (Fig. 3A, lane 4), the silver stain is almost exclusively localized to a diffuse band centered at molecular mass of 59 ± 2 kDa. The staining intensity in this region corresponds closely to the amount of GGF activity measured in the active fractions across the peak (Fig. 2, inset). The retention of this molecular mass after boiling in the presence of SDS and reducing agents suggests that this GGF species is distinct from the ~34 kDa GGF species. Based on these assignments, the two activity peaks resolved by size exclusion and reversed phase HPLC were concluded to be distinct molecular species and were named GGF-I (molecular mass, ~34 kDa) and GGF-II (molecular mass, ~59 kDa).

Similar electrophoretic analysis was extended to the minor pool 2 reversed phase peak. Although the elution position of GGF activity from this pool after reversed phase chromatography corresponds exactly to that of GGF-I (Fig. 2), no silver-stained band is observed in the region of the gel where GGF-I should be present. The most intensely silver-stained band in these fractions is centered at 45 ± 2 kDa (Fig. 3A, lane 3). The silver staining intensity of this band in the fractions across the active peak closely correlates with the amount of activity in each fraction. This electrophoretic behavior is distinct from either GGF-I or II, and we thus named this species GGF-III by SDS polyacrylamide electrophoresis. A, active fractions from reversed phase HPLC runs were pooled (see Fig. 2) and silver-stained after electrophoresis on 11% gels. The GGF species are indicated by square brackets to the left of the lanes. Lane 1, GGF-I after a single reversed phase run; lane 2, GGF-I after a reversed phase rerun; lane 3, GGF-III; lane 4, GGF-II; lane 5, GGF-II after N-glycanase digestion (the N-glycanase appears as a 35-kDa band (arrow)). B, reversed phase HPLC purified GGF-I (●), GGF-II (○), and GGF-III (×) were run in separate lanes on SDS-PAGE; supernatants after passive elution from gel slices cut across each lane were tested in the Schwann cell DNA synthesis assay. GGF activity in cpm has been normalized to aid comparison of the three samples tested.

**Fig. 3.** A comparison of the molecular masses of GGF species by SDS polyacrylamide electrophoresis. A, active fractions from reversed phase HPLC runs were pooled (see Fig. 2) and silver-stained after electrophoresis on 11% gels. The GGF species are indicated by square brackets to the left of the lanes. Lane 1, GGF-I after a single reversed phase run; lane 2, GGF-I after a reversed phase rerun; lane 3, GGF-III; lane 4, GGF-II; lane 5, GGF-II after N-glycanase digestion (the N-glycanase appears as a 35-kDa band (arrow)). B, reversed phase HPLC purified GGF-I (●), GGF-II (○), and GGF-III (×) were run in separate lanes on SDS-PAGE; supernatants after passive elution from gel slices cut across each lane were tested in the Schwann cell DNA synthesis assay. GGF activity in cpm has been normalized to aid comparison of the three samples tested.
activity is observed in regions of the gel corresponding to structural characteristic shared by all three GGF species. GGF containing inhibitory activity.

Further confirmation of the assignment of GGF activity to these bands was obtained from SDS-PAGE activity elution experiments. GGF reversed phase HPLC purified samples were subjected to SDS-PAGE in the absence of reducing agents; proteins were eluted from gel slices of each lane into 1% bovine serum albumin in phosphate-buffered saline and eluates assayed as shown in Fig. 3B. In each case, GGF activity was only eluted from one region of the gel, indicating that a single GGF species is present in each preparation. The molecular masses observed are consistent with those measured by size exclusion and silver-stained SDS-PAGE.

**Purification Summary**—A summary for the purification of GGFs-I, -II, and -III is shown in Table I. Although the column protein and activity elution profiles are generally reproducible for different preparations, and the activity in column fractions is stable at -20 °C following the carboxymethylcellulose step, the GGFs are isolated in low yields and in low quantities. A precise estimate of the final yield is difficult, since the relative quantities of the three species are unknown, but it is probably less than 1% if there are no other Schwann cell mitogens in the carboxymethylcellulose fraction. However, the preparation yields quantities of material sufficient to enable initial studies of structure and cell biology.

**Treatment of GGFs with N-Glycanase**—The diffuse silver-stained bands on SDS-PAGE assigned to all three GGFs may reflect some proteolytic "fraying" of the active species during the lengthy purification procedure, contamination with inactive proteins, or heterogeneity might arise through glycosylation. This last possibility was tested by incubating these GGF preparations with N-glycanase, an enzyme that specifically cleaves N-linked carbohydrate groups from the peptide backbone, and analyzing changes in apparent molecular weight by SDS-PAGE. Fig. 4 shows the Schwann cell mitogenic activity recovered from gel slices before and after N-glycanase treatment. In all three cases, little GGF activity from treated samples remains in the same region of the gel as the untreated activity, indicating that N-glycosylation is a structural characteristic shared by all three GGF species. GGF activity is observed in regions of the gel corresponding to lower molecular weights, indicating that a major proportion of the N-deglycosylated material is active. The apparent molecular mass of N-deglycosylated GGF-I is some 5 kDa less than the native molecule (Fig. 4A, shift from 34 to 29 kDa). The N-glycanase treatment of GGF-II causes a more substantial activity shift from a molecular mass of 59 to 45 kDa (Fig. 4B), while N-deglycosylation of GGF-III causes a smaller mobility shift from 44 to ~40 kDa (Fig. 4C). A poor recovery of mitogenic activity from both control and deglycosylated GGF-III was observed compared with GGF-I and GGF-II, reflecting an activity that is less stable. For GGF-I and GGF-III, similar results were obtained when samples were analyzed by silver staining of SDS polyacrylamide gels run under reducing conditions (data not shown). However, N-glycanase treatment of GGF-II yields two silver-stained bands, a major species of ~41 kDa and a minor band of ~50 kDa (Fig. 3A, lane 5). Whether the activity peak observed in the gel elution experiments (Fig. 4D) corresponds to only one of these silver-stained bands or whether the activity of these two species has not been resolved under the nonreducing conditions used for the activity elution experiments is not clear.

**Mitogenic Effects with Rat Schwann Cells**—The apparently homogeneous preparations of the GGFs described here provided an opportunity to define their mitogenic effects in the absence of both contaminants and of platelet-derived mitogens by using plasma instead of serum in the activity assay. GGF-I, -II, and -III activities were compared in the Schwann cell mitogenesis assay using plasma instead of serum in the activity assay. GGF-I, -II, and -III activities were compared in the Schwann cell [125I]UDR incorporation assay, carried out in DMEM, 10% fetal calf plasma (Fig. 5). Each of these molecules shows a sigmoidal stimulation of DNA synthesis in this assay, saturating at concentrations around 10 nM, with half-maximal effects at approximately 1 nM. These dose responses are not affected by the substitution of plasma for serum in the assay, indicating that the presence of platelet factors is not a prerequisite for the mitogenic effects of the GGFs in vitro. Indeed, all three GGF species can stimulate significant Schwann cell division in defined medium in the presence or absence of forskolin.

Partially purified bovine pituitary GGF carboxymethylcellulose fraction (see "Materials and Methods") can synergize with agents that elevate intracellular levels of cAMP such as forskolin (Davis and Stroobant, 1990). Fig. 5 shows that highly purified preparations of GGFs-I, -II, and -III all retain this property, since the addition of 5 μM forskolin causes a 3-

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**Table I**

| Fraction          | Total protein | Total volume | Total activity* | Specific activity | Cumulative yield* |
|-------------------|---------------|--------------|----------------|------------------|-------------------|
| Pituitary extract | 167,900       | 4,000        | 3,322,000      | 19.8             |                   |
| Carboxymethyl cellulose | 1,114 | 470          | 1,134,760      | 1,037             | 100               |
| Hydroxylapatite   | 210           | 171          | 285,087        | 1,357             | 24.7              |
| Mono S            | 6.88          | 27.5         | 63,965         | 2,927             | 5.5               |
| Superdex 75 GGF-I | 0.076         | 91.9         | 17,230         | 227,500           | 1.5               |
| Superdex 75 GGF-II/III | 0.012 | 63.8         | 4,530          | 377,500           | 0.39              |
| Reversed phase GGF-I | 0.019*        | 2.4          | 1,999          | 105,210           | 0.17              |
| Reversed phase GGF-II | 0.0074*       | 1.8          | 947            | 127,973           | 0.08              |
| Reversed phase GGF-III | 0.0055*       | 1.8          | 340            | 64,151            | 0.03              |

* One unit of activity gives half-maximal stimulation of DNA synthesis under the assay conditions described.

* The yield calculation is performed relative to the carboxymethylcellulose-purified material, since the extract is a complex mixture containing inhibitory activity.

* Prepared from a batch of 4,000 pituitaries.

* The size exclusion column shown here was used rather than the Superose 12 FPLC column used in earlier preparations, to improve the resolution. This substitution does not significantly affect the yield at this stage in the purification.

* Protein concentration estimated from amino acid analysis.

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4 A sigmoidal stimulation of DNA synthesis in this assay, saturating at concentrations around 10 nM, with half-maximal effects at approximately 1 nM. These dose responses are not affected by the substitution of plasma for serum in the assay, indicating that the presence of platelet factors is not a prerequisite for the mitogenic effects of the GGFs in vitro. Indeed, all three GGF species can stimulate significant Schwann cell division in defined medium in the presence or absence of forskolin.
been observed with all three GGFs (data not shown). No consistent differences have been observed in the extent of these factors, confirming that each GGF species stimulates Schwann cells, more substantial shifts of up to 15-fold have occurred in uitro.

Since the mitogenic assay used to monitor the purification of the GGFs measures cell [\(^{125}\)I]UdR incorporation rather than actual proliferation, a direct measurement of cell growth was carried out in which the numbers of Schwann cells were counted at various time points after administration of GGFs. Table II illustrates the increases in cell number during relatively short periods of secondary culture in uitro. Forskolin (5 \(\mu M\)) renders the Schwann cells more sensitive to all three GGFs. It has been shown that the stimulation of proliferation by forskolin in these cells (Porter et al., 1987) is due to elevation of intracellular cAMP concentrations (Davis and Stroobant, 1990), raising the question of whether cAMP plays a role as a second messenger in the mitogenic signaling of the GGFs. Although forskolin alone has some mitogenic effect in the assay conditions used here, maximal doses of the GGFs are >5-fold more effective in stimulating DNA synthe-

![Graph A](image1)

**Graph A**

Electrophoretic mobility shift of GGF activity eluted from SDS polyacrylamide gels before and after N-glycanase digestion. GGF-I (A), -II (B), and -III (C) were incubated in the absence (\(\odot\)) or presence (\(\bullet\)) of N-glycanase overnight. After SDS-PAGE, protein was passively eluted from gel slices and GGF activity was measured. This experiment was repeated twice with the same results.

![Graph B](image2)

**Graph B**

![Graph C](image3)

**DISCUSSION**

We report here the purification and characterization of three Schwann cell mitogens: GGF-I, which has properties similar to the previously identified GGF, and, in addition, two novel molecules named GGF-II and -III. The successful isolation of the GGFs was based on the very high selective sensitivity of rat Schwann cells to the mitogenic effect of these molecules (Brockes, 1987). After initial extraction of bovine pituitaries, a sequence of five chromatography steps was employed for purification. Appreciable losses of Schwann cell mitogenic activity occur at a number of the chromatographic steps giving low final yields.

This isolation procedure yields highly purified preparations of GGFs-I, -II, and -III. Silver staining of active pools shows, in each case, a diffuse band migrating at the active molecular weight, apparently representing >90% purity. Dose response curves show half-maximal effects at approximately 1 nM. This is likely to be an underestimate of the sensitivity of Schwann cells to the GGFs in uitro, given the decline in responsiveness during relatively short periods of secondary culture in uitro.

The mitogenic activity of three Schwann cell mitogens: GGF-I, which has properties similar to the previously identified GGF, and, in addition, two novel molecules named GGF-II and -III. The successful isolation of the GGFs was based on the very high selective sensitivity of rat Schwann cells to the mitogenic effect of these molecules (Brockes, 1987). After initial extraction of bovine pituitaries, a sequence of five chromatography steps was employed for purification. Appreciable losses of Schwann cell mitogenic activity occur at a number of the chromatographic steps giving low final yields.

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Forskolin (5 \(\mu M\)) renders the Schwann cells more sensitive to all three GGFs. It has been shown that the stimulation of proliferation by forskolin in these cells (Porter et al., 1987) is due to elevation of intracellular cAMP concentrations (Davis and Stroobant, 1990), raising the question of whether cAMP plays a role as a second messenger in the mitogenic signaling of the GGFs. Although forskolin alone has some mitogenic effect in the assay conditions used here, maximal doses of the GGFs are >5-fold more effective in stimulating DNA synthe-

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**Table II**

Stimulation of Schwann cell proliferation by GGFs-I, -II, and -III

| Sample  | Cells/well | S.D.  |
|---------|------------|-------|
| No addition | 57,833     | 7,318 |
| GGF-I     | 110,750    | 2,390 |
| GGF-II    | 108,083    | 10,728|
| GGF-III   | 95,708     | 5,970 |

**DISCUSSION**

We report here the purification and characterization of three Schwann cell mitogens: GGF-I, which has properties similar to the previously identified GGF, and, in addition, two novel molecules named GGF-II and -III. The successful isolation of the GGFs was based on the very high selective sensitivity of rat Schwann cells to the mitogenic effect of these molecules (Brockes, 1987). After initial extraction of bovine pituitaries, a sequence of five chromatography steps was employed for purification. Appreciable losses of Schwann cell mitogenic activity occur at a number of the chromatographic steps giving low final yields.

This isolation procedure yields highly purified preparations of GGFs-I, -II, and -III. Silver staining of active pools shows, in each case, a diffuse band migrating at the active molecular weight, apparently representing >90% purity. Dose response curves show half-maximal effects at approximately 1 nM. This is likely to be an underestimate of the sensitivity of Schwann cells to the GGFs in uitro, given the decline in responsiveness during relatively short periods of secondary culture in uitro.
sis in Schwann cells than forskolin alone. No increase in cAMP levels has been observed at saturating levels of crude GGF preparations (Davis and Stroobant, 1990). Indeed, in defined medium, elevation of CAMP by forskolin has no proliferative effects and appears to initiate processes of differentiation (Morgan et al., 1991). Thus, CAMP apparently plays no direct part in the second messenger signaling of GGF, and, therefore, forskolin synergy must arise through the modulation of a rate-limiting step in GGF signaling, which could occur at the cell surface or further downstream. Convincing evidence suggests that the effects of forskolin on at least one rat Schwann cell growth factor are likely to occur by modulation of the numbers of cell surface receptors. Forskolin elevates expression of platelet-derived growth factor receptors (Weinmaster and Lemke, 1990) through transcriptional activation, enhancing the sensitivity of Schwann cells to B-chain containing platelet-derived growth factor dimers by several orders of magnitude (Davis and Stroobant, 1990).

Modulation of a rate-limiting step in GGF signaling, which GGF, and, therefore, forskolin synergy must arise through the defined medium, elevation of cAMP by forskolin has no differentiation (Morgan et al., 1991), suggesting that the N-linked carbohydrate is not essential for mitogenic activity. The GGFs differ in the extent to which their molecular masses decrease upon deglycosylation, ranging from 4 kDa for GGF-III and 5 kDa for GGF-I to 14 kDa for GGF-II, presumably reflecting the different quantities of N-linked carbohydrate present in each factor. No two deglycosylated GGFs have the same relative molecular weight, demonstrating that differential N-deglycosylation alone does not account for the different apparent molecular weights of the native molecules.

The GGF-I molecule (34 kDa) identified here has very similar properties to the 31-kDa GGF species previously isolated (Lemke and Brockes, 1984). Apart from the small discrepancy in molecular mass, the main difference is the reported instability of GGF under acidic conditions (Brockes, 1987). In our hands, GGF-I is stable in 0.1% trifluoroacetic acid, pH 2.0, with no loss of activity over a period of more than 6 months. Although GGF activity has been observed to elute from a size exclusion column at around 60 kDa (Brockes et al., 1989), this activity migrated on SDS-PAGE at ~31 kDa, unlike the GGF-II and -III species reported here, suggesting that GGFs-II and -III were not present in these earlier studies. These differences may reflect the use of anterior lobes of pituitaries (Brockes, 1987) as opposed to the whole pituitaries employed here. The age of the cattle at the time the pituitaries were isolated is also a potential factor influencing the results obtained in this laboratory using relatively large pituitaries (presumably from older cattle) contained no detectable amounts of GGF-II or -III.

During development, neurons stimulate Schwann cell division (Agauyo et al., 1976), an effect that has been reproduced in vitro with co-cultures of neurons and Schwann cells (Wood and B urge, 1975; Salzer and B urge, 1980; Ratner et al., 1987). This effect appears to require axonal contact (Salzer et al., 1980a), an observation that led to the identification of Schwann cell mitogenic activity in membrane extracts of dorsal root ganglion neurons (Salzer et al., 1980b), and in neonatal rat brain (Ratner et al., 1988). This latter activity is a heparin-binding protein with an apparent molecular mass of ~50 kDa (Nordland et al., 1992), which is apparently distinct from any of the GGF molecules isolated here. A less well characterized Schwann cell mitogen has also been identified in axolemmal-enriched extracts of whole rat brain and cultured cerebellar granule cells (Mason et al., 1989). The interesting question as to whether any of the GGFs can associate with axonal cell surfaces awaits the development of GGF-specific reagents.

The main differences between GGFs-I, -II, and -III are their molecular weights and their affinities for reversed phase HPLC matrices, features that were successfully applied to chromatographically resolve the three proteins. Although structurally distinct, the GGFs clearly share common properties. They bind to and elute from carboxymethylcellulose, hydroxyapatite HPLC, and Mono S FPLC chromatography columns under identical conditions. Each is constituted in part by N-linked carbohydrate, which can be enzymatically removed without eliminating biological activity. All the GGFs stimulate DNA synthesis in Schwann cells in vitro with a similar high potency, which is increased by agents that elevate CAMP. Recent protein sequence analysis of the GGFs following scale-up of the purification protocols described here has detected a closely related peptide in all three species, indicating that they are structurally related, although there were insufficient data to distinguish between the possibilities that they are either homologous but distinct species, or proteolytically derived forms of the same protein (Marchionni et al., 1993). Furthermore, following cloning studies and database searches, this report postulates that GGFs-I, -II, and -III are derived from a single copy gene, which itself has been shown to encode erbB2 ligands (Holmes et al., 1992; Wen et al., 1992) and the acetylcholine receptor inducing protein known as ARIA (Falls et al., 1993). The GGFs are thus members of a family of related factors isolated as proteins from diverse sources that are probably derived from the same gene by alternative splicing.

Subsequent studies using antibody and oligonucleotide probes will be employed to investigate the roles that these apparently novel growth factors may play in the processes of development, regeneration, and tumor biology.

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