Transforming growth factor α and a PC12-derived growth factor induce neurites in PC12 cells and enhance the survival of embryonic brain neurons

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We have identified and characterized a 5000-Da protein that induces neurite outgrowth from PC12 pheochromocytoma cells, enhances the survival of embryonic rat brain neurons in primary culture, and induces the multiplication of embryonic rat brain astrocytes in primary culture. The factor is produced by a flat cell PC12 variant that expresses the activated *ras* oncogene after transfection of the gene. The factor resembles transforming growth factor α (TGF α) and epidermal growth factor (EGF) in that it induces anchorage-independent colony formation of normal rat kidney cells in soft agar and competes with EGF for binding to the EGF receptor. Rat TGF α and human TGF α also induce neurite outgrowth from PC12 and enhance the survival of embryonic brain neurons. The PC12 variant-derived factor can be distinguished from TGF α and EGF immunologically and by migration rates on reversed-phase high-performance liquid chromatography.

Introduction

PC12 rat pheochromocytoma cells that express the activated *ras* oncogene cease multiplication, extend long axonlike processes (neurites), and acquire the appearance of neurons (Noda *et al.*, 1985; Guerrero *et al.*, 1988; Sugimoto *et al.*, 1988; Sassone-Corsi *et al.*, 1989). Our laboratory has isolated a flat cell PC12 variant (Andersen *et al.*, 1990) that continues to multiply and only extends short processes when it expresses active Harvey (Ha) *ras* after transfection of the gene (Rozenberg, Bitler, and Howard, manuscript in preparation). In a search for an

autocrine factor that might cause the extension of processes from the transfected variant, we have identified a 5000-Da polypeptide that induces neurite outgrowth from wild-type PC12 cells.

As reported here, this factor was found to be similar to the previously described transforming growth factor α (TGF α) and epidermal growth factor (EGF), each of which is a 6-kDa polypeptide that reversibly confers a transformed phenotype on untransformed cells (Roberts *et al.*, 1983; Deuel, 1987; Derynck, 1988). For example, TGF α induces the anchorage-independent multiplication of normal rat kidney (NRK) cells embedded in soft agar, an effect that also requires the presence of TGF β , another transforming factor of larger size. TGF α was first identified in the conditioned medium of neoplastic cells including those expressing active *ras*, but is now known to be produced by non-neoplastic cells as well (Roberts *et al.*, 1983; Derynck, 1988). TGF α binds to the EGF receptor and activates its tyrosine kinase (Massague, 1983; Derynck, 1988).

We also report here that TGF α induces neurite outgrowth from PC12 cells and that both TGF α and the factor derived from the PC12 variant enhance the survival of embryonic brain neurons in primary culture. Although similar to TGF α and EGF, the factor derived from the PC12 variant can be distinguished from the other two factors by immunologic and chromatographic criteria.

Results

Neurite-promoting activity

6B3 is a PC12 flat-cell variant (Andersen *et al.*, 1990) that had been transfected with the pMTEJ plasmid (Trimble *et al.*, 1986). This plasmid contains the cDNA for Ha-*ras* under transcriptional control of the metallothionein promoter. pMTEJ also contains the gene for geneticin resistance to allow selection of cells transfected with the plasmid. 6B3 exhibits an increased level of the p21 *ras* protein after exposure to a heavy metal (Rozenberg *et al.*, manuscript in preparation).

Figure 1. Factor-induced neurite outgrowth from PC12 cells. Wild-type PC12 cells were incubated for 3 d in standard culture medium containing no added factor (A), a fourfold dilution of conditioned medium from 6B3 cells (B), 60 ng/ml of rat TGF α (C), or 60 ng/ml of human TGF α (D).

As shown in Figure 1, A and B, 6B3 cells release into the culture medium a factor that induces the extension of neurites from wild-type PC12 cells and a flattening of the bodies of these cells. Neurite extension could be detected within 24 h of treatment, but maximum neurite extension typically occurred after 3–4 d of treatment. Figure 1B shows the maximum extension of neurites induced by the 6B3-derived factor. The neurites shown in Figure 1B resemble those induced in PC12 by cAMP (Gunning *et al.*, 1981) but are much shorter and less branched than those induced by nerve growth factor (Greene and Tischler, 1976) or fibroblast growth factor (Rydel and Greene, 1987).

6B3 cells synthesize the neurite-promoting factor even when a heavy metal was not added to the culture medium to induce *ras* transcription from the transfected plasmid. It is known that cells expressing a transfected metallothionein gene exhibit substantial baseline metallothionein synthesis without induction (Hamer, 1986). Addition of 50 μM Zn^{2+} to the culture medium resulted in a threefold increase in the level of neurite-promoting activity in the medium, presumably because of an induction of *ras* synthesis. PC12-variant cells transfected with the plasmid pSV₂, which contains only the genetecin resistance gene, did not secrete neurite-promoting activity.

The activity of the neurite-promoting factor was abolished by treatment with proteinase K (0.1 $\mu\text{g}/\text{ml}$; 40 min), phenol, or 0.2 mM β -mercaptoethanol. However, it was resistant to boiling (10 min) and 1 M acetic acid. Preliminary studies indicated that its molecular weight was <10,000 Da. These properties indicate that the factor released by 6B3 cells is not any of several known growth factors, including fibroblast growth factor, nerve growth factor, insulin-like growth factor or platelet-derived growth factor. However, as shown below, it has properties similar but not identical to those of TGF α and EGF. We have chosen to refer to this 6B3-derived factor as growth factor-6B3 (GF-6B3) until it can be shown to be either unique or a modified form of one of the previously described factors. The increased production of GF-6B3 by a cell expressing active *ras* is a property shared with TGF α ; thus, we have more fully compared GF-6B3 with TGF α than with EGF.

As shown in Figure 1, C and D, rat TGF α and human TGF α also induce the extension of neurites by PC12 cells. Maximum neurite extension occurred 3–4 d after TGF α treatment. We have found that murine EGF at 0.2 nM also induces

PC12 neurites similar to those in Figure 1, B–D (not shown).

Partial purification of GF-6B3

Fractionation of GF-6B3 by gel filtration chromatography (Figure 2a) resulted in three peaks of neurite-promoting activity (a–c) that eluted as molecules of 30 000, 10 000, and 5000 Da, respectively. These peaks could also be resolved by ion exchange chromatography (Figure 2b). In this case identification of the peaks of activity was determined by rechromatographing each peak through a gel filtration column. The first eluted peak of activity shown in Figure 2b

Figure 2. Chromatography elution patterns of GF-6B3. Conditioned medium (300 ml) from 6B3 cells was precipitated with acetone, and 2 mg of protein was subjected to gel filtration chromatography on a Superose 12 HR 10/30 column (a) or ion-exchange chromatography on a Mono Q HR 5/5 column (b) as described in Materials and methods. The fractions were examined for neurite-promoting activity in PC12 cell cultures. Fractions containing such activity are indicated by hatched bars. Arrows indicate the positions of elution of the molecular weight standards: 1) blue dextran (2000 kDa); 2) bovine serum albumin (66 kDa); 3) carbonic anhydrase (29 kDa); 4) cytochrome C (12 kDa); and 5) apoferritin (6.5 kDa). (Inset to a) Protein (15 μg) from fractions 35–38 from the gel filtration column was subjected in duplicate lanes to SDS-gel electrophoresis. One of the lanes (lane B) and a lane containing molecular weight standards (lane A) were stained with Coomassie Blue. The other lane was sliced and each slice was extracted in buffer, which was then assayed for neurite-promoting activity (lane C). The positions of the slice are indicated by boxes; hatch boxes contained activity.

corresponds to peak b of Figure 2a, the second peak to peak c, and the third peak to peak a. The active species of peak b of Figure 2a converts to a lower molecular weight form on standing or in the presence of a 0.1% sodium dodecyl sulfate (SDS), so it may be a dimer of the active protein in peak c.

We have confined our studies of GF-6B3 to the 5000-Da species, so subsequent studies refer to the polypeptide from peak c of Figure 2a. The factor from this peak retained neurite-promoting activity after electrophoresis through an SDS-polyacrylamide gel (containing no β -mercaptoethanol). Assay of extracts of gel slices for neurite-promoting activity (Figure 2a, inset) showed that GF-6B3 migrated as a protein of 5000 Da.

GF-6B3 was further purified by reverse-phase high-performance liquid chromatography (HPLC) (Figure 3a). GF-6B3 eluted at 36% acetonitrile, whereas under the same conditions, human TGF α and rat TGF α eluted at 25% acetonitrile (Figure 3b). GF-6B3 partially purified as described for Figure 3a was used for all of the subsequent studies presented below. A silver-stained SDS-polyacrylamide gel of the preparation from Figure 3a shows that it still contained some contaminating peptides (Figure 3a, inset).

Colony-forming and mitogenic activities

GF-6B3 has transforming activity. Like TGF α it induced the anchorage-independent multiplication of NRK-49F cells embedded in soft agar (Figure 4). We counted the number of colonies containing >20 cells in eight randomly chosen fields for each condition of culture. Control cultures containing no added factor had <1 colony/cm², whereas cultures containing TGF α or GF-6B3 had, respectively, 360 or 290 colonies/cm². GF-6B3, like TGF α , also stimulated multiplication of the NRK cells grown on petri dishes, as judged by the incorporation of [³H]thymidine into DNA of the cells (Figure 5a).

Binding to the EGF receptor

Figure 5b shows that GF-6B3 competes with EGF for binding to the EGF receptor. For these studies, we incubated ¹²⁵I-labeled EGF and EGF receptor with varying concentrations of unlabeled EGF, TGF α , or GF-6B3. Figure 5 describes the concentrations of these factors as units. One unit of GF-6B3 is 0.8 μ g/ml, the concentration of GF-6B3 that gives a half-maximal effect on neurite outgrowth from PC12 cells as is

Figure 3. Reversed-phase chromatographic elution profile of GF-6B3 and human TGF α . Fractions containing neurite-promoting activity in PC12 cultures are indicated by hatched bars. (a) Protein from peak C from the gel filtration column of Figure 2a was subjected to ion-exchange chromatography and protein (0.2 mg) from fractions containing neurite-promoting activity were pooled and subjected to reversed-phase HPLC on a Vydac C₄ column, as described in Materials and methods. (Inset to a) Protein from fractions 29–32 was subjected to SDS-gel electrophoresis (lane B). Lane A contains molecular weight standards. Proteins in the gel were silver stained. (b) Human TGF α (2 μ g) was subjected to reverse-phase HPLC under the same conditions. When rat TGF α was passed through this column under the same conditions, neurite-promoting activity eluted in the same fractions as did human TGF α (not shown).

shown in Figure 6a. One unit of TGF α is 6 nM (36 ng/ml), which also produced a half-maximal effect on PC12 neurite outgrowth. One unit of EGF for Figure 5b was also 6 nM. Higher concentrations of GF-6B3 (40 μ g/ml) blocked >80% of the binding of ¹²⁵I-EGF to its receptor (not shown).

The results of Figure 5b indicate that, per mole, TGF α has about one-half of the receptor-binding activity of EGF, consistent with the previous finding of Winkler *et al.* (1986). The results of Figure 5b also indicate that, per unit of neurite promoting activity, GF-6B3 had about one-third of the receptor-binding activity of TGF α and about one-sixth of that of EGF. However, because GF-6B3 has not been purified, we cannot determine whether the neurite-promoting activity and/or the receptor-binding activity per mole of GF-6B3 differs from that of TGF α or EGF.

Dose-response curves for neurite-promoting activity

Figure 6a shows dose-response curves for the neurite-promoting activities of TGF-6B3 and

Figure 4. Effect of GF-6B3 or TGF α on colony formation by NRK-49F cells in soft agar. NRK-49F cells were embedded in medium containing 0.3% agar and no other factor (A) 2 μ g/ml GF-6B3 from a reversed-phase column under conditions described for Figure 3a (B), or 60 ng/ml rat TGF α (C). The cultures were incubated for 7 d.

TGF α . Both factors show saturation, and the curves for the two factors roughly parallel each other. Neurite extension was seen by treatment with as little as 1 nM (6 ng/ml) pure TGF α or as little as 120 ng/ml impure GF-6B3. Maximum neurite outgrowth occurred when the cells were exposed to 30 nM TGF α or 4 μ g/ml GF-6B3. If TGF α and GF-6B3 have the same specific activity with respect to neurite induction, we can

conclude that the GF-6B3 used in this experiment is 5% pure. For each dose of GF-6B3, the maximum percentage of PC12 cells with neurites was observed after 3 d of treatment (Figure 6b). A similar result was obtained with TGF α (not shown).

In addition to inducing neurites, GF-6B3 allows survival of PC12 cells cultured in serum-free medium (Figure 7); neurite induction also

Figure 5. The abilities of TGF α and GF-6B3 to induce [3 H]thymidine incorporation and to bind to the EGF receptor. One unit of unlabeled EGF (■) or TGF α (●) was 6 nM and one unit of TGF-6B3 (○) was 0.8 μ g/ml. (a) Incorporation of [3 H]thymidine into the DNA of NRK-49F cells was measured as described in Materials and methods. The results, which are the means for duplicate incubations, are expressed as the percent of [3 H]thymidine incorporated by control cells incubated in the absence of added TGF. Control cells had $24\,000 \pm 1000$ acid-precipitable cpm per dish. (b) Competition by TGF α , GF-6B3, or unlabeled EGF of the binding of 125 I-labeled EGF to the EGF receptor was measured as described in Materials and methods. Control receptor-containing cells (incubated in the absence of added unlabeled ligand) bound 1850 ± 120 dpm of 125 I-EGF ($n = 3$).

occurs in serum-free medium. Similar results were obtained with TGF α (not shown).

Effects of factors in embryonic brain cells

Because GF-6B3 and TGF α exhibited neurite-promoting activity on PC12 cells and allowed survival of these cells in serum-free medium, we examined their effects on cells from brain tissue. When incubated with embryonic rat brain in culture for 5 d, GF-6B3 enhanced the survival of neurons (Figure 8, A and B); few intact neurons could be seen in the absence of GF-6B3. This factor also induced the multiplication of astrocytes (Figure 8, C and D). Rat TGF α at 10 ng/ml produced a similar enhancement of neu-

ron survival (not shown); the effect of TGF α on astrocytes was not determined.

Immunologic studies

GF-6B3 is immunologically distinct from both rat TGF α and murine EGF. Polyclonal antibody raised against human TGF α immunoprecipitated rat TGF α but not GF-6B3. For this study a solution of GF-6B3 or rat TGF α (containing equal levels of neurite-promoting activity) was incubated with anti-TGF α antibody overnight at 0°C and then for 2 h at room temperature with fixed *Staphylococcus aureus* cell suspension (Pansorbin), which contains protein A and thus binds antibody and antibody-antigen complexes. After sedimentation and washing, the Pansorbin was resuspended in culture medium, boiled for 5 min, and resedimented to release any bound TGF or GF-6B3 into the medium. In the case of TGF α , neurite-promoting activity

Figure 6. Dose-response curve for the neurite-promoting activity on PC12 cells of GF-6B3 and rat TGF α . The data points are the means for duplicate incubations. (a) PC12 cells were incubated for 4 d in culture medium containing either GF-6B3 (○) or rat TGF α (●) at the indicated concentration. One unit of concentration is 36 ng/ml for TGF α or 0.8 μ g/ml for GF-6B3. (b) PC12 cells were incubated for 1–4 d in culture medium containing GF-6B3 at the concentrations (μ g/ml) indicated to the right of each curve. At least 200 cells were counted in six randomly chosen fields for each data point.

Figure 7. Effect of GF-6B3 on PC12 survival in serum-free medium. Wild-type PC12 cells were cultured in standard culture medium (Δ) or in serum-free medium containing no GF-6B3 ($\circ$) or 4 $\mu\text{g/ml}$ GF-6B3 ($\bullet$). In one experiment GF-6B3 was added to the serum-free medium on the fourth day. Cell density was determined for each plate by the use of a reticle to count the cells in six randomly chosen microscopic fields. The results are the means of triplicate incubations.

was detected in a fourfold dilution of the resulting supernatant, but no neurite-promoting activity was detected in an undiluted supernatant preparation in the case of GF-6B3. TGF α was not precipitated by Pansorbin when non-immune serum was used. In similar experiments, we found that anti-EGF antibody did not precipitate nor neutralize GF-6B3, although it did precipitate and neutralize EGF.

Discussion

We have identified a protein, GF-6B3, that has many of the properties of both TGF α and EGF, and that in principle could be related to either of these other two growth factors. GF-6B3 is produced by a PC12 variant that had been transfected with the active *Ha-ras* gene under control of the metallothionein promoter. We looked for the production of neurite-promoting activity by these cells because they extended short processes on exposure to medium that contained 50 μM Zn $^{2+}$ to induce metallothionein (Rozenberg *et al.*, manuscript in preparation). Although this factor induces processes from

wild-type PC12 cells, it does not cause extension of processes from the nontransfected variant (unpublished results). GF-6B3 appears to exist in multiple molecular weight forms (Figure 2a) as does TGF α (Derynck, 1988). We have characterized only the 5000-Da species.

TGF α also was found to induce neurite outgrowth from wild-type PC12 cells; this effect could be seen within 1 d and with a concentration as low as 1 nM. Thus, PC12 neurite outgrowth provides a quick, easy, and sensitive bioassay for TGF α .

TGF α binds to the EGF receptor (Massague, 1983), and its previously described activities have been ascribed to this binding (Deuel, 1987). We have also found that GF-6B3 also binds to the EGF receptor. Nevertheless, we do not know whether the induction by TGF α and GF-6B3 of PC12 neurite outgrowth is due to binding of these factors to the EGF receptor. We have found that EGF also induces PC12 neurites, although it has been reported that PC12 cells respond to EGF but not by neurite outgrowth (Huff and Guroff, 1979; Chandler and Herschman, 1980). This apparent discrepancy may have been due to differences in the PC12 cell lines used; indeed, we have found that some PC12 lines are less responsive to TGF α than are the PC12 cells used for the experiments described here.

A finding of functional significance is that TGF α and GF-6B3 enhance the survival of embryonic brain neurons in primary culture. Morrison *et al.* (1987) have reported a similar effect for EGF. GF-6B3 also was found to induce the multiplication of embryonic astrocytes; we did not examine the effect of TGF α on astrocytes. Wilcox and Derynck (1988) and Kudlow *et al.* (1989) have shown that TGF α is expressed by neurons throughout the adult mammalian brain. They also detected TGF α expression in fetal brain. These results in conjunction with ours indicate that TGF α has a role in the development and/or maintenance of mammalian brain neurons.

Although GF-6B3 is very similar to TGF α and EGF in biological activity, size, and resistance to boiling and acid, we have detected two differences. Neither anti-TGF α antibody nor anti-EGF antibody recognized GF-6B3, and TGF α migrated differently from GF-6B3 on reversed-phase HPLC. These differences may reflect slight differences in amino acid sequence and/or posttranslational modification of the polypeptides. On the other hand, GF-6B3 may be a novel growth factor genetically unrelated to ei-

Figure 8. Effect of GF-6B3 on the survival of embryonic neurons (A and B) or astrocytes (C and D) in primary culture. As described under Materials and methods, embryonic rat brain cells were incubated for 5 d in the presence (B and D) or absence (A and C) of 2 µg/ml of GF-6B3 from a reversed-phase column under conditions described for Figure 3a.

ther TGF α or EGF. Purification of GF-6B3, in progress, will allow us to distinguish between these possibilities.

Materials and methods

Materials

Rat TGF α and antiserum to TGF α were purchased from Biotope (Redmond, WA) and human TGF α was synthesized chemically as described by Woo *et al.* (1989). Murine EGF was purified as described (Savage and Cohen, 1972). [3 H]thymidine was purchased from Amersham (Arlington Heights, IL). Antiserum to neuron specific enolase was from Polysciences (Warrington, PA), antibody to glial fibrillary acidic protein was from Boehringer Mannheim Biochemica (Indianapolis, IN), and antiserum to EGF and secondary antibodies for staining embryonic brain cells were obtained from Sigma (St. Louis, MO).

Cell lines and culture

PC12 cells were obtained from Dr. David Schubert, Salk Institute. 6B3 is a PC12 variant that had been stably transfected with the pMTEJ plasmid, which contains the cDNA for *Ha-ras* under control of the metallothionein promoter (Trimble *et al.*, 1986). NRK-49F cells were obtained from Lisa Coussens, UCLA. Cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% horse serum and 5% fetal calf serum (FCS) as described by Zhong and Howard (1990).

Collection and acetone precipitation of the 6B3 factor

Confluent cultures of 6B3 cells were washed twice with 4 ml phosphate-buffered saline (PBS) per 100-mm dish, incubated at 37°C for 4 h in serum-free DMEM containing 50 μ M ZnSO $_4$, washed again, and reincubated at 37°C in fresh serum-free DMEM containing 50 μ M ZnSO $_4$. After 3 d, the conditioned medium was collected, phenylmethyl sulfonyl fluoride was added to a concentration of 0.2 mM to inhibit proteases, and the medium was centrifuged at 4°C at 10 000 $\times g$ for 30 min to remove particulates. An equal volume of ice-cold acetone was added slowly with agitation; the mixture was allowed to stand for 30 min at room temperature, and it was centrifuged at 6000 $\times g$ for 10 min. The resulting supernatant solution was collected and more ice-cold acetone was added to a final concentration of 80% (v/v). After 30 min the precipitate was collected by centrifugation at 6000 $\times g$ for 10 min and dissolved in H $_2$ O. Particulate matter was removed by centrifugation at 12 000 $\times g$ for 30 min.

Gel filtration chromatography

The dissolved acetone precipitate from above was adjusted to 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.8), 500 mM NaCl, 10 mg/ml protein, and a 200- μ l sample was loaded onto a 10 $\times$ 300-mm prepacked Superose 12 HR 10/30 column (Pharmacia, Piscataway, NJ). Proteins were eluted with 20 mM Tris-HCl (pH 7.8), 500 mM NaCl at a flow rate of 0.6 ml/min, and 0.4 ml fractions were collected. Absorbance was monitored by an ultraviolet-light (UV) detector at 280 nm, and fractions were assayed for neurite-promoting activity.

Ion exchange chromatography

Samples from above were dialyzed against 20 mM Tris-HCl (pH 7.8), 5% betaine, passed through a 0.2- μ m pore membrane filter (Gelman, Ann Arbor, MI) and loaded onto a 5 $\times$ 50-mm Mono Q HR 5/5 column (Pharmacia). Protein was eluted with a 0- to 300-mM NaCl gradient in 20 mM Tris-HCl (pH 7.8), 5% betaine at a flow rate of 1 ml/min. Absorbance was monitored by a UV detector at 280 nm, and fractions were assayed for neurite-promoting activity.

Reversed-phase chromatography

Appropriate samples were loaded onto a 10 $\times$ 250-mm Vydac C $_4$ column. Protein was eluted with a 0–60% acetonitrile gradient in 0.1% trifluoroacetate at a flow rate of 0.7 ml/min and 1.4-ml fractions were collected. Samples were lyophilized in a Savant (Farmingdale, NY) Speed-Vac concentrator and redissolved in H $_2$ O. Absorbance was monitored by a UV detector at 230 nm, and fractions were assayed for neurite-promoting activity.

Assay of neurite-promoting activity

PC12 cells were seeded at a density of 2 $\times$ 10 4 cells/cm 2 in a 48-well cell culture dish containing 0.2 ml culture medium. The cells were allowed to attach to the plate by incubating for 3 h, and an appropriate dilution of factor in a volume no greater than 4 μ l was added to each well. The percentage of cells containing neurites greater in length than the average cell diameter was determined by phase-contrast microscopy 48 h later. The activity of a sample was quantified by determining the dilution required to give a half-maximal effect, as judged by the percentage of cells with neurites.

Colony formation in soft agar

A sample of 1 $\times$ 10 4 normal rat kidney (NRK-49F) cells was added to 2 ml of DMEM containing 10% horse serum, 5% FCS, 0.3% molten agar (Difco, Detroit, MI), and an appropriate growth factor; and the suspension was allowed to harden over a 2-ml bed of previously hardened 0.3% agar in culture medium in a 60-mm-diam dish. The samples were incubated for 7 d at 37°C under an atmosphere of 10% CO $_2$ in humidified air, and the number of colonies containing >20 cells was determined.

Thymidine incorporation into DNA

The procedure was that of Gospodarowicz *et al.* (1978) with slight modifications. NRK-49F cells were plated at a density of 2 $\times$ 10 4 cells/cm 2 in 48-well plates and incubated for 1 d in culture medium. The cells were washed twice with PBS and incubated at 37°C for 48 h in 1 ml of DMEM containing 0.1% FCS. An appropriate sample of growth factor was added, and the incubation was continued for 12 h. [3 H]thymidine (2.5 μ Ci/ml; 40 Ci/mmol) was added and, after an 8-h incubation at 37°C, the medium was aspirated and the cells were washed twice with PBS. A 0.1-ml sample of PBS was added to each well and the well was swabbed with a cotton swab. The cotton from the swab was sequentially incubated with agitation for 30 min in 10% trichloroacetic acid (TCA), 15 min in 5% TCA and 20 min in 95% ethanol. After drying, the cotton was placed in 10 ml of scintillation cocktail and the radioactivity determined by scintillation spectroscopy.

EGF receptor-binding assay

The EGF receptor-binding property of the 6B3 factor and other factors was measured by their ability to compete with ^{125}I -EGF in binding to the EGF receptor on A431 cells (ATCC) grown on 96-well plates (Corning, Corning, NY). EGF was iodinated to a specific activity of 1.5×10^6 dpm/pmol, using the Chloramin-T method (Hollenberg and Cuatrecasas, 1973). Confluent cells were washed three times at 25°C with binding buffer (a 1:1 mixture of DMEM and F12 without bicarbonate, containing 0.1% bovine serum albumin and buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.4). Cells were then incubated in triplicate with 100 μl of binding buffer containing known dilutions of the 6B3 factor or EGF or TGF α at 25°C for 30 min. After this incubation, 11 μl of 10 nM ^{125}I -EGF in binding buffer was added to each well and the samples incubated for an additional 60 min. At the end of the incubation, cells were freed of unbound ^{125}I -EGF by the use of three rapid washes with binding buffer. ^{125}I -EGF specifically associated with cell-surface EGF receptors was solubilized with 100 μL of 1 N NaOH and quantified by gamma counting.

Primary culture of embryonic brain cells

Brain was removed from a stage 15-ED rat embryo, minced in 1.5 ml DMEM/F12 medium containing 10% FCS, and gently triturated with a Pasteur pipet. After allowing the undispersed tissue to settle for 5 min, the dispersed cells were plated at a density of 2×10^5 cells/cm 2 on poly-L-lysine-coated cover slips and incubated at 37°C in 0.5 ml of the same medium under an atmosphere of 5% CO $_2$ in humidified air. After 24 h, the cells were washed with serum-free medium and incubated for an additional 5 d in 1 ml of fresh serum-free medium containing a growth factor or no growth factor.

Immunocytochemistry of embryonic brain cells

Primary cultures of embryonic brain cells were washed with PBS, fixed in 3.7% formaldehyde, and washed extensively in PBS. After incubation for 10 min in 0.1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and for 1 h in 0.05% Triton X-100, 5% normal goat serum, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, the cells were incubated for 1 h in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5% normal goat serum containing either a 1000-fold dilution of antiserum against neuron-specific enolase (NSE) to label neurons or a 200-fold dilution of monoclonal antibody against glia fibrillary acid protein (GFAP) to label astrocytes. The cells were washed nine times over 30 min with the above Tris solution lacking antibody and incubated for 45 min with a 350-fold dilution of goat anti-rabbit IgG conjugated with rhodamine for NSE staining or goat anti-mouse IgG conjugate with fluorescein for GFAP staining. Fluorescent cells were detected with a fluorescence microscope (Nikon, Garden City, NY).

Other procedures

Published procedures were used for SDS-acrylamide gel electrophoresis (Schagger and von Jagow, 1987) and protein determination (Bradford, 1976).

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