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Neuroblastoma Cells Express c-*sis* and Produce a Transforming Growth Factor Antigenically Related to the Platelet-Derived Growth Factor

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Mouse neuroblastoma Neuro-2A cells produce transforming growth factors during exponential growth in a defined hormone-free medium, which, on Bio-Gel columns in 1 M HAc, elute at a molecular size of 15 to 20 kilodaltons (kDa). These neuroblastoma-derived transforming growth factors have strong mitogenic activity, but they do not compete with epidermal growth factor for receptor binding (E. J. J. van Zoelen, D. R. Twardzik, T. M. J. van Oostwaard, P. T. van der Saag, S. W. de Laat, and G. J. Todaro, Proc. Natl. Acad. Sci. U.S.A. 81:4085–4089, 1984). In this study approximately 80% of the mitogenic activity was immunoprecipitated by antibodies raised against platelet-derived growth factor (PDGF). Immunoblotting indicated a true molecular size of 32 kDa for this PDGF-like growth factor. Analysis of poly(A)$^+$ RNA from Neuro-2A cells demonstrated the expression of the c-*sis* oncogene in this cell line, whereas in vitro translation of the RNA yielded a 20-kDa protein recognized by anti-PDGF antibodies. Separation by reverse-phase high-pressure liquid chromatography demonstrated the presence of two distinct mitogenic activities in neuroblastoma-derived transforming growth factor preparations, one of which is antigenically related to PDGF. Both activities had the ability to induce anchorage-independent growth in normal rat kidney cells, both in the presence and in the absence of epidermal growth factor. It is concluded that Neuro-2A cells express c-*sis* with concomitant production and secretion of a PDGF-like growth factor, which plays a role in the induction of phenotypic transformation on normal rat kidney cells.

Mouse neuroblastoma Neuro-2A cells can be cultured in a chemically defined serum-free medium in the absence of any externally added polypeptide growth factor. These conditions permit rapid exponential growth of this cell line with a doubling time of less than 10 h (8, 41). During proliferation in this medium, Neuro-2A cells produce and secrete transforming growth factors (TGFs) with a molecular size of 15 to 20 kilodaltons (kDa) according to their gel permeation chromatographic behavior. This so-called neuroblastoma-derived transforming growth factor (ND-TGF) is strongly mitogenic for quiescent fibroblasts and is able to induce phenotypic transformation in a nontransformed indicator cell like normal rat kidney (NRK) cells (41). Unlike the well-characterized TGFα (23, 24), however, this factor does not compete with epidermal growth factor (EGF) for receptor binding, whereas it differs from EGF-potentiated TGFβ (3) in having strong mitogenic activity. Studies on phenotypic transformation in a growth factor-defined culture medium have shown that ND-TGF induces morphological changes and soft agar growth in NRK cells independently of growth factors present in serum (42). In general, phenotypic transformation of NRK cells seems to require the combined action of a mitogen like EGF or TGFα and a modulator protein like TGFβ (1). Since ND-TGF does not compete with EGF for receptor binding, it was the aim of the present study to investigate which growth factor or combination of growth factors in our ND-TGF preparations is responsible for the induction of phenotypic transformation.

Platelet-derived growth factor (PDGF) is a strong mitogen for connective tissue-derived cells and stimulates in vitro growth of fibroblasts, smooth muscle cells, and glial cells (38, 46). It is a 28- to 32-kDa protein consisting of two structurally different polypeptide chains linked by disulfide bridges. Recently it has been shown that the 28-kDa protein encoded by the simian sarcoma virus (SSV) oncogene, v-*sis*, is structurally (11, 18, 44), antigenically (27, 34), and functionally (9, 17, 29, 43) very similar or identical to one of the chains of PDGF. A variety of transformed cell lines is known to produce PDGF-like growth factors, including glioma (28) and osteosarcoma (15) cells, a series of transformed fibroblasts (6), simian virus 40 (10)- and SSV (29)-transformed cells, embryonal carcinoma cells (13), and probably also T cells infected with human T-cell leukemia virus (47). For some of these cell lines, production of PDGF-like molecules can be correlated with expression of the c-*sis* oncogene (12, 47).

In this paper, we show that TGF activity isolated from Neuro-2A-conditioned medium contains two distinct mitogenic activities, one of which is antigenically related to PDGF. The two mitogens can be separated by reverse-phase high-pressure liquid chromatography (HPLC), and both of them are associated with soft agar growth-inducing activity in NRK cells. Analysis of poly(A)$^+$ RNA from Neuro-2A cells on Northern blots demonstrates the expression of the c-*sis* oncogene in this cell line, whereas in vitro translation of poly(A)$^+$ RNA yields a discrete protein which can be immunoprecipitated by an antibody against human PDGF. This is the first demonstration of c-*sis* expression in a murine cell line and production of a PDGF-like growth factor by a neuronal cell line. In addition, it is the first indication of the

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involvement of PDGF-like growth factors in the induction of soft agar growth in NRK cells.

MATERIALS AND METHODS

Cell culture. C1300 mouse neuroblastoma cells, clone Neuro-2A, were obtained from the American Type Culture Collection, Rockville, Md. Cells were cultured routinely in Dulbecco modified Eagle medium supplemented with 7.5% fetal calf serum (FCS; Flow Laboratories, Inc., Irvine, Scotland). Under these conditions, cells proliferated with a doubling time of approximately 8 h. For exponential growth in a chemically defined serum-free medium, cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham F12 medium supplemented with 30 nM Na_2SeO_3 (ICN Pharmaceuticals Inc., Irvine, Calif.) and 10 μg of human transferrin (Sigma Chemical Co., St. Louis, Mo.) per ml and buffered with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–17.6 mM $NaHCO_3$ (pH 7.6) at 37°C. Under these conditions, cells proliferated with a doubling time of 9 to 10 h (41).

TGF isolation. Neuro-2A cells (10^7) were plated in an 850-cm^2 plastic roller bottle (Falcon; Becton Dickinson Labware, Oxnard, Calif.) in serum-containing medium. After 24 h, the medium was exchanged for 30 ml of serum-free medium, which was discarded 8 h later. The cells were then grown for 24 h in 75 ml, and subsequently for 16 h in 100 ml, of serum-free medium. During this time, cells proliferated exponentially. The 175 ml of conditioned medium collected was passed through an AP-15 prefilter (Millipore Corp. Bedford, Mass.) and lyophilized in the presence of 1 μg of phenylmethylsulfonyl fluoride (Sigma) per ml. Lyophilized Neuro-2A-conditioned, serum-free medium was extracted with 1 M HAc, dialyzed against 0.2 M HAc in Spectrapor 3 membrane tubing (molecular weight cutoff, 3,500; Spectrum Medical Industries, Inc., Los Angeles, Calif.), and lyophilized again. Subsequently, the material was run on a Bio-Gel P-100 column (2.6 by 85 cm, 100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) in 1 M HAc. The column eluate was tested for mitogenic activity on quiescent 3T3 cells (see below), and the active fractions with apparent sizes between 15 and 20 kDa were pooled and lyophilized (for details, see reference 41). The material obtained in this way is referred to as ND-TGF and corresponded to 1 to 1.5 mg of protein per liter of conditioned medium.

Reverse-phase HPLC purification. HPLC purification of ND-TGF was performed with a model 680 automated gradient controller supplied with two 510 solvent pumps and a 441 absorbance detector (Waters Associates, Inc., Milford, Mass.). Gradients of acetonitrile (HPLC gradient grade; J. T. Baker Chemical Co., Deventer, The Netherlands) were prepared by mixtures of solvent A (10 mM trifluoroacetic acid (TFA [pH 2.2]; Baker) and solvent B (90% acetonitrile–10 mM TFA–NH_4OH [pH 2.2]). ND-TGF (1.5 mg) was dissolved in 1.5 ml of 20% solvent B and applied to a C_{18} Hi-Pore reverse-phase HPLC column (250 by 4.6 mm) (RP318; Bio-Rad) equilibrated with 20% solvent B. Material was eluted by applying a linear gradient of 0.1% acetonitrile per min at a flow rate of 1 ml/min. Fractions of 2 ml were collected in 2 ml of 2 M HAc containing 0.01% bovine serum albumin (BSA; Sigma). In the data presented in this paper, corrections were made for the delay between detector and fraction collector (1.0 ml). The indicated concentrations of acetonitrile refer to the computer setting during collection.

Immunoprecipitation of ND-TGF with anti-PDGF antibody. Protein A–Sepharose CL-4B slurry (100 μl; concentration, 1.5g/10.5 ml; Pharmacia, Uppsala, Sweden) was washed three times with ice-cold 100 mM Tris–20 mM EDTA (pH 7.4) in an Eppendorf centrifuge tube. Subsequently, 50 μl of rabbit antiserum raised against human PDGF (16) was added and incubated with the beads for 90 min at 4°C while shaking. The beads were then washed twice with the Tris-EDTA solution described above, once with 500 mM NH_4Ac–0.5% BSA (pH 7.4), and twice with 100 mM NH_4Ac (pH 7.4), all at 4°C. ND-TGF (150 μg) corresponding to 100 ml of conditioned medium was dissolved in 100 μl of 100 mM NH_4Ac (pH 7.4) and incubated with the beads for 90 min at 4°C while shaking. Subsequently, 1 ml of 100 mM NH_4Ac was added, the beads were centrifuged for 2 min in an Eppendorf centrifuge, and the supernatant of this spin, together with that of the next washing in 100 mM NH_4Ac, were collected and lyophilized. This fraction will be referred to as the protein A supernatant. The beads were then washed twice with 500 mM NH_4Ac–0.5% BSA (pH 7.4) and once again with 100 mM NH_4Ac. Subsequently, they were incubated twice with 1 M HAc for 15 min at room temperature, and the acetic acid fractions collected, which are referred to as the protein A pellet, were lyophilized. Fractions were sterilized by suspension in 1 M HAc followed by lyophilization. For control experiments, nonimmune rabbit serum was used instead of anti-PDGF antiserum, whereas the assay system was tested by using 50 ng of pure human PDGF (19) lyophilized in the presence of 100 μg of BSA.

SDS gel elctrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Laemmli (22) with a 12.5% acrylamide running gel (pH 8.8) and a 5.0% acrylamide stacking gel (pH 6.8), both at a bisacrylamide-to-acrylamide weight ratio of 0.0267. Protein samples were denaturated by heating for 5 min at 100°C in 5% SDS in the absence of reducing agents. Gels were stained with Bio-Rad silver stain.

For immunoblotting (Western blots), gels were blotted overnight onto nitrocellulose paper (4°C; 30 to 35 V; 100 mA), and antigen-antibody complexes were visualized by a modification of the procedure of Towbin et al. (40). Blot strips were incubated for 30 min at room temperature in 150 mM NaCl–20 mM Tris–0.5% Tween 20–HCl (pH 7.4) (enzyme-linked immunosorbent assay buffer) while shaking. Subsequently, the strips were transferred to a test tube containing 4 ml of enzyme-linked immunosorbent assay buffer and, in addition, 1% ovalbumin and the indicated dilution of antiserum. The tubes were sealed and incubated for 60 min at 37°C while shaking. The strips were then washed three times with tap water and enzyme-linked immunosorbent assay buffer alternately for 20 min at room temperature. Subsequently, they were transferred to test tubes containing 4 ml of enzyme-linked immunosorbent assay buffer, 1% ovalbumin, and a 1:1000 dilution of horseradish peroxidase-labeled goat anti-rabbit antibody (Nordic, Tilburg, The Netherlands) and incubated for 60 min at 37°C in sealed tubes while shaking. The strips were washed again as above, dried on filter paper, transferred to a glass plate, and incubated in TMB substrate solution (24 mg of 3,3',5,5'-tetramethylbenzidine and 80 mg of dioctyl sulfosuccinate [Sigma] dissolved together in 10 ml of ethanol and added to 30 ml of a 25 mM citric acid–50 mM Na_2HPO_4 buffer [pH 5.0] in the presence of 20 μl of a 30% H_2O_2 solution) at room temperature. The color reaction was terminated by rinsing in tap water.

Northern blotting. Poly(A)$^+$ RNA was isolated from Neuro-2A cells by the Mg precipitation method of Palmiter (30), yielding approximately 200 μg/10^9 cells. Poly(A)$^+$ RNA

(5 μg) was separated on a 1% agarose gel after denaturation in 0.5 M glyoxal–0.5% dimethyl sulfoxide. After electrophoresis, RNA was blotted onto nitrocellulose paper in 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) as previously described (25). The blot strip was incubated for 16 h at 42°C in hybridization mix (50% formamide–20 mM sodium phosphate buffer [pH 7.0]–5 mM EDTA–5× SSC–1× Denhardt solution–100 μg of denatured salmon sperm DNA [Boehringer GmbH, Mannheim, Federal Republic of Germany] per ml) containing 1×10^6 to 2×10^6 cpm of nick-translated DNA probe (see below) per ml. Subsequently, the filter was washed twice with the above hybridization mix for 1 h at 42°C and incubated in 2× SSC–0.1% SDS and 1× SSC–0.1% SDS for 15 min each. After drying, the blot strip was exposed for 2 weeks at −70°C with Kodak XAR-2 film and Dupont Cronex Lightning-Plus screen (DuPont Co., Wilmington, Del.).

The DNA probe used in this study was derived from a subclone (pA03) of the pC60 plasmid (obtained from R. Gallo), which includes a 6-kilobase-pair (kbp) *Eco*RI fragment containing the total SSV genome. pA03 is a recombinant of pSVBR94 and contains the 1.5-kbp *Sac*I (Boehringer) restriction fragment of pC60. This insert represents the total v-*sis* sequence, including a fraction of the *env* gene and long terminal repeat. The v-*sis*-specific DNA probe was isolated by digesting 100 μg of pA03 DNA with *Sac*I and *Xba*I. These restriction fragments were separated on a 0.6% agarose gel (low-melting-point agarose, SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine). The 1.0-kbp *Sac*I-*Xba*I fragment (v-*sis*) was cut out of the gel, and the DNA was isolated as described by Weislander (45) and subsequently labeled as described by Rigby et al. (32).

In vitro translation of poly(A)$^+$ RNA. Poly(A)$^+$ RNA (1 μg per 20 μl of translation mix) was translated either in a messenger-dependent reticulocyte cell-free system in the presence of [^{35}S]cysteine (660 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) under conditions described by Saris et al. (36) or in commercially available nuclease-treated wheat germ extract under conditions specified by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with [^{3}H]leucine (324 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as the radioactive amino acid. For immunoprecipitation, 10 μl of the protein sample obtained after in vitro translation was adjusted to PBSTDS (150 mM NaCl–10 mM sodium phosphate [pH 7.2]–1% Triton X-100–0.5% sodium deoxycholate–0.1% SDS) in a total volume of 100 μl and incubated for 16 h at 4°C in the presence of 1.5 μl of anti-PDGF antiserum or nonimmune rabbit serum. Subsequently, 10 μl of a 10% (wt/vol) suspension of *Staphylococcus aureus* cells (IgG-sorb; Enzyme Center Inc., Boston, Mass.) in PBSTDS containing 10 mg of BSA per ml was added, and incubation was continued for 1 h at 4°C. Samples were then centrifuged through a sucrose cushion consisting of a 0.5-ml layer of 1 M sucrose and a 0.25-ml layer of 0.5 M sucrose, both in PBSTDS, for 30 min at 17,000 × *g*. The precipitate was washed three times in PBSTDS, dissolved in 20 μl of bidistilled water, and analyzed by SDS gel electrophoresis and autoradiography.

Miscellaneous. Thymidine (TdR) incorporation into mitogenically stimulated Swiss 3T3 cells was determined as previously described (41). Cells (5×10^4) were seeded in a 1.8-cm^2 well and made quiescent by incubation for 48 h in medium containing 0.5% FCS. [^{3}H]thymidine incorporation was measured cumulatively between 8 and 24 h after mitogenic stimulus.

The assay for the induction of soft agar growth on NRK fibroblasts, clone 49F, was as previously described (41). Trypsinized cells (10^4) were incubated in 0.5 ml of serum-containing medium, including the growth factors to be tested (growth factor concentrations in the assay refer to this volume), before inoculation into a 0.375% agar layer in a 60-mm petri dish. Colonies larger than eight cells were scored after 14 days.

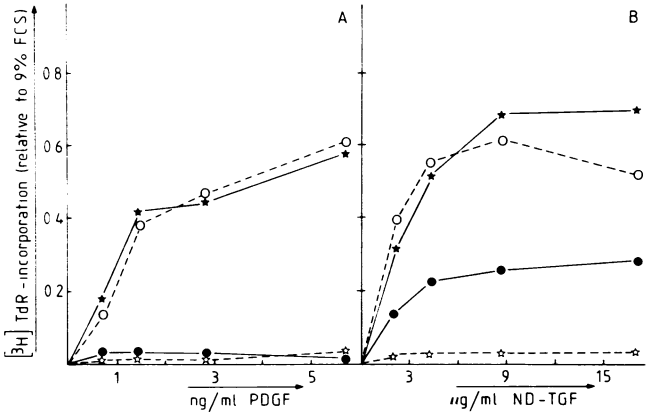

FIG. 1. Immunoprecipitation of human PDGF (A) and ND-TGF (B) with anti-PDGF antibodies. PDGF (50 ng in 100 μg of BSA) or ND-TGF (150 μg obtained from 0.1 liter of conditioned medium) was immunoprecipitated with either anti-PDGF antiserum or nonimmune rabbit serum, and both the protein A supernatant and protein A pellet were collected and lyophilized. Samples were dissolved in 800 μl of BSA-containing buffer, and 100 μl was tested for mitogenic activity in a total volume of 1.1 ml by making serial dilutions by a factor of 2. Mitogenic activity is expressed relative to that of 9% FCS (4.0×10^5 dpm of [^{3}H]TdR per well). Symbols: ○, protein A supernatant, nonimmune rabbit serum; ☆, protein A pellet, nonimmune rabbit serum; ●, protein A supernatant, anti-PDGF antiserum; ★, protein A pellet, anti-PDGF antiserum.

RESULTS

Immunoprecipitation of ND-TGF with anti-PDGF antibody. Neuro-2A cells produced and secreted a strong mitogen which, on Bio-Gel P-100 columns in 1 M HAc, eluted at a molecular size of 15 to 20 kDa. Since this mitogenic activity coeluted with soft agar growth-inducing activity on NRK cells, it was named ND-TGF. Stability tests showed that ND-TGF was stable to acid and heat, but its activity was destroyed by incubation with dithiothreitol or trypsin, demonstrating its protein nature (41). More than 70% of the mitogenic activity of ND-TGF could be immunoprecipitated with a polyclonal rabbit antiserum against human PDGF, whereas no such precipitation was seen in the presence of nonimmune rabbit serum (Fig. 1). As a control, pure PDGF from human platelets was immunoprecipitated quantitatively in this assay. The fraction of ND-TGF activity which could not be precipitated with anti-PDGF antibody (approximately 30%) showed a dose-dependent curve in the mitogenic assay, which was clearly different from that of PDGF. This indicated that our ND-TGF preparations contained at least two different mitogens, one of which was antigenically related to PDGF. Assuming that half-maximal mitogenic stimulation by the PDGF-like growth factor in ND-TGF occurs at a similar concentration as by human PDGF (1 ng/ml; see Fig. 1A), the data of Fig. 1B indicate a production of approximately 800 ng of PDGF-like growth factor per liter of Neuro-2A-conditioned, serum-free medium. A similar amount of material was detected in a radioimmunoassay for human PDGF, but significantly more competing activity was

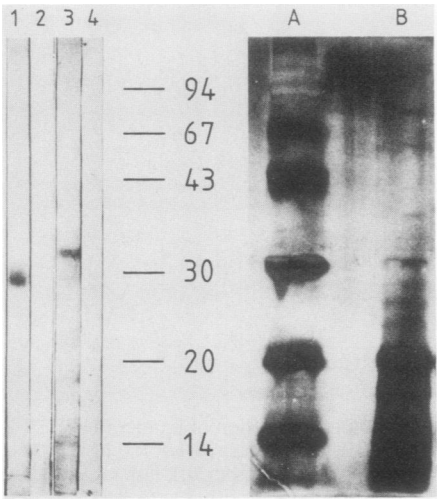

FIG. 2. SDS gel electrophoresis and immunoblotting of ND-TGF. ND-TGF (15 μg) was run on an SDS gel under nonreducing conditions and stained with Bio-Rad silver staining (B). The gel was calibrated with a Pharmacia low-molecular-weight calibration kit (A; molecular size in kDa). For immunoblotting, 20 ng of human PDGF (lanes 1 and 2) or 20 μg of ND-TGF (lanes 3 and 4) was run on the gel and stained with either anti-PDGF antiserum (lanes 1 and 3) or nonimmune rabbit serum (lanes 2 and 4), both at 200-fold dilution.

observed in a radioreceptor-binding assay for PDGF (C. H. Heldin, unpublished data), similarly as observed for a glioma-derived PDGF-like growth factor (28). Based on a protein content of 1 to 1.5 mg of ND-TGF per liter of Neuro-2A-conditioned, serum-free medium, this is equivalent to 0.05 to 0.1% PDGF-like growth factor in these preparations. To ascertain that the PDGF-like growth factor in Neuro-2A-conditioned medium did not arise from the bovine serum in which the cells were plated, Neuro-2A cells were cultured for various generations in the presence of growth factor-inactivated serum (42). Analysis of such conditioned medium showed the presence of a similar PDGF-like growth factor (data not shown).

SDS gel electrophoresis and immunoblotting of ND-TGF. Figure 2 shows an SDS gel of ND-TGF under nonreducing conditions visualized by silver staining. As expected from the molecular size cutoff of the Bio-Gel P-100 column, most proteins in this partly purified preparation ran at molecular sizes up to 20 kDa (lane B). After blotting onto nitrocellulose paper and incubation with anti-PDGF antibody (lane 3), however, staining of the PDGF-like growth factor was prominent at a molecular size of 32 kDa. No silver-stained band was seen at this molecular size, most likely because only 0.05 to 0.1% of the total protein material represents the PDGF-like growth factor. Immunostaining was not seen with nonimmune rabbit serum (lane 4), whereas pure human PDGF ran at a molecular size of 30 kDa under these experimental conditions (lanes 1 and 2). Besides the major 32-kDa band of the PDGF-like growth factor, faint bands were seen at 14, 18, and 30 kDa. The 18-kDa protein may reflect the reduced form of the PDGF-like growth factor, but the origin of the 14- and 20-kDa bands is presently unknown. These bands may be specific for PDGF-like growth factors from murine sources. When the gel was run under reducing conditions, the 32-kDa band disappeared, and there was a slight increase in the intensity of the 18-kDa band (data not shown). This indicates that this PDGF-like growth factor consists of two chains linked by disulfide bridges, just like PDGF. However, the fully reduced chains of the protein stained only poorly with the antiserum compared with the intact protein (C. H. Heldin, unpublished data). The 32-kDa molecular size of this neuroblastoma-derived, PDGF-like growth factor is close to the 30- to 34-kDa molecular size of PDGF-like growth factors produced by other cells (10, 15, 28, 29). The present data show that Neuro-2A cells secrete a 32-kDa PDGF-like growth factor which, on Bio-Gel columns in 1 M HAc, tends to elute at an aberrant molecular size of 15 to 20 kDa. Human PDGF showed similar gel permeation behavior on our Bio-Gel P-100 column (data not shown), most likely because of charge interactions between the strongly positively charged protein (46) and the slightly negatively charged column material. A similar observation has recently been made for the gel permeation characteristics of TGFβ and a purification procedure for this protein worked out based on this behavior (4).

Expression of c-*sis* in Neuro-2A cells. Figure 3 shows a Northern blot analysis of poly(A)$^+$ RNA from Neuro-2A cells hybridized with a v-*sis* probe. Hybridization occurs predominantly at RNA sizes of 4.5, 3.1, and 2.0 kbp, demonstrating the presence of RNA sequences homologous to v-*sis* DNA. No hybridization was detected with poly(A)$^+$ RNA from mouse 3T3 fibroblasts (data not shown). The major transcript of 4.5 kbp present in mouse Neuro-2A cells may be comparable to the major 4.2-kbp transcript detected in a number of human tumor cell lines (12, 47). Whether any of these transcripts in Neuro-2A cells is responsible for the translation of the PDGF-like growth factor remains to be established. Southern blot analysis of Neuro-2A DNA hybridized with the v-*sis* probe showed no qualitative or quantitative differences from that of mouse 3T3 fibroblasts, which argues against gene amplification of c-*sis* in Neuro-2A cells (W. J. M. van de Ven, unpublished data).

In vitro translation of Neuro-2A poly(A)$^+$ RNA. Poly(A)$^+$

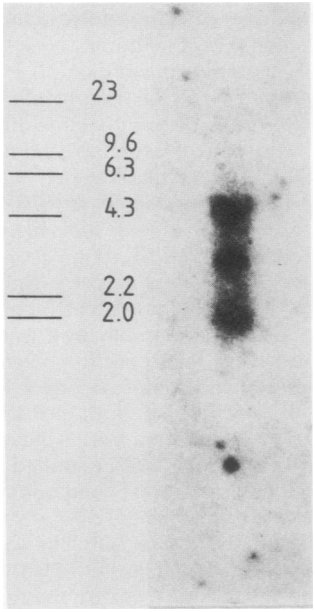

FIG. 3. Northern blot of Neuro-2A poly(A)$^+$ RNA (5 μg) hybridized with a v-*sis* probe. *Hin*dIII-generated fragments of bacteriophage λ DNA were used as molecular size standards (expressed in kbp).

RNA from Neuro-2A cells was translated in a cell-free system, and the presence of proteins antigenically related to PDGF was analyzed by immunoprecipitation of the translation products with anti-PDGF antiserum. Figure 4 shows that, both in a reticulocyte lysate (lane B) and in a wheat germ extract (lane D), Neuro-2A poly(A)$^+$ RNA codes for a protein of approximately 20 kDa, which can be immunoprecipitated with anti-PDGF antiserum but not with nonimmune rabbit serum (lane C). This demonstrates directly that Neuro-2A cells can produce a PDGF-like protein. The presence of reducing agents during SDS gel electrophoresis did not affect the apparent size of the protein (data not shown). It should be realized, however, that to optimize reliable translation, the reticulocyte lysate and the wheat germ extract contained 2 and 5 mM dithiothreitol, respectively. Furthermore, the *sis* oncogene product is known to undergo a series of discrete processing steps within the cell, including dimer formation and proteolytic digestion (34), which will certainly not take place similarly after cell-free translation. The relation between this 20-kDa protein and the 20-kDa protein immunoprecipitated with anti-PDGF antiserum from lysates of SSV-transformed NIH-3T3 cells (9) therefore requires further investigation.

Reverse-phase HPLC purification of ND-TGF. Reverse-phase HPLC has turned out to be a powerful technique for purification of polypeptide growth factors, since these factors are stable at low pH and in organic solvents like acetonitrile and propanol (2, 24). We used a Hi-Pore C_{18} column, since it gives a better recovery of proteins with molecular sizes above 10 kDa than conventional C_{18} columns. Figure 5 shows the elution profile of ND-TGF on this column with an acetonitrile gradient. Clearly, two mitogenic activities are observed well separated from the major protein peaks. The first activity, which eluted between 27 and 29% acetonitrile, was a very strong mitogen for 3T3 cells. In general, the activity tended to elute as a double peak, which may indicate protein heterogeneity. The second activity eluted as a broad band between 32 and 37% acetonitrile and was mitogenically much less active than the first one. Although each of the two peaks may well contain various growth factor activities, these results demonstrated that Neuro-2A cells produce at least two different mitogens which, on Bio-Gel columns, both elute at 15 to 20 kDa. We will refer to the first, more hydrophilic, activity as ND-TGF I and to the second, more hydrophobic, one as ND-TGF II.

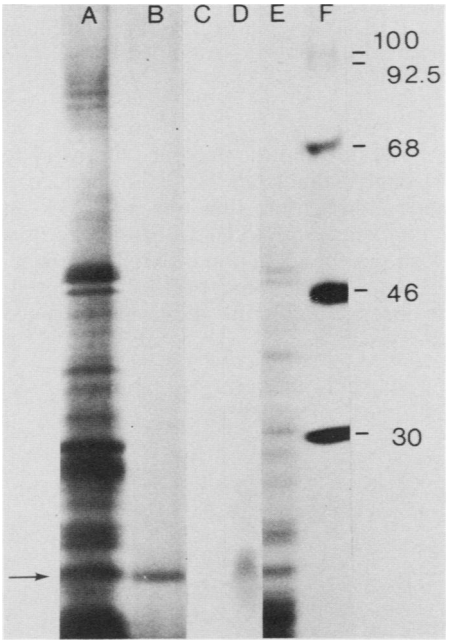

FIG. 4. In vitro translation of Neuro-2A poly(A)$^+$ RNA in reticulocyte lysate (lanes A and B) in the presence of [^{35}S]cysteine or in wheat germ extract (lanes C, D, and E) with [^{3}H]leucine as the radioactive amino acid and 1 μg of RNA per 20 μl of translation mix. Total translational products (3 μl of translation mix) were analyzed on a 15% SDS-polyacrylamide gel (lanes A and E). For immunoprecipitation, 10-μl portions of translation products were treated with nonimmune rabbit serum (lane C) or anti-PDGF antiserum (lanes B and D), as described above, and subsequently analyzed on SDS gels. Molecular sizes were calibrated (lane F) with ^{14}C-labeled methylated phosphorylase *b* (100 and 92.5 kDa), BSA (68 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa), all from Amersham. Molecular sizes of the marker proteins are expressed in kDa. The arrow denotes the size of the major immunoprecipitated product.

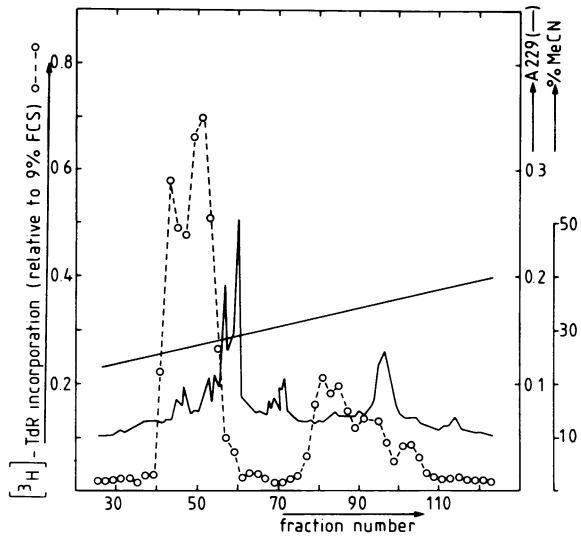

FIG. 5. Protein profile (absorbance at 229 nm) and mitogenic activity of ND-TGF (1.5 mg) separated on a Hi-Pore C_{18} reverse-phase HPLC column by applying a linear acetonitrile gradient (0.1%/min). Fractions of 2 ml were collected in 2 ml of 2 M HAc-0.01% BSA, and 400-μl samples were tested for mitogenic activity. Activity is expressed relative to that of 9% FCS (2.6×10^5 dpm of [^{3}H]TdR per well).

Figure 6 shows immunoblotting of ND-TGF I and ND-TGF II stained with either anti-PDGF antiserum or nonimmune rabbit serum. The data clearly show that ND-TGF I is antigenically related to PDGF and has a molecular size of 32 kDa (lane A). We consistently observed that human PDGF eluted from this HPLC column at 28 to 30% acetonitrile (data not shown). The nature of ND-TGF II was much less clear. Unlike that of ND-TGF I, the activity of ND-TGF II could not be immunoprecipitated with anti-PDGF antiserum (data not shown). Surprisingly, however, a 14-kDa protein was observed by immunoblotting (lane C). The origin of this protein, which may be similar to one which shows up faintly in the blotting of Fig. 2, is unknown. It may represent a proteolytic cleavage product of nondimerized c-*sis* oncogene product, which is mitogenically inactive, since the activity of ND-TGF II could not be immunoprecipitated with anti-PDGF antiserum. To what extent this product may be an artifact of the HPLC procedure used requires further inves-

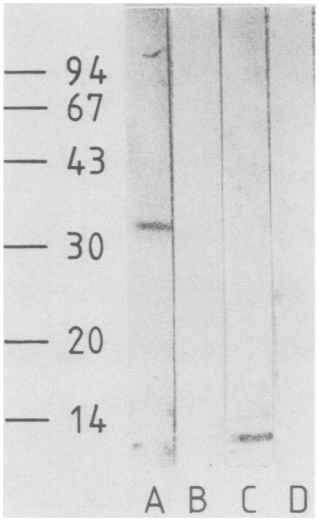

FIG. 6. Immunoblotting of ND-TGF I (lanes A and B) and ND-TGF II (lanes C and D) with anti-PDGF antiserum (lanes A and C) and nonimmune rabbit serum (lanes B and D). Pooled fractions of ND-TGF I and ND-TGF II derived from 150 μg of ND-TGF were applied to the gel under nonreducing conditions and stained with diluted (1:200) antiserum or nonimmune serum. Molecular sizes of marker proteins are expressed in kDa.

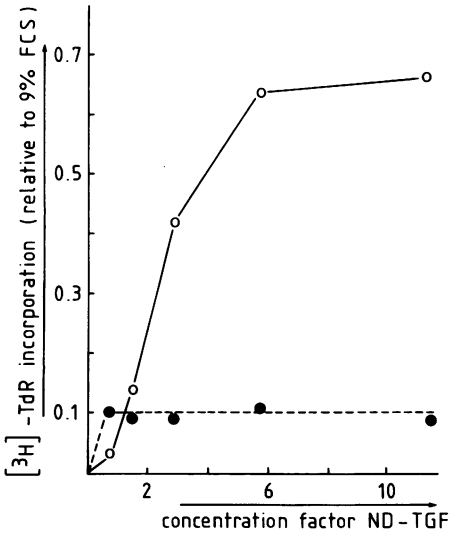

FIG. 7. Dose-response curves for mitogenic activities of ND-TGF I (○) and ND-TGF II (●). Pooled fractions of ND-TGF I and ND-TGF II derived from 300 μg of ND-TGF were lyophilized and suspended in 800 μl of BSA-containing buffer. From these samples, 100 μl was tested for mitogenic activity by using serial dilutions by a factor of 2. Stimuli are expressed as a concentration factor, i.e., the concentration of mitogen in the cellular DNA synthesis assay (volume, 1.1 ml) relative to that in Neuro-2A-conditioned medium. A concentration factor of 1 corresponds to pooled fractions obtained from 1.65 μg of ND-TGF tested in 1.1 ml of test medium. Mitogenic activity is expressed relative to that of 9% FCS (3.2×10^5 dpm of [^{3}H]TdR per well).

tigation. Preliminary studies on the nature of ND-TGF II have shown that it requires intact disulfide bridges for activity and that it has mitogenic activity toward NR-6 cells, a 3T3 mutant lacking an EGF receptor, indicating that ND-TGF II differs from both EGF and TGFα.

Figure 7 shows dose-response curves for the mitogenic activity of ND-TGF I and ND-TGF II. ND-TGF I stimulated TdR incorporation into quiescent 3T3 cells to a similar extent as did human PDGF (see Fig. 1A). ND-TGF II was a relatively poor mitogen for this cell line but showed saturating activity at more dilute concentrations than did ND-TGF I, demonstrating again the different characters of these two activities. NG-TGF II accounted for approximately 20% of the total mitogenic activity present in ND-TGF, which might correspond to the value of 30% for activity which could not be immunoprecipitated with anti-PDGF antiserum (Fig. 1B). Assuming that ND-TGF I shows similar half-maximal mitogenic stimulation as does human PDGF (1 ng/ml), the dose-response curve of Fig. 7 suggests the presence of 450 ng of PDGF-like growth factor per liter of Neuro-2A-conditioned medium, indicating the 60% recovery of mitogenic activity during this HPLC purification step. Based on the overlap of mitogenic activity and protein absorbance of Fig. 5, a 20- to 50-fold increase in specific activity of this PDGF-like growth factor was observed compared with the material obtained by gel permeation. No substantial increase in specific activity was obtained by applying an additional propanol gradient to the same column, as has been shown for other TGFs (24).

Phenotypic transformation induced by ND-TGF. It has been demonstrated that ND-TGF is able to induce soft agar growth and morphological transformation of NRK cells both in serum-containing media (41) and in media supplemented with growth factor-inactivated serum (42). Since ND-TGF does not compete with EGF for receptor binding, it was concluded that, in contrast to data of Anzano et al. (1), phenotypic transformation of NRK cells can be induced in the absence of a ligand binding to the EGF receptor. It was observed, however, that the presence of low concentrations of EGF strongly stimulated ND-TGF-induced phenotypic transformation, indicating that our ND-TGF preparations may have contained additional TGFβ-like activity (41).

Figure 8 shows the induction of NRK soft agar growth by the various column fractions of HPLC-separated ND-TGF, both in the absence and in the additional presence of 2 ng of

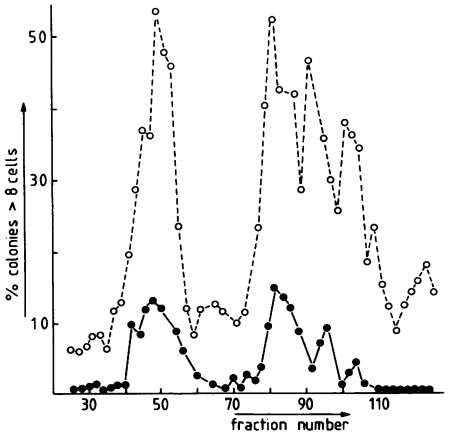

FIG. 8. Induction of NRK soft agar growth by column fractions of reverse-phase HPLC-separated ND-TGF (see Fig. 5) tested in the absence (●; 4-ml fraction tested) and presence (○; 2-ml fractions tested) of 2 ng of EGF (receptor grade; Collaborative Research, Inc., Waltham, Mass.) per ml. Colonies were scored, unfixed and unstained, after 14 days and expressed as the number of colonies larger than eight cells relative to the number of inoculated cells.

EGF per ml. In the absence of EGF, a maximum of approximately 15% of the cells could be induced to form progressively growing colonies in agar, which could reach sizes of up to 5,000 μm^2. In the additional presence of EGF, however, 55% of the cells could be induced to form colonies of up to 13,000 μm^2, in agreement with previous data (41). In the presence of EGF alone, colonies larger than 1,500 μm^2 were generally not observed. When comparing the profile of Fig. 8 with the elution pattern of mitogenic activity shown in Fig. 5, it is striking that mitogenic and soft agar growth-inducing activities were fully coincident on this column, both in the presence and absence of additional EGF. It is also remarkable that ND-TGF II, clearly heterogeneous in nature, was as potent in inducing NRK soft agar growth as was ND-TGF I. TGFβ from human platelets, purified as described by Assoian et al. (4), eluted from this column with a major peak at 37% acetonitrile (fraction numbers 100 to 110), the ascending slope starting at 34% acetonitrile (data not shown). TGFβ from a transformed murine cell line eluted at a similar percentage of acetonitrile on a μBondapak C_{18} column (Waters Associates, Inc., Milford, Mass.) (2). It is therefore well possible that EGF-potentiated induction of soft agar growth by ND-TGF II was mediated by TGFβ, although this does not explain why the activity should be coincident with mitogenic activity. It is important to note that there was no soft agar growth-inducing activity coincident with the major protein peaks around fraction 60, indicating that growth factors did not tend to elute aspecifically with other proteins from this column. Therefore, we have no indication at this stage that the EGF-potentiated soft agar growth-inducing activity coincident with PDGF-like growth factor ND-TGF I was mediated by TGFβ.

The above data strongly suggest that mitogens present in ND-TGF were directly involved in the induction of NRK soft agar growth, in spite of the fact that these assays were carried out in the presence of 10% calf serum. To what extent specific modulator proteins were involved is presently unknown. Human PDGF was by itself unable to induce NRK soft agar growth when assayed in the presence of 10% bovine serum (data not shown), nor was it able to potentiate TGFβ (3, 35). So, unless PDGF-like growth factors have properties different from those of human PDGF, our results may point toward a specific PDGF-potentiated modulator protein. Preliminary data have shown that EGF-potentiated soft agar growth-inducing activity can be immunoprecipitated from ND-TGF I by anti-PDGF antiserum, but not by nonimmune rabbit serum. These experiments are hampered by the fact that remnants of rabbit serum, which itself contains high levels of TGFβ activity (31), may have been present, but they also show that PDGF-like growth factors may have been directly involved in the induction of NRK soft agar growth. The consequences of these observations for the mechanism of phenotypic transformation will be discussed.

DISCUSSION

In this paper, we showed that mouse neuroblastoma Neuro-2A cells show expression of the c-*sis* oncogene and that they both produce and secrete a PDGF-like growth factor. During all purification steps so far, the mitogenic activity of this PDGF-like growth factor was coincident with soft agar growth-inducing activity. This is the first demonstration of c-*sis* expression in a murine cell line and of production of PDGF-like growth factors by a neuronal cell line, and it is the first indication of involvement of PDGF-like growth factors in the induction of soft agar growth in NRK cells. We are interested in the regulation of proliferation and differentiation of neuroblastoma cells, and in particular in the extracellular stimuli which govern these processes (8). The observation that this cell line proliferates very rapidly in the absence of externally added growth factors suggests that cellular proliferation may be regulated by the autocrine production of polypeptide growth factors. Most likely, however, complex interactions between multiple growth factors are required for this process (26). In this study we focused on one of the possible factors involved.

The recent observation that the *sis* oncogene codes for one of the chains of PDGF has given a strong impetus to studies on the production of PDGF-like growth factors by transformed cells (14). The amount of immunoprecipitable PDGF-like growth factor (0.8 to 1.0 μg/liter of conditioned medium) is of the same order of magnitude as that shown for other cells, although competing activity with ^{125}I-PDGF for receptor binding may suggest much higher amounts (28). It is interesting that c-*sis* expression and production of PDGF-like growth factors correlates for the most part with mesenchymally derived tumor lines such as transformed fibroblasts and osteosarcoma cells, whose normal cell counterparts are mitogenically stimulated by PDGF. However, T cells infected with human T-cell leukemia virus also show c-*sis* expression, whereas lymphocytes show no mitogenic response to PDGF (47). Similarly, there are no published reports indicating the presence of PDGF receptors in either neuroblasts or neuroblastoma cells. Whether *sis* expression is a contributing factor or a consequence of transformation in Neuro-2A cells is therefore unknown. Addition of anti-PDGF antibodies to the culture medium of these cells does not slow down their proliferation (unpublished observation). A similar observation has been made, however, for other PDGF-like growth factor-producing cells which have normal counterparts carrying PDGF receptors (5, 14). Therefore, this result does not rule out a critical role for PDGF-like growth factors in growth regulation of Neuro-2A cells. Alternatively, production of PDGF-like growth factors may be relevant in vivo for paracrine stimulation of nearby glia cells, which do have functional PDGF receptors. In work to be published elsewhere, we will show that a clone obtained from another mouse neuroblastoma cell line, N1E-115, which is able to proliferate in the absence of external growth factors, also produces PDGF-like growth factors, demonstrating that our observations are not restricted to the Neuro-2A cell line.

In recent years, evidence has accumulated for strong parallels between the mode of action of polypeptide growth factors and that of oncogene products (reviewed in reference 15). Based on the observation that Neuro-2A cells secrete a PDGF-like growth factor, we tested this cell line for the expression of the c-*sis* oncogene. Of other oncogenes tested, c-*myc* in particular showed prominent expression in Neuro-2A cells (W. J. M. van de Ven, unpublished data). Recently, a number of neuroblastoma cell lines have been shown to express N-*ras* (39) and N-*myc* (37). Furthermore, mitogenic stimulation of cells by PDGF has been shown to involve transient expression of c-*myc* (21).

Transforming growth factors are, by definition, mitogenic polypeptide hormones with the additional ability to induce phenotypic transformation in nontransformed indicator cells. In general, however, phenotypic transformation requires the combined action of multiple growth factors. Based on data for rat embryo fibroblasts, Kaplan and Ozanne (20) have proposed that anchorage-independent growth reflects

the total growth factor concentration in the medium, not the presence of a specific type of growth factor. In contrast, Sporn and co-workers (1, 2, 4, 35) have demonstrated that induction of soft agar growth on NRK cells, when tested in the presence of 10% bovine serum, requires the combined action of TGFβ as modulator protein and either EGF or TGFα as a mitogen binding to the EGF receptor. When assayed in the presence of platelet-poor, plasma-derived serum, anchorage-independent growth of this cell line shows an additional requirement for PDGF (3). In a variety of studies (7, 26 [and references therein], 33, 41), however, TGFs have been identified which are able to induce NRK cells to grow in soft agar in the absence of a ligand binding to the EGF receptor. In general, these preparations have not been tested for mitogenic activity, and therefore no information is available on the possible mitogens and modulator proteins involved. On the other hand, studies on the production of PDGF-like growth factors by transformed cells generally do not deal with induction of anchorage-independent growth. With respect to the capacity of pure human PDGF to induce phenotypic transformation, it has been established that PDGF is able to induce morphological changes in 3T3 cells, giving them the appearance of transformed fibroblasts (46). Moreover, PDGF is able to induce anchorage-independent growth of rat embryo fibroblasts (20) but not of NRK cells in serum-containing media (3, 35). Recent experiments indicated, however, that in the presence of retinoic acid, PDGF is able to induce phenotypic transformation of NRK cells (E. J. J. van Zoelen, T. M. J. van Oostwaard, and S. W. de Laat, manuscript in preparation).

In this study, we showed that the mitogenic activity of ND-TGF separated on a reverse-phase HPLC column was fully coincident with soft agar growth-inducing activity on NRK cells, both in the absence and presence of additional EGF. This suggests strongly that the mitogens in ND-TGF are directly involved in the induction of NRK soft agar growth, in agreement with considerations in reference 41. When concentrating on the activity of ND-TGF I, the possibility should be considered that, with respect to the induction of soft agar growth, PDGF-like growth factors have properties different from those of human PDGF. The c-*sis* oncogene codes for only one of the chains of PDGF (18), and therefore it is not clear whether PDGF-like growth factors are composed of two identical chains linked by disulfide bridges (homodimer) or of two different chains (heterodimer), as has been proposed for human PDGF (46). With respect to all properties tested so far, however, PDGF-like growth factors and PDGF have behaved identically. If PDGF-like growth factors could indeed induce soft agar growth by themselves, the stimulation of soft agar growth by EGF could simply result from optimalization of growth factor conditions in the medium, in line with the model of Kaplan and Ozanne (20). Alternatively, induction of soft agar growth by PDGF-like growth factors may require specific modulator proteins present in ND-TGF I, for example, a PDGF-potentiated TGF. It will require further purification of the various factors involved to answer these questions.

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ADDENDUM IN PROOF

Slamon and Cline (D. J. Slamon and M. J. Cline, Proc. Natl. Acad. Sci. USA **81**:7141–7145, 1984) recently detected a 3.9-kbp c-*sis* transcript in mouse embryos.

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