Growth Control in Cultured 3T3 Fibroblasts. V. Purification of an M_r 13,000 Polypeptide Responsible for Growth Inhibitory Activity

Yen-Ming Hsu and John L. Wang

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. Dr. Hsu's present address is Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138.

Abstract. A growth regulatory factor, which reversibly inhibits DNA synthesis and proliferation of fibroblasts, has been isolated from medium conditioned by exposure to density-inhibited mouse 3T3 cells. This factor, termed FGR-s (13K), yielded a single polypeptide (M_r 13,000) when analyzed by SDS PAGE under both reducing and nonreducing conditions. The dose-response curve of growth inhibition by FGR-s (13K) showed that 50% inhibition of 3T3 cell proliferation was achieved at a concentration of ~3 ng/ml, corresponding to ~0.23 nM. The activity of FGR-s (13K) was depleted by passing the material over an affinity column containing the monoclonal antibody 2A4; this monoclonal antibody had been previously characterized to bind to the M_r 13,000 polypeptide. These results indicate that we have purified a growth regulatory factor that acts to inhibit the proliferation of cells in an autocrine pathway.

THE mouse 3T3 fibroblast line exhibits a form of growth control in vitro in that the cells reach only a low saturation density and can remain for long periods in a viable, monolayer state (21, 22). Treatment of sparse, proliferating cultures of 3T3 cells with medium conditioned by exposure to density-inhibited 3T3 cultures resulted in an inhibition of growth and division in the target cells as compared with similar treatment with unconditioned medium (20). This inhibitory activity was fractionated, yielding one preparation that exhibited reversible inhibition of growth and direct interactions with target 3T3 cells (19, 25). This fraction, designated fibroblast growth regulator, soluble form (FGR-s),[1] contained two major polypeptides (M_r's 10,000 and 13,000) (19, 27). Using similar procedures, Wells and Mallucci have shown that secondary cultures of mouse embryo fibroblasts release into the medium a growth inhibitory activity whose polypeptide composition and physiocochemical behavior closely parallel those of FGR-s (28).

More recently, we have generated and characterized a monoclonal antibody (antibody 2A4) that neutralized the activity of FGR-s (8). This monoclonal antibody specifically bound the M_r 13,000 polypeptide, hereafter designated as FGR-s (13K). Based on these data, we concluded that FGR-s (13K) is responsible for at least part of the observed growth inhibitory activity. This assignment of biological activity to FGR-s (13K) does not exclude the possibility that other components of the FGR-s fraction (e.g. M_r 10,000 polypeptide) may also be biologicaly active. Moreover, we could not ascertain whether FGR-s (13K) was active independent of the presence of other polypeptides (8, 26). In the present communication, we report the purification to apparent homogeneity of FGR-s (13K) on a preparative scale. This has allowed us to assay the individual polypeptide for biological activity.

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FGR-s, fibroblast growth regulator, soluble form; TGF-β, transforming growth factor-β.

Materials and Methods

Cell Culture and Preparation of FGR-s

Swiss 3T3 cells (American Type Culture Collection, CCL 92) were grown at 37°C in Dulbecco's modified Eagle's medium (DME, KC Biological Inc., Lenexa, KS) containing 10% calf serum (Microbiological Associates, Walkersville, MD). The detailed protocol for the preparation of FGR-s has been described (19, 20). In brief, confluent monolayers of 3T3 cells were washed twice with DME; fresh, serum-free DME was then added to the cultures (10 ml/150 cm^2 of growth area). After an overnight incubation, the medium was collected as serum-free conditioned medium. This medium was centrifuged at 1,470 *g* for 10 min and the supernatant was subjected to ammonium sulfate precipitation (80% of saturation at room temperature). The precipitate was redissolved in 5 mM Tris, pH 8.0 (2.5 ml of buffer per liter of conditioned medium) and fractionated by Sephadex G-50 chromatography (1.4 × 110 cm) in the same buffer. The material eluting at a position corresponding to polypeptides of molecular weight 10,000 to 15,000 was pooled as the FGR-s fraction.

FGR-s labeled with [^{35}S]methionine was prepared as described, using 100 μCi of radioactive methionine per ml (1,014 Ci/mmol, Amersham Corp., Arlington Heights, IL) and DME containing unlabeled methionine at 3 μg/ml (one tenth of the concentration normally found in DME) (19).

Ion-Exchange Chromatography on DEAE-Cellulose

A column (0.8 × 2 cm) of DEAE-cellulose (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated with 5 mM Tris, pH 8.0. The pooled material corresponding to the FGR-s fraction of the Sephadex G-50 column (19) was applied to the ion-exchange column. After a wash with starting buffer, a gradient (0 to 0.5 M NaCl in 100 ml of 5 mM Tris, pH 8.0) was used to develop the column. 1.7-ml fractions were collected.

Monoclonal Antibody 2A4

The generation and characterization of hybridoma clone 2A4 have been previously described (8). Rat spleen cells were immunized in vitro (12) with the FGR-s fraction. After 96 h of culture, the rat lymphocytes were fused with the mouse myeloma cell line NS-1-Ag4/1 (NS-1 line, Salk Institute), using polyethylene glycol 1500 (5). Hybridoma cultures that produced antibodies reacting with FGR-s were detected by the binding of rat immunoglobulin in the culture supernatants to FGR-s adsorbed onto Immulon-2 plates (Dynatech Laboratories, Alexandria, VA) (8).

The supernatant from hybridoma clone 2A4 was fractionated on an affinity column of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) covalently derivatized (9) with rabbit antibodies directed against rat immunoglobulin (Sigma Chemical Co., St. Louis, MO). Material bound to the column was eluted with 0.1 M citrate buffer (pH 3.0). This material was a rat immunoglobulin designated as antibody 2A4 (8).

Depletion of FGR-s (13K) over an Antibody 2A4 Column

Rabbit antibodies directed against rat immunoglobulin (40 mg) were coupled to Affi-Gel 10 (2 ml of beads) (9). Supernatant (50 ml) from the hybridoma clone 2A4 was passed through the column (0.4 × 2.5 cm) three times. The supernatant of the parent myeloma NS-1 cell line was similarly passed through an identical column for controls. Material bound nonspecifically was removed by washing with 0.1 M phosphate buffer (pH 8.0). Finally, a preparation of FGR-s (13K) (8 ng/ml, 2 ml) was percolated through either the antibody 2A4 column or the NS-1 control column. The activities of the original FGR-s (13K) preparation, as well as the pooled flow-through fractions, representing material not bound by the respective affinity columns, were assayed for growth inhibitory activity.

Assays of DNA Synthesis

Target cells used to test the growth inhibitory activity were routinely seeded at an initial density of 5×10^3 cells/cm^2 in a 96-well culture dish (Costar, Cambridge, MA). For experiments in which the density of the target cells was varied, the cells were seeded at one half the desired density. After overnight incubation, the cells were deprived of serum for 24 h. Then the medium was removed and the test fraction was added (75 μl) along with 150 μl DME containing 5% (vol/vol) calf serum.

DNA synthesis was assayed 24 h later with a pulse of [^{3}H]thymidine (1 μCi/culture, 1.9 Ci/mmol, Schwarz-Mann, Spring Valley, NJ) for 3 h at 37°C. After the pulse, the radioactive medium was removed and the cells were washed three times with cold phosphate-buffered saline (PBS) and once with 10% trichloroacetic acid. The cells were then solubilized with 0.2 ml of 1% SDS in 0.1 N NaOH. After incubation at 37°C for 10 min, cell lysates were added to 2 ml of scintillation cocktail (1 g dimethyl-1,4-bis[2-5-phenyloxazoly]benzene; 8 g 2.5-diphenyl-oxazole; 1.33 ml Triton X-100; and 2.666 ml of toluene) for scintillation counting.

In reversibility studies, cultures were treated with fractions derived from FGR-s. After 22 h, the medium was removed and replaced with an equal volume of fresh DME containing 5% (vol/vol) calf serum. DNA synthesis in these cultures was measured 16 and 42 h later.

Assays of Cell Number and Cell Viability

Cultures were treated with fractions derived from FGR-s or a control fraction. At various times thereafter, the cells were washed three times with PBS and were removed from the growth surface by trypsin treatment. The cells were then centrifuged, resuspended in DME, diluted with trypan blue, and counted in a corpuscle counting chamber (Hausser Scientific, Blue Bell, PA) (20).

The viability of cells treated with FGR-s-derived fractions was determined while the cells remained attached to the growth surface. After removal of growth medium, the cells were incubated with trypan blue (0.08% wt/vol in PBS) for 10 min at room temperature. The staining solution was then removed and the viable cells were counted using an Olympus inverted microscope.

PAGE

SDS PAGE was carried out according to the procedure of Laemmli (11), using 10 or 16% acrylamide running gels and 5% acrylamide stacking gels. For gels electrophoresed under reducing conditions, 2-mercaptoethanol (4%, vol/vol) was included in the sample buffer. The gels were fixed and stained with silver reagent (15). With radioactive samples, the gel was subjected to fluorography as described by Bonner and Laskey (4) using Kodak X-Omat R (XAR-5) film.

Results

Ion-Exchange Chromatography of FGR-s

In previous experiments, we demonstrated that the growth inhibitory in conditioned medium of 3T3 cells can be fractionated by ammonium sulfate precipitation and gel filtration on Sephadex G-50 columns (19). The material eluting from the Sephadex G-50 column at a position corresponding to a molecular weight range of 10,000–15,000 showed enrichment in specific biological activity (growth inhibition assay) and was designated FGR-s. Isoelectric focusing and PAGE analysis showed that the principal components of the FGR-s fraction were polypeptides of M_r 10,000 (pI ~7.5) and 13,000 (pI ~10) (27).

Because the ampholines used to establish the pH gradient were toxic to the target 3T3 cells, we could not demonstrate growth inhibitory activity in the individual polypeptides. Isoelectric focusing did not appear, therefore, to be a promising preparative procedure for the purification of the inhibitory polypeptide. Nonetheless, the high isoelectric point of FGR-s (13K), which was implicated by monoclonal antibody neutralization experiments to be at least partially responsible for growth inhibitory activity (8), suggested ion-exchange chromatography as a preparative procedure.

To carry out ion-exchange chromatography, two minor modifications of the original procedure (19) for the preparation of FGR-s were made. First, gel filtration on Sephadex G-50 columns was performed in 5 mM Tris, pH 8.0, instead of DME. This allowed the direct application of the effluent from the Sephadex column onto the ion-exchange column. Second, a wider range of fractions, centered approximately at FGR-s, was pooled and subjected to ion-exchange chromatography. The rationale for this was that we did not assay the individual fractions from the Sephadex G-50 column for activity or for polypeptide content (SDS gels). Therefore, the precise position corresponding to the FGR-s fractions was not determined and was compensated for by including material in fractions adjacent to FGR-s. This allowed us to save material, which would have been used in the assays, and to save time, thereby minimizing losses of material due to adsorption to test tubes.

DEAE-cellulose chromatography of a [^{35}S]methionine-labeled preparation of FGR-s resulted in the separation of several components (components *A–F*, Fig. 1 *b*). When the fractions eluting from the ion-exchange column were assayed for growth inhibitory activity, only component A (Fig. 1 *b*) exhibited activity; the remainder of the components failed to show any appreciable activity (Fig. 1 *a*). The sum of the growth inhibitory activity in component A (Fig. 1 *b*) accounted for ~80% of the total activity applied to the column. There was a sixfold enrichment in terms of specific activity in this fractionation step.

PAGE and fluorography were carried out on the fractions derived from the DEAE-cellulose column (Fig. 2). Component A (Fig. 1 *b*) yielded a single polypeptide, migrating at a position corresponding to a molecular weight of 13,000 (Fig. 2, lane 2). Identical results were obtained irrespective of whether the PAGE was carried out under reducing (with β-mercaptoethanol) or nonreducing conditions (Fig. 3 *A*). In addition to fluorography, we have also subjected the polyacrylamide gel to staining with the silver technique. Again, component A yielded predominantly a single polypeptide of M_r 13,000 (Fig. 3 *B*, lane *2*). Using known amounts of cyto-

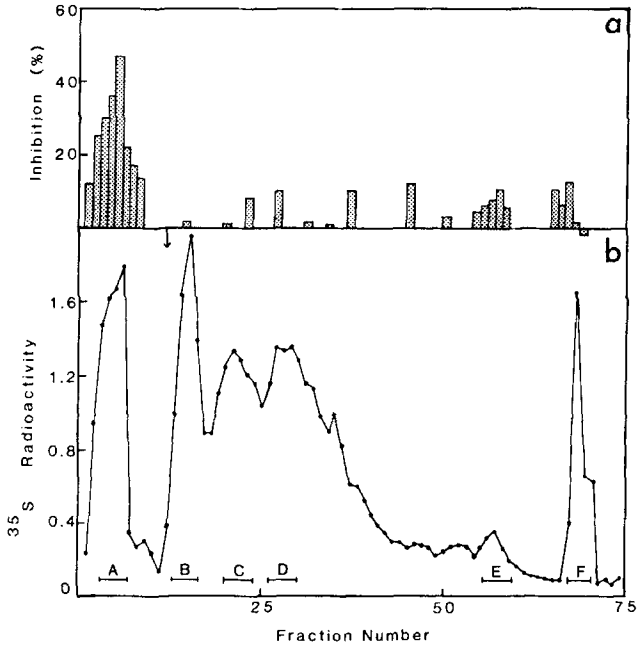

Figure 1. Ion-exchange chromatography of a [^{35}S]methionine-labeled FGR-s preparation (1.5×10^6 cpm) on a column (0.8×2 cm) of DEAE-cellulose equilibrated with 5 mM Tris, pH 8.0. At the point indicated by the arrow, a linear gradient (0–0.5 M NaCl, 100 ml total volume) was used to elute the material bound in the column. 17-ml fractions were collected. (*a*) Profile of the growth inhibitory activity assayed by the inhibition of [^{3}H]thymidine incorporation into target cells. The data on the ordinate axis are expressed as the percentage of inhibition relative to control cultures and represent the averages of triplicate determinations. (*b*) Profile of the protein content assayed by counting the radioactivity due to [^{35}S]methionine. The horizontal bars marked A–F denote the fractions that were pooled for further analysis.

chrome *c* (M_r 12,500) as a standard for the silver staining technique, we estimated that 1 liter of conditioned medium derived from 3T3 cultures yielded ~1 μg of the 13-kD polypeptide. The material in component A (Fig. 1 *b*) is designated FGR-s (13K).

Depletion of Growth Inhibitory Activity from FGR-s (13K) on an Affinity Column Containing Antibody 2A4

To show that the M_r 13,000 polypeptide was directly responsible for the biological activity, we attempted to deplete, using an affinity column containing antibody 2A4, the growth inhibitory activity of preparation of FGR-s (13K). The affinity column was prepared by first coupling the immunoglobulin fraction of rabbit antibodies directed against rat immunoglobulin to Affi-Gel 10. This derivatized gel was used to prepare two different columns in the following ways: (*a*) the supernatant of hybridoma clone 2A4 was passed over one column to bind antibody 2A4 (antibody 2A4 column); and (*b*) the supernatant of parent myeloma NS-1 cell line was passed over the other column (NS-1 control column). Finally, a preparation of FGR-s (13K) was percolated through either the antibody 2A4 column or the NS-1 control column. The activities of the original FGR-s (13K) preparation as well as the flow-through fractions (representing unbound material) of the two respective affinity columns were compared in growth inhibition assays.

The results showed that the growth inhibitory activity of FGR-s (13K) was depleted in the material passed through the antibody 2A4 column (Fig. 4). In contrast, the NS-1 control column had little effect on the growth inhibitory activity. These results, in conjunction with the previous demonstration that antibody 2A4 affinity columns deplete FGR-s of the 13,000-kD polypeptide, strongly suggest that FGR-s (13K) is

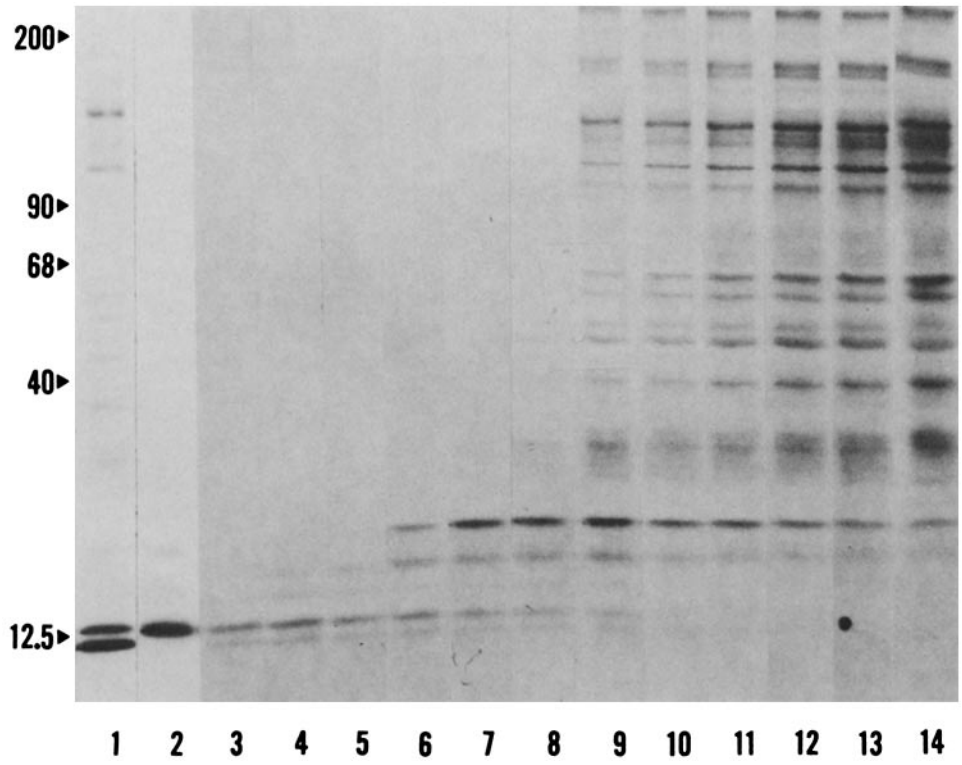

Figure 2. SDS PAGE of a [^{35}S]-methionine-labeled preparation of FGR-s and components derived from fractionation of FGR-s by ion-exchange chromatography (Fig. 1). The acrylamide concentration of the running gel was 16%. The samples applied to the gel contained β-mercaptoethanol (4% vol/vol). Approximately 5,000 cpm were applied to each lane, and the radioactive polypeptides were revealed by fluorography (2 d exposure). Lanes *1* and *2* were developed on a different fluorogram than the remainder of the lanes. The arrows indicate positions of migration of molecular weight (in thousands) markers. Lanes: *1*, [^{35}S]methionine-labeled FGR-s; *2*, fraction 6; *3*, fraction 9; *4*, fraction 15; *5*, fraction 21; *6*, fraction 27; *7*, fraction 29; *8*, fraction 33; *9*, fraction 35; *10*, fraction 38; *11*, fraction 46; *12*, fraction 52; *13*, fraction 57; *14*, fraction 68. All fractions refer to those fractions in Fig. 1 *b*.

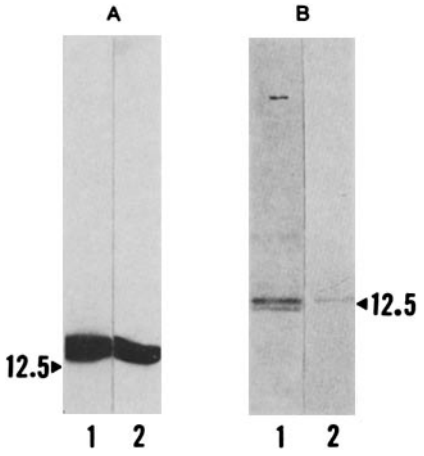

Figure 3. (*A*) SDS PAGE of a [^{35}S]methionine-labeled preparation of FGR-s (13K) under nonreducing (lane *1*) and reducing (4% vol/vol, 2-mercaptoethanol, lane *2*) conditions. The acrylamide concentration of the running gel was 16%. Approximately 2,000 cpm were applied to each lane, and the radioactive polypeptides were revealed by fluorography (14 d). (*B*) Analysis of the polypeptide conpositions of FGR-s (lane *1*) and FGR-s (13K) (lane *2*) by the silver staining technique after SDS gel electrophoresis. The acrylamide concentration of the running gel was 16%. The samples applied to the gel contained 2-mercaptoethanol (4% vol/vol).

directly responsible for the observed growth inhibitory activity. The data also argue against any indirect mechanisms, such as antibody 2A4 stimulating a growth factor receptor, in the observed neutralization of FGR-s effects in the growth inhibition assays (8).

Therefore, FGR-s (13K) contains a single polypeptide (M_r 13,000) and exhibits growth inhibitory activity. We can now conclude that this M_r 13,000 polypeptide is active in growth inhibition assays independent of the presence of any other polypeptide. Moreover, the other polypeptides do not appear to contain growth inhibitory activity inasmuch as the remainder of the fractions from the DEAE-cellulose column (components *B–F*, Fig. 1 *b*) failed to show activity.

The Effects of FGR-s (13K) on Target Cells: Dose-Response, Viability, and Reversibility

The inhibitory effect of FGR-s (13K) on [^{3}H]thymidine incorporation into target cells was dependent on the concentration of ligand added (Fig. 5). Over the concentration range of 0.1–10 ng/ml, there was a monotonic increase in inhibitory activity with increasing concentration of FGR-s (13K) added to the cultures. The concentration of inhibitor required for 50% inhibition was ~3 ng/ml (Fig. 5, inset).

Three series of experiments were performed to ascertain that the inhibition of [^{3}H]thymidine incorporation in target cells by FGR-s (13K) was due to a true suppression of cell growth rather than to any cytotoxic effects of the inhibitory fractions. First, the viabilities of the cells, assayed by trypan blue exclusion tests, were identical for target cultures treated with FGR-s (13K) and with a control fraction from the DEAE-cellulose column. Second, to demonstrate that the inhibitory effect of FGR-s on DNA synthesis was reversible, parallel cultures were treated with FGR-s (13K) for 22 h. The medium was then removed and replaced with an equal volume of fresh growth medium. At various times thereafter, DNA synthesis was assayed by the incorporation of [^{3}H]thymidine. The data showed that the inhibition was reversible within 20 h after the removal of FGR-s (13K) (Fig. 6). Taken together with the demonstration that the viability of target cells was not affected by FGR-s, these results suggest that the inhibitory activity of FGR-s cannot be ascribed to cytotoxicity.

Finally, the inhibitory effect of FGR-s (13K) on cell proliferation is also reflected in assays of cell number after treatment with the inhibitory fraction. Target cultures treated with a control fraction continued to proliferate; in contrast, cultures treated with FGR-s (13K) failed to increase in cell number at the same rate (Fig. 7). These data provide a confirmation of the growth inhibitory activity of FGR-s (13K) using an assay that is independent of [^{3}H]thymidine incorporation.

The Effects of FGR-s (13K) on Target Cells: Density and Serum Dependence

In this series of experiments, the effect of varying conditions of the assay during the period of exposure of target cells to FGR-s (13K) was tested using a single preparation of the inhibitor. The effect of variation in the density of the target cells on the inhibition by FGR-s (13K) is shown in Fig. 8. At target cell densities of $\geq 5 \times 10^3$ cells/cm^2, the inhibitory activity of FGR-s (13K) was consistently observed. In contrast, when the density of the target cells was $<2.5 \times 10^3$ cells/cm^2, there was a dramatic decrease in the potency of FGR-s

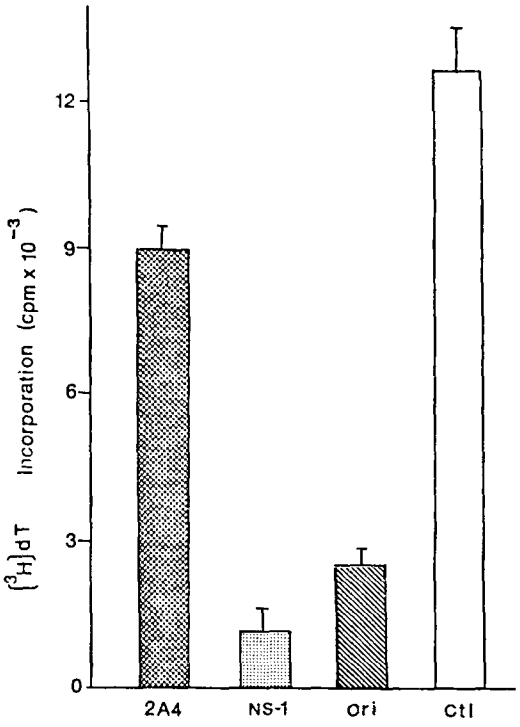

Figure 4. Depletion of the growth inhibitory activity of FGR-s (13K) by an antibody 2A4 column. Affinity columns (0.4 × 2.5 cm) were prepared by coupling rabbit anti-rat immunoglobulin to Affi-Gel 10. The supernatant (50 ml) of hybridoma clone 2A4 or parent myeloma line NS-1 was passed over the affinity column. A preparation of FGR-s (13K) (8 ng/ml, 2 ml) was percolated through these affinity columns. The growth inhibitory activities of the flow-through fractions were compared with those of the original FGR-s (13K) preparation (Ori) and control cultures treated with DME (Ctl). The data are expressed in terms of [^{3}H]thymidine ([^{3}H] *dT*) incorporation into target cells and represent the averages of triplicate determinations (± standard error of the mean).

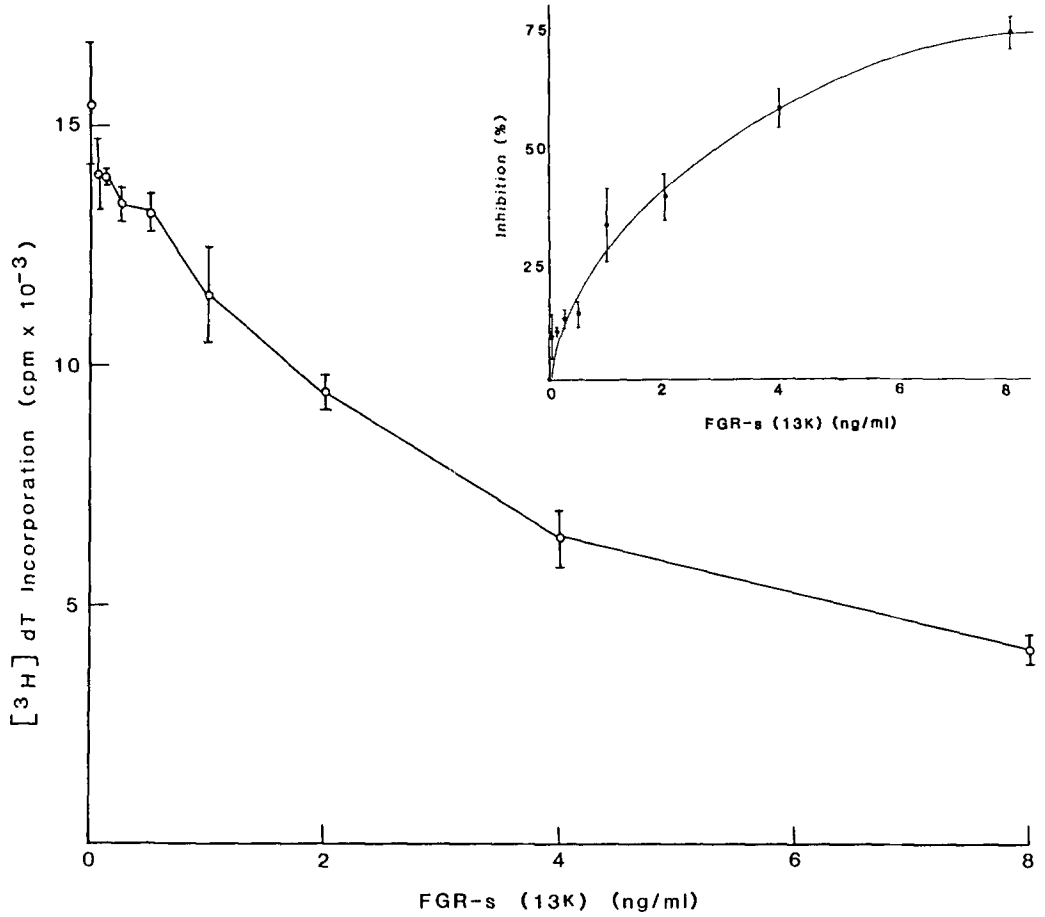

Figure 5. Dose-response curve of the growth inhibitory activity of FGR-s (13K) on target 3T3 cells (2×10^4 cells/cm^2). The protein concentration of FGR-s (13K) was determined by the silver staining technique on SDS gels. The target cells were treated with FGR-s (13K) for 20 h and then assayed for the incorporation of [^{3}H]thymidine ([^{3}H] *dT*) as described in Materials and Methods. The data represent the averages of triplicate determinations (± standard error of the mean). The inset shows the same data plotted with percent inhibition on the ordinate.

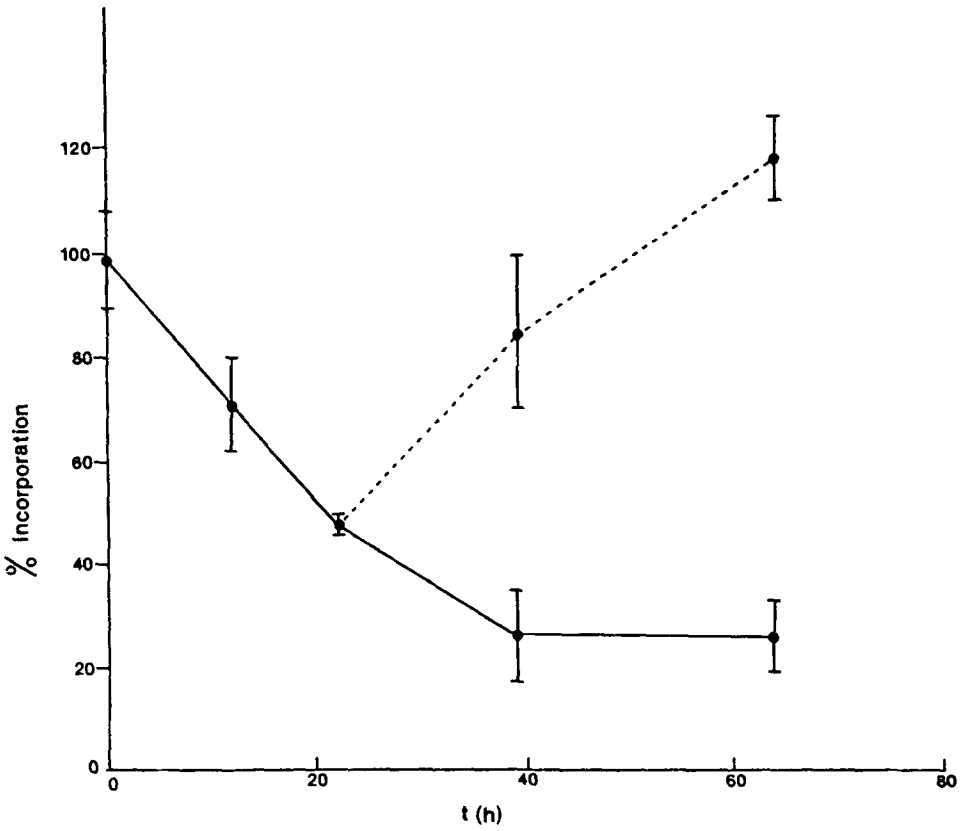

Figure 6. The reversibility of the effect of FGR-s (13K) on 3T3 cells. Target cells (5×10^3 cells/cm^2) were treated with FGR-s (13K) (3 ng/ml). The kinetics of DNA synthesis, assayed by thymidine incorporation and expressed as a percent of control cultures is shown by the solid line. The dotted line indicates the level of DNA synthesis in cultures after the removal of FGR-s (13K), relative to control cultures, which also received a medium change.

(13K) to inhibit 3T3 cells growth. This is consistent with our previous observation that a minimum target cell density may be required before the inhibitory effect of conditioned medium on cell proliferation can be observed (20).

The growth inhibitory activity of FGR-s (13K) was dependent on the serum concentration used in the assay (Fig. 9). In the present experiment, FGR-s (13K) (8 ng/ml) yielded ~20% inhibition when the serum concentration was ≥5%. With a decreasing serum concentration, the same concentration of FGR-s (13K) exhibited higher levels of growth inhibition (Fig. 9). In previous experiments, we showed that the binding of [^{35}S]methionine-labeled FGR-s was inhibited by serum (25). Therefore, the present result, showing higher levels of activity of FGR-s (13K) as a function of decreasing concentration of serum, would be expected on the basis of higher levels of binding of the inhibitor.

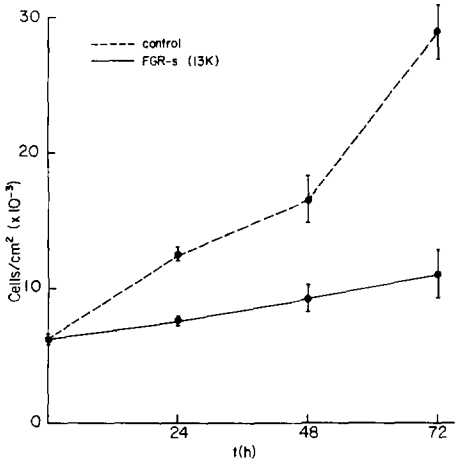

Figure 7. The kinetics of the increase in cell density of 3T3 cultures in the presence of FGR-s (13K) and control. At various times, parallel cultures were trypsinized and the cells were collected for the determination of the total cell number in the cultures. Data points are the averages of quadruplicate determinations (± standard error of the mean).

Discussion

The key conclusion derived from the present study is that FGR-s (13K) exhibits growth inhibitory activity. In previous studies (8, 26), we had generated a monoclonal antibody (2A4) that specifically bound FGR-s (13K). This monoclonal antibody also neutralized the growth inhibitory activity of the partially purified FGR-s fraction. We inferred, therefore, that FGR-s (13K) must be at least partly responsible for the biological activity. The present results on the purification of FGR-s (13K) and the demonstration of its biological activity indicate that this polypeptide can exhibit growth inhibitory activity independent of the presence of any other polypeptide.

The main features of the inhibition of 3T3 cell proliferation by FGR-s (13K) include: (*a*) The dose response curve indicates that 50% inhibition is obtained at a concentration of ~3 ng/ml, corresponding to 0.23 nM. (*b*) The effect of FGR-s (13K) on 3T3 cells is reversible and cannot be ascribed to cytotoxicity. (*c*) The effect of FGR-s (13K) on DNA synthesis was most prominent at high (>5 × 10^3 cells/cm^2) target cell density, consistent with previous observations, made with conditioned medium (20), that a minimum target cell density

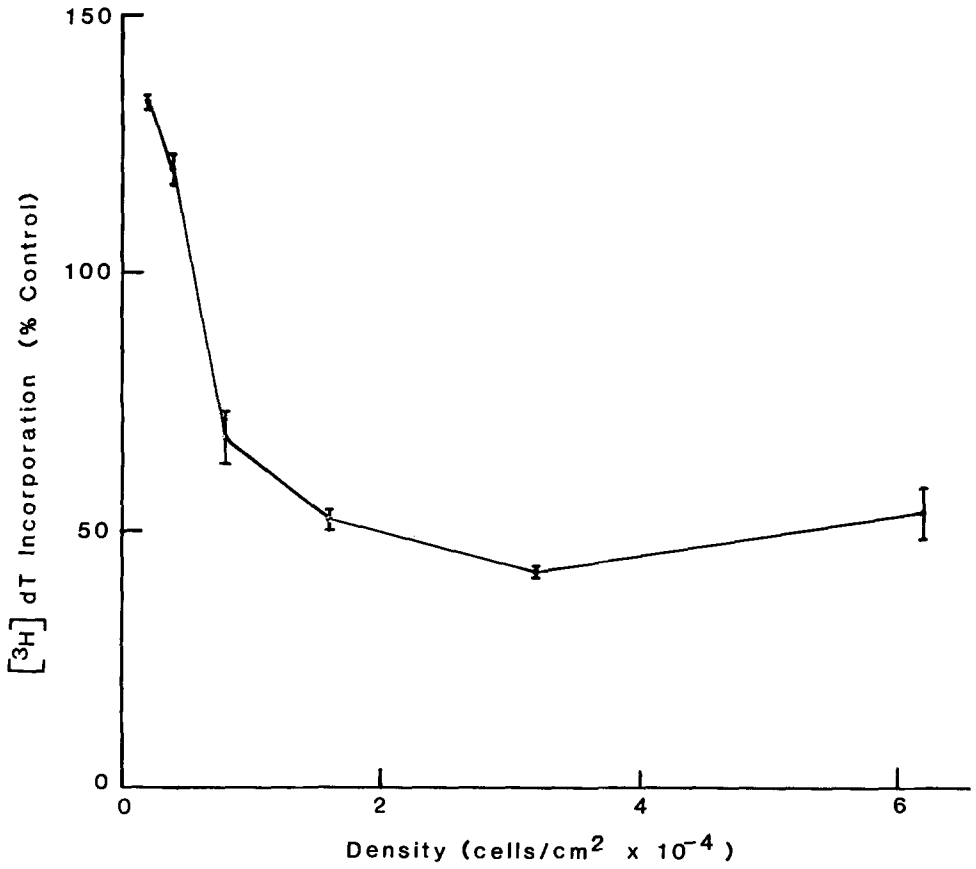

Figure 8. The effect of varying the density of the target cells on the inhibitory activity of FGR-s (13K). Treatment of target cells seeded at different densities with FGR-s (13K) for 24 h was followed by a 3-h pulse of [^{3}H]thymidine (*[^{3}H] dT*). Data points represent the averages of triplicate determinations (± standard error of the mean).

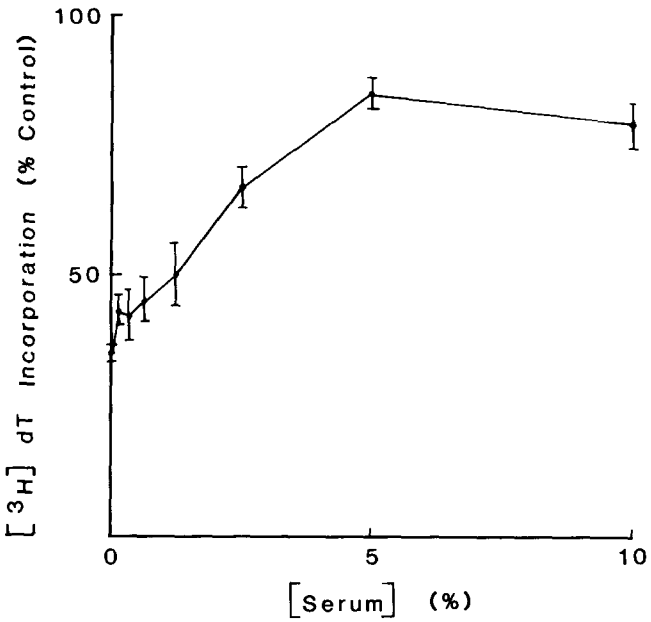

Figure 9. The effect of serum concentration on the growth inhibitory activity of FGR-s (13K) assayed on target 3T3 cells (2×10^4 cells/cm^2). The target cells were treated with FGR-s (13K) for 24 h in the presence of different concentrations of calf serum. The cells were pulsed with [^{3}H]thymidine ([^{3}H] *dT*) for 3 h. Data points represent the averages of triplicate determinations (± standard error of the mean).

may be required to observe the inhibitory effect. (*d*) The inhibition by FGR-s (13K) on 3T3 cells was most potent at low (<5%) serum concentrations, in agreement with previous results that the binding of radioactive FGR-s was decreased by increasing concentrations of serum (25).

Analysis of the molecular properties of FGR-s (13K) indicates that it is active as a single polypeptide (M_r 13,000). This polypeptide remains as a monomer in SDS gel electrophoresis under both reducing and nonreducing conditions. Moreover, FGR-s (13K) migrates on gel filtration columns at a position corresponding to a molecular weight of 13,000. This information is useful in a comparison of the properties of FGR-s (13K) with corresponding properties of other negative regulators of cell growth.

Tucker et al. (23) have recently shown that transforming growth factor-β (TGF-β) and a growth inhibitor isolated from conditioned medium of African green monkey kidney epithelial cells (BSC-1) shared many properties: (*a*) both have M_r's of 25,000 under nonreducing, nondenaturing conditions and M_r's of 12,500 under reducing SDS gels (1, 7, 24); (*b*) both TGF-β and the BSC-1 growth inhibitor stimulate colony growth of AKR-2B cells in soft agar; (*c*) both molecules inhibit the monolayer growth of a variety of cell types including BSC-1, AKR-2B, and mink lung (CLL64) cells; and (*d*) both appear to compete for the same receptors on the cell surface of responsive cells. Therefore, it appears that TGF-β and the BSC-1 growth inhibitor may be similar if not identical molecules (23). Corroborating these results, Roberts et al. (18) have reported that TGF-β is a bifunctional molecule, acting either as a growth stimulatory factor or as a growth inhibitory molecule. The conditions that determine which of these two activities is expressed is not solely dependent on the cell type or the condition of anchorage-dependent versus anchorage-independent growth but is also modulated by the action of other growth factors (and their receptors) that may be present (18). TGF-β has been purified to homogeneity, and, therefore, it stands as a paradigm for negative regulators of cell growth and autocrine regulation.

Our present results on the purification of FGR-s (13K) suggests that it may join TGF-β as another example of negative regulator that functions in an autocrine pathway. In comparing the properties of FGR-s (13K) and TGF-β it should be noted that the latter is active as a dimer (M_r 25,000) under nonreducing conditions. Upon reduction, the polypeptide molecular weight of TGF-β becomes 12,500, and the biological activity (stimulation of anchorage independent growth in soft agar cultures) is lost (1, 24). In contrast, FGR-s (13K) is active as a single polypeptide (M_r 13,000), as noted earlier.

Using procedures similar to our previous studies (19, 20), Wells and Malucci (28) have shown that secondary cultures of mouse embryo fibroblasts release into the medium a growth inhibitory activity whose physicochemical behavior and polypeptide composition closely parallel those of FGR-s. The molecular weights of the polypeptides in their active fractions were 11,000 and 14,000. In this connection, it should be noted that both soluble and plasma membrane-associated growth inhibitory fractions, derived from 3T3 cells and with properties similar to FGR-s have been reported by several laboratories (6, 16, 17, 29). The molecular properties of these active fractions and their relationship to FGR-s (13K) remain to be elucidated.

Three additional growth inhibitory activities have been characterized. (*a*) A hepatocyte proliferation inhibitor has been purified from rat liver; this inhibitor yielded a polypeptide (M_r 26,000) in SDS gels under reducing conditions and an isoelectric point of 4.56 (13, 14). It reversibly inhibited the proliferation of nonmalignant rat liver cells in culture but exerted no effect on the proliferation of hepatoma cells. (*b*) A glycopeptide fraction that inhibits protein synthesis and cell growth of normal but not transformed cells has been partially purified from bovine cerebral cortex cells (10). This preparation contained polypeptides of molecular weights 18,000 and 16,000. (*c*) Finally, a growth inhibitor (M_r 13,000) for Ehrlich ascites mammary carcinoma cells has been purified from the bovine mammary gland (2). Polyclonal antiserum raised against the inhibitor immunoprecipitated the M_r 13,000 polypeptide and neutralized its growth inhibitory activity as well (3). It is quite striking that the constituent polypeptide chains of several growth inhibitors have molecular weights of ~13,000 and 26,000. Perhaps a family of growth inhibitors may be defined when structural information becomes available for this list of negative regulators of cell growth.

We thank Mrs. Teresa Vollmer for her help in preparing the manuscript.

This work was supported by grants GM 27203 and GM 32310 from the National Institutes of Health. J. L. Wang was supported by Faculty Research Award FRA-221 from the American Cancer Society.

Received for publication 29 July 1985, and in revised form 7 October 1985.

References

1. Anzano, M. A., A. B. Roberts, J. M. Smith, M. B. Sporn, and J. E. DeLarco. 1983. Sarcoma growth factor from conditioned medium of virally

transformed cells is composed of both type α and type β transforming growth factors. *Proc. Natl. Acad. Sci. USA.* 80:6264–6268.

2. Böhmer, F. D., W. Lehmann, H. E. Schmidt, P. Langen, and R. Grosse. 1984. Purification of a growth inhibitor for Ehrlich ascites mammary carcinoma cells from bovine mammary gland. *Exp. Cell Res.* 150:466–476.

3. Böhmer, F. D., W. Lehmann, F. Noll, R. Samtleben, P. Langen, and R. Grosse. 1985. Specific neutralizing antiserum against a polypeptide growth inhibitor for mammary cells purified from bovine mammary gland. *Biochim. Biophys. Acta.* 846:145–154.

4. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.

5. Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73:1–46.

6. Harel, L., G. Chatelain, and A. Golde. 1984. Density-dependent inhibition of growth: inhibitory diffusible factors from 3T3- and Rous Sarcoma Virus (RSV)-transformed 3T3 cells. *J. Cell Physiol.* 119:101–106.

7. Holley, R. W., R. Armour, J. H. Baldwin, and S. Greenfield. 1983. Activity of a kidney epithelial cell growth inhibitor on lung and mammary cells. *Cell Biol. Int. Rep.* 7:141–147.

8. Hsu, Y.-M., J. M. Barry, and J. L. Wang. 1984. Growth control in cultured 3T3 fibroblasts: neutralization and identification of a growth-inhibitory factor by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 81:2107–2111.

9. Ikeda, Y., and M. Steiner. 1976. Isolation of platelet microtubule protein by an immunosorptive method. *J. Biol. Chem.* 251:6135–6141.

10. Kinders, R. J., and T. C. Johnson. 1982. Isolation of cell surface glycopeptides from bovine cerebral cortex that inhibit cell growth and protein synthesis in normal but not in transformed cells. *Biochem. J.* 206:527–534.

11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

12. Luben, R. A., and M. A. Mohler. 1980. *In vitro* immunization as an adjunct to the production of hybridomas producing antibodies against the lymphokine osteoclast activating factor. *Mol. Immunol.* 17:635–639.

13. McMahon, J. B., and P. T. Iype. 1980. Specific inhibition of proliferation of nonmalignant rat hepatic cells by a factor from rat liver. *Cancer Res.* 40:1249–1254.

14. McMahon, J. B., J. G. Farrelly, and P. T. Iype. 1982. Purification and properties of a rat liver protein that specifically inhibits the proliferation of nonmalignant epithelial cells from rat liver. *Proc. Natl. Acad. Sci. USA.* 79:456–460.

15. Merril, C. R., M. L. Dunau, and D. Goldman. 1981. A rapid sensitive silver stain for polypeptides in polyacrylamide gels. *Anal. Biochem.* 110:201–207.

16. Natraj, C. V., and P. Datta. 1978. Control of DNA synthesis in growing Balb/c 3T3 mouse cells by a fibroblast growth regulatory factor. *Proc. Natl. Acad. Sci. USA.* 75:6115–6119.

17. Peterson, S. W., V. Lerch, M. E. Moynaham, M. P. Carson, and R. Vale. 1982. Partial characterization of a growth-inhibitory protein in 3T3 cell plasma membranes. *Exp. Cell Res.* 142:447–451.

18. Roberts, A. B., M. A. Anzano, L. M. Wakefield, N. S. Roche, D. F. Stern, and M. B. Sporn. 1985. Type β transforming growth factor: a bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA.* 82:119–123.

19. Steck, P. A., J. Blenis, P. G. Voss, and J. L. Wang. 1982. Growth control in cultured 3T3 fibroblasts. II. Molecular properties of a fraction enriched in growth inhibitory activity. *J. Cell Biol.* 92:523–530.

20. Steck, P. A., P. G. Voss, and J. L. Wang. 1979. Growth control in cultured 3T3 fibroblasts. Assays of cell proliferation and demonstration of a growth inhibitory activity. *J. Cell Biol.* 83:562–575.

21. Stoker, M. G. P., and H. Rubin. 1967. Density-dependent inhibition of cell growth in culture. *Nature (Lond.).* 215:171–172.

22. Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17:299–313.

23. Tucker, R. F., G. D. Shipley, H. L. Moses, and R. W. Holley. 1984. Growth inhibitor from BSC-1 cells closely related to platelet type β transforming growth factor. *Science (Wash. DC).* 226:705–707.

24. Tucker, R. F., M. E. Volkenant, E. L. Branum, and H. L. Moses. 1983. Comparison of intra- and extracellular transforming growth factors from non-transformed and chemically transformed mouse embryo cells. *Cancer Res.* 43:1581–1586.

25. Voss, P. G., P. A. Steck, J. C. Calamia, and J. L. Wang. 1982. Growth control in cultured 3T3 fibroblasts. III. Binding interactions of a growth inhibitory activity with target cells. *Exp. Cell Res.* 138:397–407.

26. Wang, J. L., and Y.-M. Hsu. 1984. Isolation and characterization of a growth regulatory factor from 3T3 cells. *In* Growth, Cancer, and the Cell Cycle. P. Skehan and S. J. Friedman, editors. Humana Press, Clifton, NJ. 177–186.

27. Wang, J. L., P. A. Steck, and J. W. Kurtz. 1982. Growth control in cultured 3T3 fibroblasts: molecular properties of a growth regulatory factor isolated from conditioned medium. *Cold Spring Harbor Conf. Cell Proliferation.* 9:305–317.

28. Wells, V., and L. Mallucci. 1983. Properties of a cell growth inhibitor produced by mouse embryo fibroblasts. *J. Cell Physiol.* 117:148–154.

29. Whittenberger, B., D. Raben, M. A. Lieberman, and L. Glaser. 1978. Inhibition of growth of 3T3 cells by extract of surface membranes. *Proc. Natl. Acad. Sci. USA.* 75:5457–5461.