Purification and Characterization of a Human Pituitary Growth Factor†

John M. Rowe,* Stephen F. Henry, and Henry G. Friesen

Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

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ABSTRACT: A growth factor has been purified to homogeneity from human pituitary glands. The pituitary growth factor (PGF) is trypsin-sensitive and acid- and heat-labile and has a molecular weight of 18 000 and an isoelectric point of 7.5. PGF was purified by heparin and copper affinity chromatography followed by carboxymethylcellulose 52. The amino-terminal amino acid sequence of PGF was established as PALPEXGGXGA and is identical with that of basic fibroblast growth factor at the identified amino acid residues. PGF was mitogenic for rabbit fetal chondrocytes and bovine corneal endothelial cells in the range of 0.015–15 ng mL^{-1}. Heparin alone at low concentrations (0.5 μg mL^{-1}) was found to be weakly mitogenic for rabbit fetal chondrocytes. In combination with PGF a marked increase in cell growth was observed, which was inhibited by protamine sulfate. These data demonstrate the presence of a potent mitogen in human pituitaries that is structurally related to basic fibroblast growth factor and synergizes with heparin to promote cell growth.

In addition to the well-recognized trophic hormones, the pituitary gland has been recognized as a source of novel growth factors such as fibroblast growth factor (FGF)[1] (Gospodarowicz, 1974), chondrocyte growth factor (CGF) (Kasper et al., 1982), and adipocyte growth factor (AGF) (Lau et al., 1983). Heparin affinity chromatography has been shown to greatly facilitate the purification of endothelial cell growth factors from a variety of sources. On the basis of a variety of biochemical criteria these growth factors fall into one of two categories of heparin-binding growth factors (HBGF's) (Lobb et al., 1986). Class I HBGF's are anionic peptides which elute from heparin–Sepharose at about 1 M NaCl and include mitogens isolated from neural tissue such as acidic FGF (Lobb & Fett, 1984; Thomas et al., 1984; Böhlen et al., 1985; Gimenez-Gallego et al., 1985) and endothelial cell growth factor (Maciag et al., 1984; Klagsbrun & Shing, 1985) as well as retina-derived growth factor (D'Amore et al., 1981; D'Amore & Klagsbrun, 1984). Class II HBGF's are cationic peptides which elute from heparin–Sepharose at about 1.5 M NaCl and include basic FGF isolated from the pituitary and brain (Gospodarowicz et al., 1984; Lobb & Fett, 1984), macrophage (Baird et al., 1985a), corpus luteum (Gospodarowicz et al., 1985), kidney (Baird et al., 1985b), and placenta (Gospodarowicz e t al., 1985) as well as a rat chondrosarcoma derived endothelial cell growth factor (Shing et al., 1984) and cartilage-derived growth factor (Davidson et al., 1985). Additional HBGF's have been reported and include human prostate growth factor (Nishi et al., 1985), astroglial growth factors (Pettmann et al., 1985), and human mammary tumor growth factor (Rowe et al., 1986). Comparisons of the complete amino acid sequence of acidic FGF/class I HBGF with basic FGF/class II HBGF shows approximately 50% homology (Esch et al., 1985; Gimenez-Gallego et al., 1985; Strydom et al., 1986). In this report we describe the isolation of a basic growth factor from the human pituitary gland that is identical with basic FGF at its amino terminal (Esch et al., 1985).

Experimental Procedures

Materials. Human pituitary glands were collected at autopsy and kept at −70 °C until extraction. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, penicillin, streptomycin, and trypsin were obtained from Grand Island Biological Co. All culture plastic ware was obtained from either Falcon or Corning. Sephadex G-100, heparin-Sepharose, chelating Sepharose, and electrophoresis calibrating kits were obtained from Pharmacia Fine Chemicals. Whatman carboxymethylcellulose CM52 was obtained from Mandel Scientific Co. Ltd.

BSA, myoglobin, and cytochrome *c* were obtained from Sigma Chemical Co. Spectrapor dialysis tubing (M_r cutoff 6000–8000) was obtained from Spectrum Medical Industries, and dithiothreitol was obtained from Aldrich Chemical Co. SDS, Tris, ammonium persulfate, acrylamide, and bis(acrylamide) were obtained from Bio-Rad Laboratories. All other standard laboratory reagents were obtained from Fisher.

Bioassay of Pituitary Growth Factor. Mitogenic activity of column fractions was determined by using rabbit fetal chondrocytes (RFC) obtained as described (Kellett et al., 1981). Briefly, RFC (1×10^4 cells/dish) were plated in 35-mm dishes in F10 medium supplemented with 10% FBS (growth medium), day 0. Samples to be assayed were diluted in F10 growth medium, filter-sterilized, and added to duplicate cultures on days 1 and 3. Cell number was determined by counting in a Coulter counter on day 5.

Other Cell Types. Human mammary tumor cells (T-47D, BT20, and BT474) were obtained from Mason Research Institute, Rockville, MD. Stock cultures were maintained in T-75 flasks containing Dulbecco's modified Eagle's medium (DMEM) containing 100 units of penicillin and 100 μg of streptomycin/mL and supplemented with 10% FBS, 140 ng/mL glucose, and 1 μg/mL porcine insulin (DMEM growth

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* Address correspondence to this author at Oncogen, Seattle, WA 98121.

[1] Abbreviations: hPGF, human pituitary growth factor; CM52, carboxymethylcellulose 52; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; RFC, rabbit fetal chondrocytes; BCE, bovine corneal endothelial cells; Tris, tris(hydroxymethyl)aminomethane.

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medium). The growth medium was changed twice weekly, and the cells were passaged at confluency.

Bovine corneal endothelial cells (BCE) were prepared as described (Gospodarowicz, 1977) and maintained in medium 199 supplemented with antibiotics and 20% FBS (M-199 growth medium).

All cells were plated in 35-mm dishes in their respective growth medium at 1×10^4 cells per dish, day 0. On day 1, the human mammary tumor cells were washed with serum-free medium and incubated in serum-free medium. Samples to be assayed were diluted in F10 growth medium, sterilized, and added to cultures on alternate days. Cells were counted in a Coulter counter five or seven days later.

Purification of Pituitary-Derived Growth Factors. Heparin–Sepharose A. All procedures were carried out at 4 °C unless otherwise stated. Human pituitaries (1200) were homogenized in 2.5 L of 0.15 M NH_4SO_4 at pH 5.5 and allowed to stir for 2 h. The homogenate was centrifuged (40 min at 30000*g*) and the supernatant combined with 75 mL of heparin–Sepharose and stirred for 1 h. After the resin settled, the supernatant was decanted and the resin poured into a column (2.5 × 16 cm) and equilibrated with 300 mL (flow rate 20 mL/h) of 10 mM phosphate buffer, pH 7.4, containing NaCl (0.5 M) and ethylene glycol (20% v/v) (equilibration buffer D). The column was then developed with a gradient of 0.5–3.0 M NaCl in equilibration buffer D (total volume 800 mL). Fractions (9 mL) were collected into siliconized glass tubes, and those which contained biological activity (fractions 45–75) were pooled and dialyzed twice against 2 L of 10 mM phosphate buffer, pH 7.4, containing 20% ethylene glycol (equilibration buffer C) for 24 h.

Heparin–Sepharose B. The dialyzed heparin–Sepharose pool was reapplied (flow rate 20 mL/h) to the heparin–Sepharose column equilibrated in equilibration buffer C. The column was then washed with 90 mL of equilibration buffer D and finally developed with a gradient of NaCl (0.5–3.0 M) in equilibration buffer D (total volume 800 mL). Fractions (10 mL) were collected into siliconized glass tubes, and those which contained biological activity (fractions 120–155) were pooled and applied to a column of copper chelated to Sepharose.

Copper–Sepharose Affinity Chromatography. A column (17 × 0.7 cm) of copper chelated to Sepharose was prepared according to Pharmacia guidelines and equilibrated in equilibration buffer D. The heparin–Sepharose pool was applied at a flow rate of 3 mL/h, and then the column was washed with equilibration buffer D (30 mL) containing imidazole (1 mM) and finally developed with a gradient of imidazole (1–10 mM) in equilibration buffer D (total volume 140 mL). Fractions (3.0 mL) were collected into siliconized glass tubes, and those which contained biological activity (fractions 15–17) were pooled and applied to a column of CM52.

CM52 Chromatography. Pooled fractions from the copper affinity step were diluted 1:10 with ammonium formate (0.05 M, pH 6.5) and applied to CM52 (200 μL) equilibrated in the same buffer. The column was sequentially eluted with 2.0 mL of 0.1–0.7 M ammonium formate, and fractions were collected into siliconized glass tubes.

Structural Characterization. Automated Edman degradation was performed with approximately 500 ng of the unmodified pituitary growth factor in a gas-phase sequencer (Model 470A, Applied Biosystems, Inc., Foster City, CA). The phenylthiohydantoin-modified amino acids were analyzed by reverse-phase HPLC (detection unit 1 pmol) with an IBM

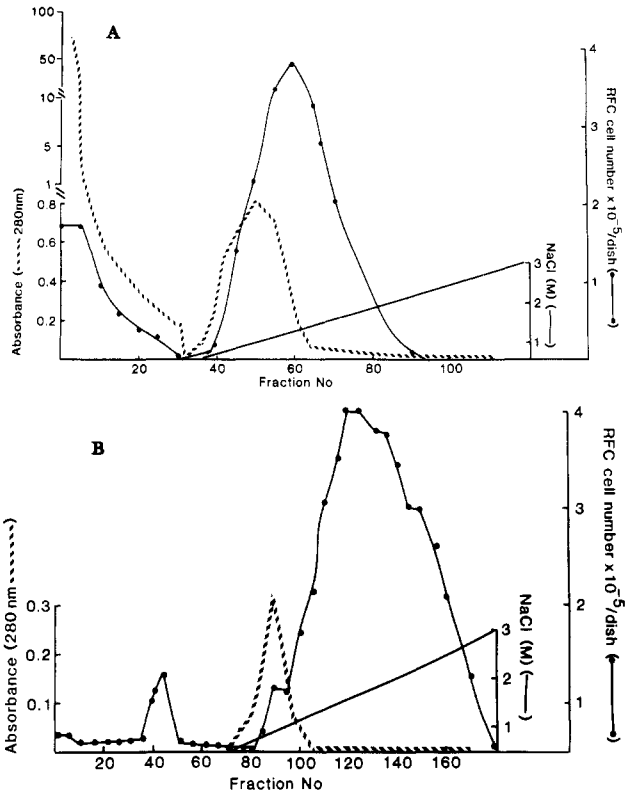

FIGURE 1: Chromatography of the pituitary extract on heparin–Sepharose: (A) extract prepared from 1200 human pituitaries was adsorbed to heparin–Sepharose and poured into a column as described in Experimental Procedures; (B) pooled fractions (fractions 45–75) containing mitogenic activity were dialyzed and reapplied to heparin–Sepharose. Absorbance at 280 nm (---) was determined. Aliquots (100 μL) were diluted in F10 growth medium (2 mL), and 100 μL was added to duplicate cultures in the RFC bioassay (●).

Instruments Cyano column using a sodium acetate buffer/tetrahydrofuran/acetonitrile gradient for elution (Applied Biosystems, Protein Sequence Users Bulletin No. 2, 1984).

Other Techniques. Analytical SDS–PAGE was carried out as described (Laemmli, 1970) in the presence of thiol reducing agents and stained with silver nitrate (Wray et al., 1981). Horizontal slab gel isoelectric focusing was carried out on an LKB Multiphore unit (Model 2117) using prepreapred polyacrylamide gels (LKB). Samples were applied to the gel at a distance of 1.5 cm from the anode. Focusing was carried out at a constant power of 4 W for 2 h by using an LKB constant power supply. At the completion of electrofocusing the gel was sliced into consecutive 0.5-cm segments and eluted in either F10 growth medium for bioassay or distilled water for the determination of pH.

Gel permeation chromatography was carried out by using a column of Sephadex G-100 superfine (0.7 × 71 cm), equilibrated in equilibration buffer D. The flow rate was adjusted to 2 mL/h, and fractions (1 mL) were collected into siliconized glass tubes.

RESULTS

Purification of Mitogenic Activity. The first step in the purification involved batchwise adsorption of the crude pituitary extract to heparin–Sepharose. The unadsorbed proteins and proteins which eluted at 0.5 M NaCl represented greater than 99% of the applied protein (Figure 1A). Mitogenic activity eluted from the column between 1 and 2 M NaCl with a major protein peak at 1 M NaCl. The pooled fractions were dialyzed against equilibration buffer C and reapplied to the heparin–Sepharose column equilibrated in buffer C. Re-

Table I: Purification of Human Pituitary Growth Factor

purification	total protein (mg)	specific activity (μg/mg of protein)	total units	total purification	% recovery
crude extract[a]	6.5×10^4	33	2.1×10^6	1	100
heparin–Sepharose (1)	81	5.0×10^3	4.1×10^5	152	20
heparin–Sepharose (2)	3.7	6.6×10^4	2.5×10^5	2.0×10^3	12
copper–Sepharose	1.08	1.4×10^5	1.5×10^5	4.2×10^3	7
CM52	0.045	6.6×10^5	3×10^4	2×10^4	1.4

[a] One unit of activity was determined from the half-maximal response of the dose–response curve for the crude extract.

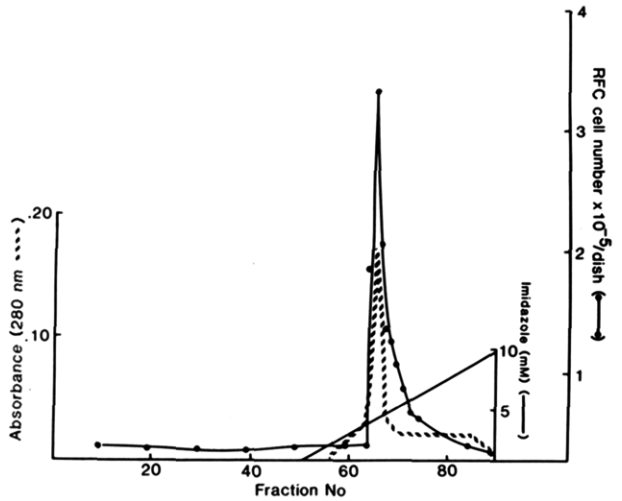

FIGURE 2: Chromatography of heparin-binding growth factor on copper–Sepharose. The pooled fractions from the heparin–Sepharose step B were applied to a column of copper chelated to Sepharose. Absorbance at 280 nm (---) was determined. Aliquots (25 μL) were diluted in F10 growth medium (2 mL), and 100 μL was added to duplicate cultures in the RFC bioassay (●).

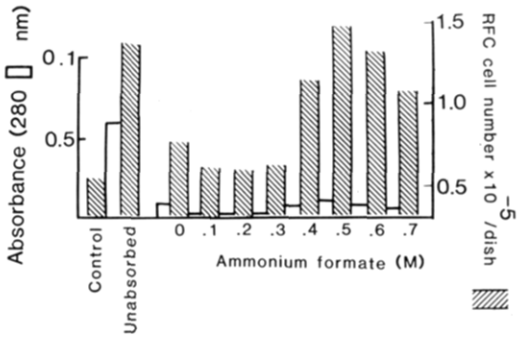

FIGURE 3: Chromatography of the copper–Sepharose pool on CM52. Pooled fractions (65–67) following the copper affinity purification were applied to CM52 as described, and adsorbed proteins were eluted with increasing concentrations of ammonium formate. Fractions were monitored for absorbance at 280 nm (open bars) and bioactivity (crosshatched bars) as described in Figure 2. Control represents unstimulated cultures.

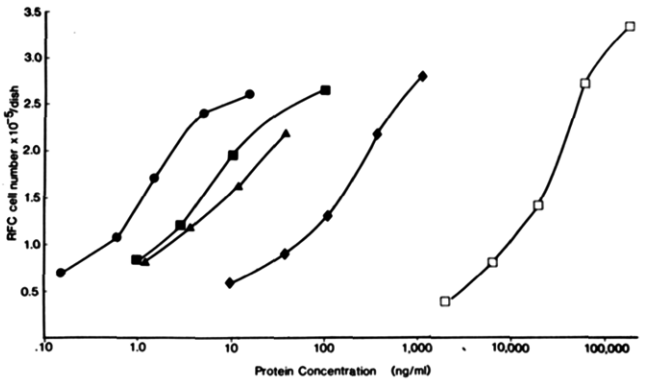

FIGURE 4: Dose–response analysis of the various steps in the purification of the pituitary growth factor. Each pool in the purification was tested at multiple dilutions in the RFC bioassay: crude extract (□); heparin–Sepharose A (◆); heparin–Sepharose B (▲); copper–Sepharose (■); CM52 (●).

fractionation of the adsorbed proteins with a linear gradient of 0.5–3 M NaCl resulted in an improved separation of the mitogenic activity from the major protein peak (Figure 1B). Further purification of the growth factor activity was achieved following metal chelating affinity chromatography (Figure 2). The majority of the biological activity eluted from the column at 2–4 mM imidazole.

The final step in the purification of the pituitary growth factor was acieved by using CM52 exchange resin. The majority of the mitogenic activity adsorbed to and eluted from the column between 0.4 and 0.7 M ammonium formate (Figure 3). Less than 10% of the applied activity remained unadsorbed to the column. Dose–response studies showed that the CM52 pool (0.4–0.7 M) stimulated a dose-dependent increase in chondrocyte proliferation in the range of 0.1–15 ng/mL (Figure 4). The purification scheme outlined yielded 45 μg of growth factor from 1200 frozen human pituitary glands with an overall 20000-fold increase in the specific activity (Table I).

Characterization of Pituitary Growth Factor. Analysis of the CM52 0.4–0.7 M pool by SDS–PAGE revealed a single band of molecular weight 18K (Figure 5). Activity associated with this band has not been ascertained due to its instability in the presence of SDS. However, gel permeation chromatography using Sephadex G-100 superfine of the CM52 0.4–0.7 M pool showed activity in fractions corresponding to a molecular weight of 18000–20000 (Figure 6A). The correlation between the molecular weight determined by gel filtration and SDS–PAGE suggests the band migrating at M_r 18K is the pituitary growth factor.

Isoelectric focusing of the copper–Sepharose pool was performed by using analytical polyacrylamide slab gels in the

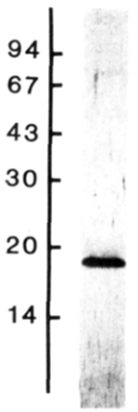

FIGURE 5: SDS–PAGE of the CM52 pool (0.4–0.7 M). Approximately 500 ng of the CM52 pool was applied to a 15% SDS–PAGE and stained with silver nitrate. Molecular weight standards: phosphorylase *b*, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; trypsin inhibitor, 20.1K; α–lactalbumin, 14.4K.

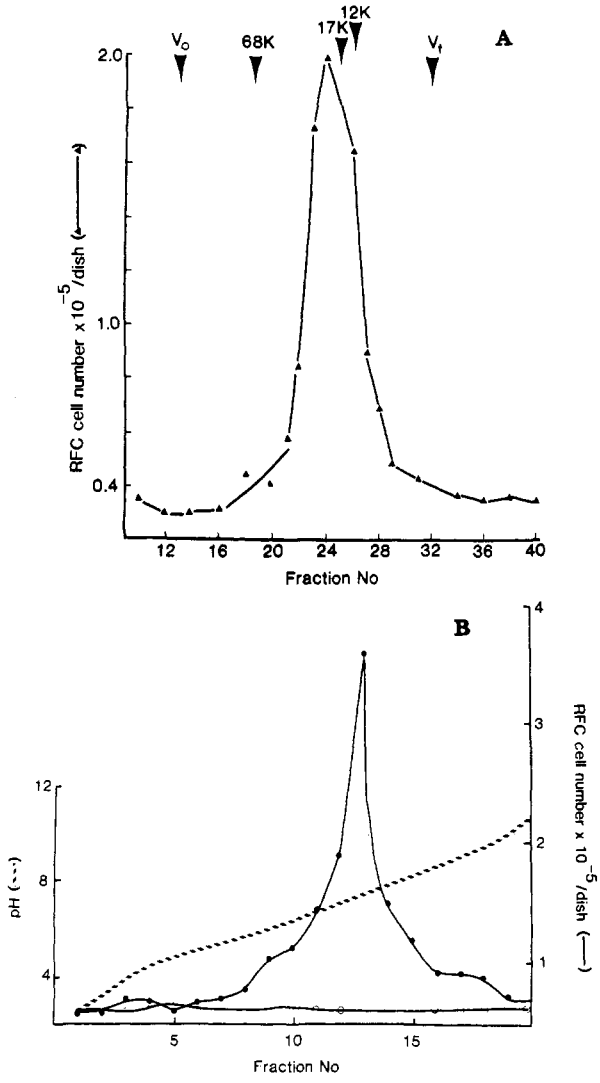

FIGURE 6: (A) Gel permeation chromatography of the CM52 pool using Sephadex G-100 superfine. Approximately 500 ng of the CM52 pool was applied to a column of Sephadex G-100 superfine as described under Experimental Procedures. Five hundred microliters of each fraction was diluted with 1.5 mL of F10 growth medium, and biological activity (▲) was determined by adding 100 μL to the RFC bioassay. (B) Isoelectric focusing of the MCAC pool. Approximately 5 μg of the MCAC pool was electrofocused by using a slab PAGE in the pH range of 3.5–10 described under Experimental Procedures. Gel segments were eluted and the pH (---) and biological activity in the sample lane (●) and control lane (○) were determined in the RFC bioassay.

pH range of 3.5–10. One peak of activity was detected in gel eluants corresponding to a p*I* of 7.5 (Figure 6B). The biological activity of the pituitary growth factor was abolished by trypsin digestion, pH 2.0, and by boiling (data not shown). Amino-terminal sequence analysis of the native PGF allowed assignment of 9 of the first 11 residues (PALPEXGGXGA) which were identical with basic FGF (Esch et al., 1985).

When tested in the RFC bioassay, PGF (0.75 ng mL^{-1}) or heparin (0.5 μg mL^{-1}) alone produced an approximate 2-fold increase in cell growth above control cultures (data not shown). However, when tested in combination, PGF/heparin stimulated cell growth more than 5-fold above control values. In the presence of protamine (10 μM), the PGF/heparin-induced cell growth was inhibited by more than 50%. Protamine did not inhibit cell growth when tested alone or in combination with either heparin or PGF (data not shown).

PGF was also mitogenic for bovine corneal endothelial cells in the range of 0.015–1.5 ng/mL (Figure 7). However, PGF

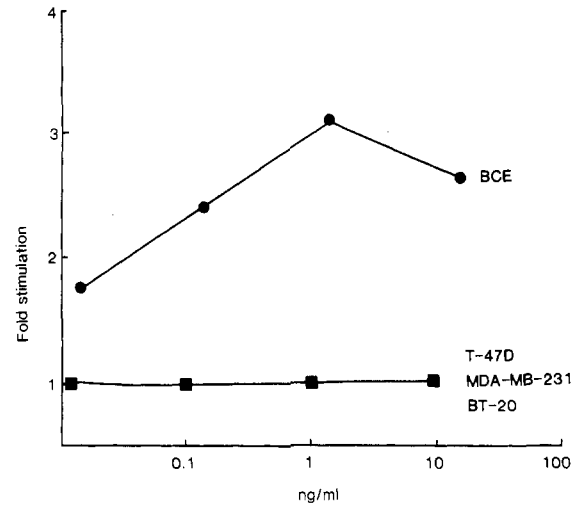

FIGURE 7: Effect of PGF on other cell types. Purified PGF was tested at various doses for its ability to stimulate the proliferation of bovine corneal endothelial cells (BCE) (●) and three human breast cancer cell lines, T-47D, MDA-MB-231, and BT-20 (■).

was not stimulatory when tested on human breast cancer cells in monolayer culture (Figure 7).

Discussion

In this study we have purified a growth factor (molecular weight 18 000, p*I* = 7.5) from human pituitaries which exhibited an affinity for heparin. The purified growth factor was homogeneous as judged by a single band on SDS–PAGE. On the basis of the similarity in the retention behavior on heparin–Sepharose and the amino-terminal amino acid sequence, PGF and basic FGF are closely related if not identical. However, PGF and basic FGF differ in their reported molecular weights (M_r 18 000 and 16 000) and p*I* (7.5 and 9.5), respectively (Gospodarowicz et al., 1985). Whether these differences reflect variation in experimental procedures or are a consequence of structural differences in the basic FGF gene family between the species remains to be determined.

Heparin has been shown to stimulate or inhibit cell proliferation in vitro and in vivo (Costachel et al., 1964; Clowes & Karnovsky, 1977; Yang & Jenkin, 1978; Taylor & Folkman, 1982; Folkman et al., 1983; Castellot et aal., 1984). These effects are due in part to the dose of heparin and the cell type used. In our study, heparin (0.5 μg/mL) was found to stimulate RFC proliferation, while at higher concentrations heparin was inhibitory (data not shown). Earlier studies using heparin at a concentration of 0.05% (a dose which is inhibitory to RFC cell growth) demonstrated inhibition of mitosis in the fertilized eggs of chaetopterus (Heilbrunn & Wilson, 1949). The effect of heparin was attributed to the inhibition of protoplasmic gelation which precedes the appearance of the mitotic spindle. At present the mechanism of synergism between heparin and PGF on stimulating RFC growth observed here is unknown. These effects may be mediated through cell surface receptors since specific binding sites for heparin (Kjellen et al., 1977; Glimelius et al., 1978) and basic FGF (Neufeld & Gospodarowicz, 1985) have been reported.

It has been speculated that the effect of heparin in regulating cell growth may be due to an increase in the accessibility of membrane receptors for other external stimuli (Kraemer & Smith, 1974). Modulation of the EGF and somatomedin C receptor by PDGF has been demonstrated (Fox et al., 1979; Clemmons et al., 1980) and might support the notion that the effect of heparin observed here is mediated through alteration in the PGF receptor. Alternatively, the close structural re-

lationship between heparin and the natural predominant heparan sulfate (a sulfated glycosaminoglycan of the pericellular glycocalyx) might suggest that heparin is acting by either increasing the local pericellular concentration of PGF or increasing the intrinsic potency of PGF as a consequence of binding to heparin. The observed synergism between PGF and heparin was attenuated by protamine sulfate at concentrations that were not inhibitory to RFC stimulated with PGF or heparin alone, ruling out a cytotoxic effect of protamine. Since protamine was not retained on the heparin–Sepharose affinity column (data not shown) and did not inhibit the PGF-induced cell growth, it is unlikely that the protamine effect involves competitive interaction with PGF for its binding to either heparin or the putative PGF receptor. Protamine has been shown to inhibit the heparin-enhanced tumor angiogenesis response (Taylor & Folkman, 1982) as well as the ECGF-enhanced response to heparin by using cultured human umbilical vein endothelial cells Thornton et al., 1983).

The physiological role of growth factors that bind to heparin is unknown. It is conceivable that in vivo the action of these mitogens may be modified by components of the extracellular matrix, which might act either to enhance or to reduce their biological activity. The availability of purified preparations of these growth factors should now permit a more careful examination of their physiological role in vivo.

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