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# Release of Cell Surface-Associated Basic Fibroblast Growth Factor by Glycosylphosphatidylinositol-Specific Phospholipase C

PNINA BASHKIN, GERA NEUFELD, HELA GITAY-GOREN, AND ISRAEL VLODAVSKY*
*Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem 91120 (P.B., I.V.) and Department of Cell Biology, Technion, Haifa (G.N., H.G.-G.), Israel*

Heparan sulfate proteoglycans (HSPG) are ubiquitous constituents of mammalian cell surfaces and most extracellular matrices. A portion of the cell surface HSPG is anchored via a covalently linked glycosyl-phosphatidylinositol (PI) residue, which can be released by treatment with a glycosyl-PI specific phospholipase C (PI-PLC). We report that exposure of bovine aortic endothelial and smooth muscle cells to PI-PLC resulted in release of cell surface-associated, growth-promoting activity that was neutralized by antibasic fibroblast growth factor (bFGF) antibodies. Active bFGF was also released by treating the cells with bacterial heparitinase. Under the same conditions there was no release of mitogenic activity from cells (BHK-21, NIH/3T3, PF-HR9) that expressed little or no bFGF, as opposed to PI-PLC-mediated release of active bFGF from the same cells transfected with the bFGF gene. The released bFGF competed with recombinant bFGF in a radioreceptor assay. Addition of PI-PLC to sparsely seeded vascular endothelial cells resulted in a marked stimulation of cell proliferation, but there was no mitogenic effect of PI-PLC on 3T3 fibroblasts. Studies with exogenously added $^{125}$I-bFGF revealed that about 6.5% and 20% of the cell surface-bound bFGF were released by treatment with PI-PLC and heparitinase, respectively. Both enzymes also released sulfate-labeled heparan sulfate from metabolically labeled 3T3 fibroblasts. PI-PLC failed to release $^{125}$I-bFGF from the subendothelial extracellular matrix (ECM), as compared to release of 60% of the ECM-bound bFGF by heparitinase. Our results indicate that 3–8% of the total cellular content of bFGF is associated with glycosyl-PI anchored cell surface HSPG. This FGF may exert both autocrine and paracrine effects, provided that it is released by PI-PLC and adequately presented to high affinity bFGF cell surface receptor sites. © 1992 Wiley–Liss, Inc.

Basic fibroblast growth factor (bFGF) is a multipotential, heparin-binding, angiogenic growth factor that stimulates and supports cell proliferation, migration, and differentiation (Folkman and Klagsbrun, 1987; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). Unlike most other polypeptide growth factors, bFGF is a nonglycosylated, primarily cell-associated protein, consistent with the lack of a conventional signal sequence for secretion (Abraham et al., 1986). Basic FGF has been extracted from the extracellular matrix (ECM) produced by cultured endothelial cells (Baird and Ling, 1987; Vlodavsky et al., 1987) and was visualized by immunostaining in basement membranes of blood vessels (Cardon-Cardo et al., 1990), cornea (Folkman et al., 1988), and several other tissues (Casscells et al., 1990; Gonzalez et al., 1990). These results and the finding that anti-bFGF antibodies inhibit the proliferation and migration of vascular EC in the absence of added bFGF (Sato and Rifkin, 1988; Rifkin, 1991) suggest that under certain conditions bFGF is released from cells via an unusual route and may then exert an extracellular autocrine activity.

Heparan sulfate proteoglycans (HSPG) are ubiquitous constituents of mammalian cell surfaces (Hook et al., 1984; Gallagher et al., 1986) and most extracellular matrices. They play a role in cellular interactions and in growth control during morphogenesis, cell differentiation, and proliferation (Castellot et al., 1987; San Antonio et al., 1987; Thesleff et al., 1989) and may modulate growth factor activities (Ruoslahti and Yamaguchi, 1991). Basic FGF binds with low affinity to HSPG on the cell surface and ECM (Moscatelli, 1987; Saksela et al., 1988; Bashkin et al., 1989; Kiefer et al., 1990; Saksela and Rifkin, 1990; Yayon et al., 1991). This binding appears to protect the growth factor from degradation (Saksela et al., 1988) and may be required for subsequent interaction of bFGF with its high affinity tyrosine kinase receptor (Yayon et al., 1991) and signal transduction. It has been reported that a portion

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*To whom reprint requests/correspondence should be addressed.

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of the cell surface associated HSPG is anchored via a covalently linked glycosyl-phosphatidylinositol (glycosyl-PI) residue that has its fatty acyl chains buried in the lipid bilayer. This HSPG can be released from the cell surface by treatment of cultured cells (i.e., hepatocytes, Schwann cells, rat ovarian granulosa cells) with a glycosyl-phosphatidylinositol-specific phospholipase C (PI-PLC) (Ishihara et al., 1987; Low and Saltiel, 1988; Carey and Evans, 1989; Yanagishita and McQuillan, 1989). In the present study we demonstrate that a small proportion of the cellular content of bFGF is associated with glycosyl-PI-anchored cell surface HSPG and may function in an autocrine manner when it is released by PI-PLC.

## MATERIALS AND METHODS

### Materials

Highly purified PI-PLC from Bacillus thuringiensis and recombinant PI-PLC, isolated from Bacillus subtilis, were a kind gift of Dr. Martin Low (College of Physicians and Surgeons, Columbia University, New York) (Low et al., 1988). No proteolytic activity could be detected in these preparations of PI-PLC (Knudsen et al., 1989). Bacterial heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) (Flavobacterium heparinum) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Chondroitinase ABC and trypsin were purchased from Sigma Chemicals (St. Louis, MO), and hyaluronidase from Worthington Biochem. Corp. (Freehold, NJ). Recombinant human bFGF was kindly provided by Takeda Chemical Industries (Osaka, Japan). Heparin-Sepharose was from Pharmacia (Uppsala, Sweden). Heparin and low Mr heparin (Mr ~4500) were kindly provided by Kabi Pharmacia (Stockholm, Sweden). Dulbecco's modified Eagle's medium (DMEM), Nutrient mixture F-12, calf serum, fetal calf serum (FCS), penicillin, and streptomycin were obtained from GIBCO (Grand Island, NY). Saline containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02% EDTA (STV) was obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes and 96-well plates were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA). $^{3}$H-Thymidine (5,000 mCi/mmol), $Na_2(^{35}S)O_4$ (540–590 mCi/mmol), and Na-$^{125}$I were obtained from Amersham (Buckinghamshire, England). Methylene blue, cetyl pyridinium chloride, and all other chemicals were of reagent grade, purchased from Sigma (St. Louis, MO).

### Cell cultures

Balb/c 3T3 cells were maintained in DMEM (4.5 g glucose/liter), supplemented with 10% FCS, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37°C in 10% $CO_2$ humidified incubators. Cultures of bovine corneal endothelial cells were established from steer eyes as previously described (Gospodarowicz et al., 1977). Stock cultures were maintained in DMEM (1 g glucose/liter) supplemented with 10% bovine calf serum, 5% FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37°C in 10% $CO_2$ humidified incubators. Partially purified brain derived bFGF (100 ng/ml) was added every other day during the phase of active cell growth. Bovine aortic endothelial cells (EC) and smooth muscle cells (SMC) were isolated and cultured in DMEM (1 g glucose/liter) supplemented with 10% bovine calf serum, as described (Gospodarowicz et al., 1976). Lines of PF-HR9 cells and NIH-3T3 fibroblasts transfected with the bFGF gene were kindly provided by Dr. S. Rogelj and Dr. M. Klagsbrun (Children's Hospital, Harvard Medical School, Boston, MA). The PF-HR9 cell lines were obtained by co-transfection of PF-HR9 mouse endodermal cells with a construct containing both the bFGF cDNA and the dominant selectable marker pSV2-neo (HR9/bFGF), or the pSV2-neo alone (HR9/neo) (Rogelj et al., 1989). We have also used NIH-3T3 cells transfected with a construct in which the bFGF cDNA was fused with the amino-terminal immunoglobulin signal peptide cDNA (3T3-pIg-bFGF). This construct also contained the dominant selectable marker pSV2-neo (Rogelj et al., 1988). All cell lines were maintained in DMEM (4.5 g glucose/liter) supplemented with 10% FCS and antibiotics, as described above. Stock cultures of BHK-21 cells and BHK-21 cells co-transfected with pbFGF and the selectable marker pSV2-neo (Neufeld et al., 1988) were maintained in a 1:1 mixture of DMEM:F-12 media (DF medium) supplemented with 5% calf serum.

### Growth factor activity

Assay for DNA synthesis in 3T3 cells was performed as described (Vlodavsky et al., 1987). Briefly, Balb/c 3T3 cells were seeded at half confluence into 0.3 cm$^2$ microtiter wells in DMEM supplemented with 10% fetal calf serum. After reaching confluence (2–3 days), the cells were further incubated for a minimum of 5 days. Samples and $^{3}$H-thymidine (1 μCi/well) were then added to the quiescent cells and after an incubation period of 32–40 h, DNA synthesis was assayed by measuring the radioactivity incorporated into TCA insoluble material. For measurements of EC proliferation, cells were seeded at low density ($2 \times 10^2$ cells per well of a 96-well plate) in 0.2 ml DMEM containing 10% heat inactivated calf serum. Samples (10–20 μl) were added on days 2 and 4 and the cultures fixed (2.5% formaldehyde in PBS) on days 6–8. The plates were immersed in 10 mM borate buffer (pH 8.5), stained (10 min, 24°C) with 0.1 ml/well methylene blue (1% in 0.1 M borate buffer, pH 8.5) and washed four times in borate buffer. This procedure removed practically all noncell-bound dye. Specific cell-incorporated methylene blue was dissolved with 0.2 ml of 0.1 N HCl (60 min, 37°C) and determined by reading the absorbance at 600 nm. Uptake of methylene blue was linearly correlated to the number of viable cells (Goldman and Bar-Shavit, 1979).

### Iodination of bFGF

Recombinant bFGF was iodinated with Na$^{125}$I and IodoGen (Pierce Chemical Co.) as described (Neufeld and Gospodarowicz, 1985; Bashkin et al., 1989). Briefly, bFGF (3.3 μg in 50 μl of 10 mM Tris-HCl, pH 7.1 and 2 M NaCl) together with 60 μl of 0.2 M sodium phosphate pH 7.2, was added to a glass tube containing 1.6 μg Iodogen. The reaction was started by the addition of a twofold molar excess of Na$^{125}$I and stopped after 15 min at room temperature by addition of 60 μl of 0.1% sodium metabisulfite and 30 μl of 0.1 mM KI. The reaction mixture was applied onto a small (0.3 ml)

heparin-Sepharose column and the $^{125}$I-bFGF eluted with 1.5 ml buffer containing 20 mM sodium phosphate pH 7.2, 2 M NaCl and 0.2% gelatin. The specific activity was usually $1.2 \times 10^5$ cpm/ng bFGF and the labeled preparation was kept for up to 3 weeks at 4°C. The iodinated material yielded a single band (18.4 kDa) when subjected to $NaDodSO_4$-PAGE and autoradiography. In some experiments bFGF was iodinated with $^{125}$I and chloramin T. Results were essentially the same.

### Preparation of dishes coated with ECM

Bovine corneal endothelial cells were dissociated from stock cultures (second to fifth passage) with STV and plated into 35 mm dishes at an initial density of $5 \times 10^4$ cells/ml. Cells were maintained as described above, except that 5% dextran T-40 was included in the growth medium. Six to 8 days after the cells reached confluency, the subendothelial ECM was exposed by dissolving (3 min, 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM $NH_4OH$ in PBS, followed by four washes in PBS (Vlodavsky et al., 1987). The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish. The presence of nuclei or cytoskeletal elements could not be detected in the denuded ECM. No serum proteins could be identified in the ECM (Gospodarowicz et al., 1983).

### Treatment of cells with PI-PLC

Cultured cell monolayers were washed (4 times) with serum-free medium to remove serum traces. The cells were detached by incubation (15 min, 37°C) with 0.5 mM EDTA in PBS, centrifuged, and washed twice in DMEM containing 0.05% BSA. Cells were suspended in this medium to a concentration of $3 \times 10^6$ cells/0.5 ml, and incubated (1 h, 37°C) with PI-PLC (0.7–0.8 units/ml) with occasional shaking. The reaction was stopped by centrifugation of the cells ($1000 \times g$, 10 min, 4°C) and aliquots of the supernatants were applied to the proliferation assays. Alternatively, cells were grown in ECM-coated 35 mm dishes to enforce firm cell attachment. The cell monolayers were washed six times in DMEM containing 0.05% BSA and incubated (1 h, 37°C) with PI-PLC in the same medium. After incubation, the medium was collected, centrifuged ($1{,}000 \times g$, 10 min, 4°C) and samples tested for induction of cell proliferation.

### Affinity purified anti-bFGF antibodies

Rabbit anti-bFGF antibodies were obtained as previously described (Schweigerer et al., 1987). In brief, rabbit anti-bFGF antiserum obtained by subcutaneous and intracutaneous injections of pure pituitary bFGF was purified by protein A-Sepharose affinity chromatography. The antibodies in the IgG fraction recognized in a radioimmunoassay bFGF ($ED_{50}$ = 2 ng/ml), but not aFGF, EGF, insulin, transferin, cytochrome c, or thyroglobulin (at concentrations up to 0.4 μg/ml). These antibodies inhibited bFGF- but not aFGF- stimulated proliferation of adrenal cortex- or brain- derived capillary endothelial cells (Schweigerer et al., 1987), nor did they inhibit the bioactivities of serum, EGF, or PDGF. The anti-bFGF antibodies failed to react with any other species of the FGF family, except for slight recognition of FGF-5.

For affinity purification of the anti-bFGF antibodies, human recombinant bFGF (200 μg protein) was chromatographed on a 15% SDS-PAGE gel. Electrophoretic transfer to nitrocellulose was performed and the proteins on the nitrocellulose were crosslinked using glutaraldehyde and stained with Ponceau-s. The nitrocellulose strip containing the bFGF was excised and incubated at room temperature for 2 h with wash buffer consisting of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5% bovine serum albumin (BSA). The strips were then incubated (2 h) in wash buffer with the antihuman bFGF antibodies (415 μg) as described above (Schweigerer et al., 1987), followed by three washes in the same buffer. To elute the anti-bFGF antibodies, the strips were incubated with 0.1 M glycine/HCl, pH 2.5 (3 min, 4°C), followed by neutralization to pH 8.0 with 1 M $K_2HPO_4$ (Tessler and Neufeld, 1990).

### Purification of bFGF from cell supernatants

Supernatants from cells treated or untreated with PI-PLC were collected as described above, adjusted with 10 mM Tris/HCl, pH 7.0, containing 3 M NaCl to a salt concentration of 0.3 M NaCl, and applied to a heparin-Sepharose column (100 μl bed volume) equilibrated at room temperature with 10 mM Tris/HCl, pH 7.0, containing 0.6 M NaCl. The column was washed extensively with the same buffer and the growth factor eluted ($3 \times 200$ μl fractions) with 10 mM Tris/HCl, pH 7.0, containing 2 M NaCl. The fractions were diluted with 0.2% gelatin and tested for inhibition of $^{125}$I-bFGF binding in a radioreceptor assay, as described below.

### FGF-radioreceptor assay

Radioreceptor assays were performed using BHK-21 derived cell membranes (Neufeld and Gospodarowicz, 1985); 5 μg of membrane protein at a final volume of 400 μl were used per binding assay. The binding buffer contained 20 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 140 mM NaCl, and 0.1% gelatin (w/v). $^{125}$I-bFGF was added to a concentration of 200 pg/ml and nonspecific binding to the membranes was determined in the presence of 200 ng/ml of unlabeled pituitary bFGF. Binding was started by adding the crude membrane preparation to the system and after 1 h at 24°C, 1 ml of cold wash buffer (PBS, 0.05% BSA, 0.02% $NaN_3$) was added. The membrane suspension was then filtered through HT-200 filters (Gelman Sciences, Inc., Ann Arbor, MI), which were prewashed with 1 ml cold wash buffer. Each filter was washed three times with 1 ml of ice cold wash buffer and counted in a Beckman model 5500 γ-counter. Depending on the batch of cell membrane preparation, between 5 to 15 ng of $^{125}$I-bFGF were specifically bound per mg of cell membranes protein (Neufeld and Gospodarowicz, 1985). Inhibition of $^{125}$I-bFGF binding to BHK-21 cell membranes was determined by addition of aliquots of supernatants of cells, treated or untreated with PI-PLC, with or without concentration on heparin-Sepharose, into the $^{125}$I-bFGF containing binding reaction mixture, prior to addition of the BHK-21 cell membrane preparation.

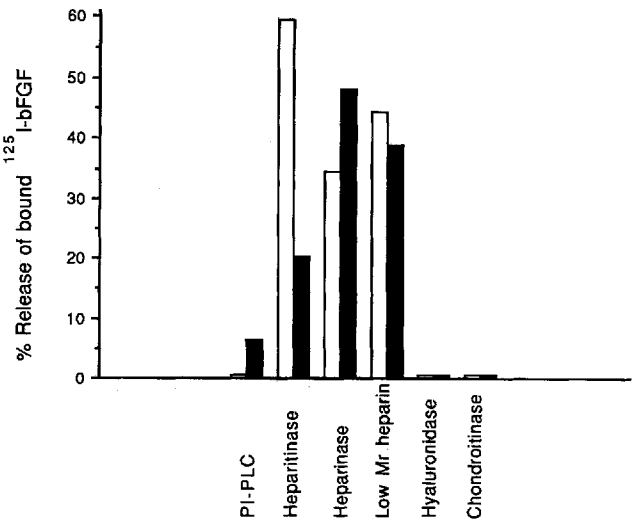


Fig. 1. PI-PLC mediated release of cell-bound and ECM-bound $^{125}$I-bFGF. 3T3 fibroblasts (solid bars) grown to confluence on fibronectin-coated dishes (60 mm) were washed two times in ice-cold PBS containing 0.05% gelatin, 1 mM $Mg^{+2}$ and 2 mM $Ca^{2+}$. $^{125}$I-bFGF was then added in the same buffer ($8 \times 10^5$ cpm/2 ml/dish) and incubated with the cells for 2 h at 4°C on an oscillating platform. The monolayers were washed extensively with cold PBS containing gelatin, $Ca^{+2}$ and $Mg^{+2}$ to remove unbound $I^{125}$-bFGF. The cells were then incubated for 1 h at 37°C in the absence or presence of 0.7 U/ml PI-PLC, 0.2 U/ml bacterial heparitinase, 0.05 U/ml bacterial heparinase, 10 μg/ml low Mr heparin, 0.1 U/ml hyaluronidase, or 0.1 U/ml chondroitinase ABC. At the end of the incubation period, the supernatants were collected and counted for estimation of released iodinated material. ECM-coated 35 mm dishes (open bars) were incubated (22°C, 2 h) with $^{125}$I-FGF ($1 \times 10^5$ cpm/ml/dish). Following incubation the medium was removed, the plates washed, and the ECM incubated with low Mr heparin and the various enzymes, as described above. The incubation media were collected and measured for the amount of released iodinated material. The values of released $^{125}$I-bFGF from untreated cells and ECM were subtracted from those of treated samples and results are expressed as percentage release from total cell- and ECM-bound $^{125}$I-bFGF (245,530 cpm and 51,355 cpm, respectively). The experiment was performed three times (in duplicates) and the variation between determinations did not exceed 15% of the mean.

### Lactic dehydrogenase (LDH) activity

LDH activity in supernatants of treated cells was determined as described (Matzner et al., 1982).

## RESULTS

### Release of exogenously added bFGF from cells and ECM

We first investigated whether exogenously added cell-bound $^{125}$I-bFGF can be released from the cell surface by treatment with PI-PLC. For this purpose 3T3 fibroblasts were incubated (2 h, 4°C) with $^{125}$I-bFGF in PBS containing 0.05% gelatin. The cells were washed to remove unbound bFGF, and exposed (1 h, 37°C) to PI-PLC. About 6.5% of the total cell-bound $^{125}$I-bFGF were released, suggesting that a small percentage of the cell surface-associated bFGF was bound to glycosyl-PI-anchored receptor molecules. There was no release of $^{125}$I-bFGF from ECM that was incubated with $^{125}$I-bFGF, washed free of the unbound factor, and exposed to PI-PLC (Fig. 1). In contrast, when bacterial heparinase (0.05 U/ml) rather than PI-PLC was applied under the same conditions, $^{125}$I-bFGF was released from both the cell surface and ECM to an extent that was about 50% and 35% of the total amount of bound bFGF, respectively (Fig. 1). Exposure (1 h, 37°C) of 3T3 cells and ECM to bacterial heparitinase (0.2 U/ml) resulted in the release of 20% and 60% of the bound $^{125}$I-bFGF, respectively. Under the same conditions, low Mr heparin (10 μg/ml) released up to 40% and 45% of the cell- and ECM- bound $^{125}$I-bFGF, respectively (Fig. 1). Exposure to hyaluronidase or chondroitinase ABC induced little or no release of $^{125}$I-bFGF from both the cell surface and ECM.

These results indicate that PI-PLC cleaves a specific component on the cell membrane, most likely glycosyl-PI anchored HSPG, that is not present in the subendothelial ECM. The results also indicate that the effect of PI-PLC cannot be ascribed to an undetectable amount of proteolytic activity in the PI-PLC preparation, since the enzyme failed to release $^{125}$I-bFGF that was first bound to ECM. We have previously demonstrated that ECM-bound bFGF is readily accessible to release by proteases (Bashkin et al., 1989). In other experiments, cells were metabolically labeled (48 h, 37°C) with $Na_2{}^{35}SO_4$ (10 μCi/ml), washed free of unincorporated radioactivity, and exposed to PI-PLC. Almost all the sulfate labeled material released in the presence of PI-PLC was precipitable by 0.05% CPC in 0.6 M NaCl (Fig. 2). Under these conditions CPC precipitates mostly HS glycosaminoglycans (Roden et al., 1972). Treatment with bacterial heparitinase released about twice as much sulfate labeled material as compared to PI-PLC, of which 55% were precipitable with CPC (Fig. 2). In contrast, less than 10% of the labeled material released by bacterial heparinase was precipitated by CPC. Exposure of cells to heparin or hyaluronidase resulted in little or no release of CPC precipitable sulfate labeled material (Fig. 2).

### Mitogenic effect of PI-PLC on vascular endothelial cells

Based on the above described results, we examined whether PI-PLC elicits a mitogenic response in vascular endothelial cells (EC) by means of releasing bFGF-HS complexes from the cell surface. For this purpose, EC were seeded at low density (200 cells per well of a 96-well plate), allowed to adhere for 2 h at 37°C, and then exposed to increasing concentrations of PI-PLC (0.01–0.3 U/ml). PI-PLC was added to the cells once again after 2 days in culture. Control cells were seeded and incubated under the same conditions, except that the enzyme was not added. After 8 days in culture, the extent of cell proliferation was evaluated using the methylene blue uptake assay as a measure for cell number. Treatment of sparsely seeded EC with PI-PLC (B. thuringiensis) resulted in cell proliferation that was about 20-fold higher as compared to control cultures that were not incubated with the enzyme. The mitogenic effect increased as a function of the amount of enzyme added, reaching a plateau at 0.1–0.15 U/ml (Fig. 3A). An increased effect was observed as a function of the number of cells seeded per well (Fig. 3B). Unlike its effect on EC, addition of PI-PLC to 3T3 fibroblasts had no mitogenic effect on the cells. Another highly purified preparation of PI-PLC isolated from B. thuringiensis, as well as recombinant PI-PLC purified

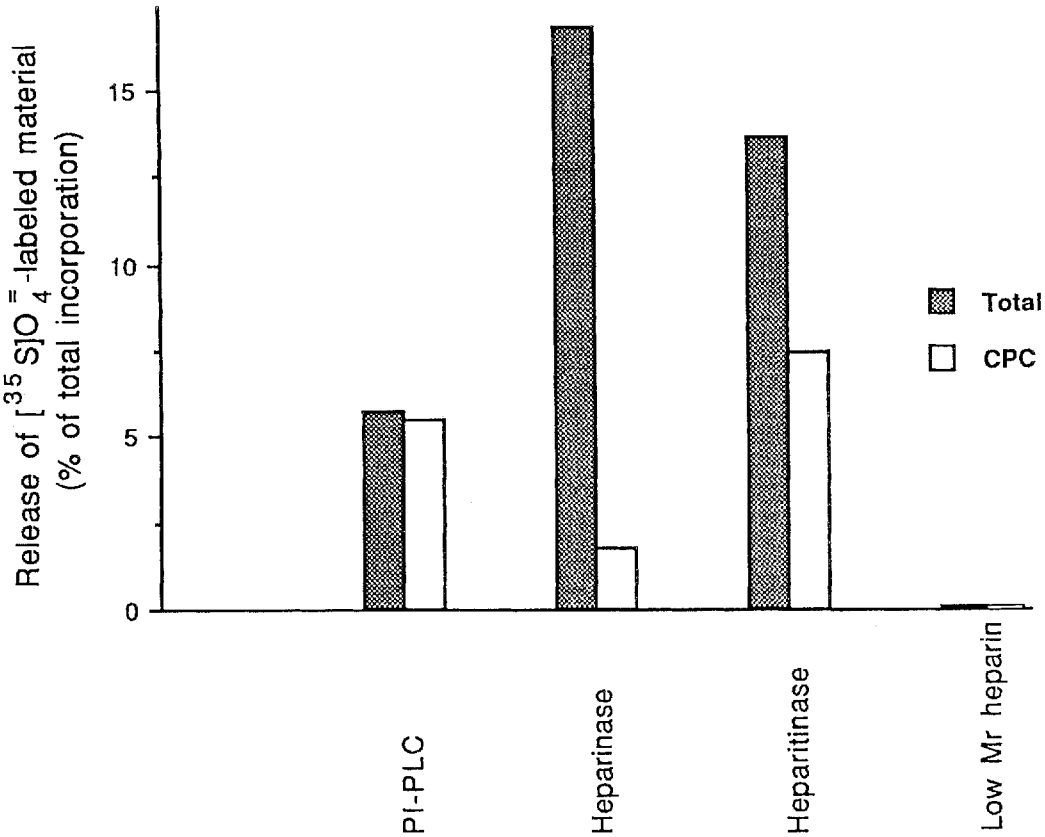


Fig. 2. PI-PLC induced release of sulfate labeled material from 3T3 fibroblasts. 3T3 fibroblasts were grown on fibronectin-coated, 60 mm culture dishes. Preconfluent cultures were metabolically labeled with $Na_2{}^{35}SO_4$ (10 μCi/ml) for 48 h. Prior to treatment, dishes were rinsed extensively with DMEM to remove unincorporated sulfate. The cell monolayers were then incubated (1 h, 37°C) in DMEM containing 0.1% gelatin, in the absence or presence of either PI-PLC (0.7 U/ml), bacterial heparitinase (0.2 U/ml), bacterial heparinase (0.05 U/ml), or low Mr heparin (10 μg/ml). The supernatants were then collected for measurement of total (shaded bars) and CPC (open bars) precipitable released sulfate labeled material. The cell pellet was dissolved in 0.1 N NaOH for determination of the total incorporated sulfate radioactivity. CPC precipitation was performed on GFA Whatman filters as described (Roden et al., 1972). Release of sulfate-labeled material from untreated cells was subtracted from the values of the treated samples and results are expressed as percentage release from total cell incorporated sulfate-labeled material (100% = 95,733 cpm). The variation between triplicate determinations did not exceed ±15%.

from B. subtilis, exhibited a mitogenic effect, which was similar to that of the original batch used throughout the experiments (Fig. 3A). Since PI-PLC was added to EC maintained in complete growth medium containing 10% calf serum, it is unlikely that its mitogenic effect is due to any trace amount of proteolytic activity present in the PI-PLC preparation. Trypsin, heparinase, or chondroitinase ABC added to EC under the same conditions had no mitogenic effect on the cells (Fig. 3A).

### PI-PLC treated cells release biologically active bFGF into the incubation medium

We postulated that the mitogenic effect exerted by PI-PLC on vascular EC resulted from release of endogenous bFGF-like growth factor bound to the cell membrane through glycosyl-PI anchored HSPG (Ishihara et al., 1987). We therefore examined whether the ability of PI-PLC to release growth-promoting activity from various cell types correlates with the expression of bFGF by these cells. For this purpose cells were incubated with PI-PLC (0.7 U/ml, 1 h, 37°C) and samples of the incubation medium were tested for induction of $^3$H-thymidine incorporation in 3T3 fibroblasts (Fig. 4) and stimulation of EC proliferation (Fig. 5).

In preliminary experiments we noticed that following incubation of PI-PLC with cells that express little or no FGF (i.e., 3T3 fibroblasts, BHK-21 cells, PF-HR9 endodermal cells), the enzyme lost its ability to induce EC proliferation as shown by lack of mitogenic response when aliquots of the incubation medium were added to EC. This loss of mitogenic effect was attributed to inactivation and/or sequestration of PI-PLC by the cells. In contrast, as demonstrated in Figure 4, mitogenic effect toward 3T3 fibroblasts was identified in supernatants of PI-PLC treated smooth muscle cells (SMC), vascular EC, and cells transfected with the bFGF cDNA, either without (BHK-21 and PF-HR9 cells) or with (3T3 fibroblasts) fusion to a signal sequence. There was no release of detectable mitogenic activity from nontransfected 3T3 fibroblasts, BHK-21 cells, and PF-HR9 cells and from cells transfected with pSV2-neo alone (Fig. 4), suggesting that the mitogenic activity released from PI-PLC treated cells was induced by bFGF.

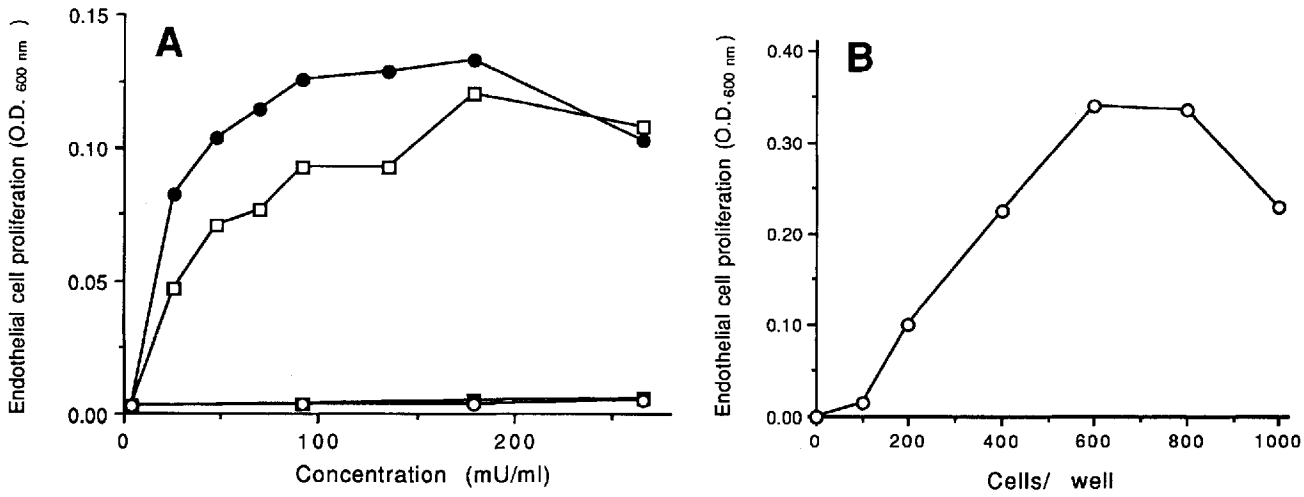


Fig. 3. Mitogenic effect of PI-PLC on endothelial cells. **A.** Cells were seeded at low density (200 cells/well of a 96-well plate) in 0.2 ml DMEM supplemented with 10% heat-inactivated calf serum. Increasing amounts of PI-PLC (●), recombinant PI-PLC (□), heparinase (○), or chondroitinase ABC (■) were added on day 2 and 4. The number of cells was determined on day 8 by the methylene blue uptake assay, as described in Materials and Methods. **B.** EC were seeded at increasing densities in a 96-well plate. PI-PLC (350 mU/ml) was added on day 2 and 4 and the cells were tested for uptake of methylene blue, 8 days after seeding. Mitogenic activity (0.02 O.D.) in the presence of medium alone was subtracted. Each data point represents the mean of triplicate wells and the variation between different determinations did not exceed 10% of the mean.

The experimental conditions of the experiments described above (i.e., dissociation of cells with EDTA, centrifugation, and incubation for 1 h at 37°C in serum-free medium) resulted in a certain level of spontaneous release of mitogenic activity, most likely due to some cell damage and lysis. However, as demonstrated in Figure 4, mitogenic activity released from PI-PLC treated cells and tested on 3T3 fibroblasts was up to threefold higher than that released from untreated cells. In other experiments, cells were seeded on ECM to enforce firm cell attachment and treated with PI-PLC when attached to the ECM. This procedure reduced the level of nonspecific release of growth promoting activity caused by cell damage. Results (Fig. 4) were similar to those obtained with cells in suspension, in that a two- to fivefold higher mitogenic activity toward 3T3 fibroblasts was determined in aliquots of the incubation medium following treatment of cells with PI-PLC, as compared to control untreated cultures.

In some experiments the cellular content of bFGF was estimated by measuring the mitogenic activity in aliquots of cell lysates. It was found that, depending on the cell type, treatment with PI-PLC resulted in release of 3–8% of the total cellular content of bFGF. Measurements of LDH activity revealed that under the experimental conditions applied in this study, the amount of LDH released from the cells (4–5% of the cellular content) was similar, regardless of whether the cells were treated or untreated with PI-PLC. These results indicate that a large proportion of the observed mitogenic activity was released in response to treatment with PI-PLC rather than to leakage from damaged cells.

Supernatants from PI-PLC-treated cells were also tested for induction of EC proliferation. These cells respond to FGF but not to other growth factors, such as PDGF and EGF. As demonstrated in Figure 5, medium taken from PI-PLC-treated SMC stimulated the proliferation of vascular EC to a greater extent (twofold) than medium taken from untreated SMC, indicating that the mitogenic factor released in response to treatment with PI-PLC is an endothelial cell mitogen, most likely FGF. More conclusive identification of the mitogenic factor in supernatants of PI-PLC-treated cells was achieved by treating aliquots of the incubation medium with affinity purified anti-bFGF antibodies that inhibit the growth-promoting activity of bFGF but not of aFGF (Schweigerer et al., 1987). These antibodies yielded a nearly complete inhibition (70–90%) of the growth-promoting activity released by PI-PLC from EC, SMC, bFGF transfected PF-HR9 cells and 3T3 fibroblasts (Fig. 6). There was no effect to nonimmune rabbit IgG. This result indicates that bFGF is the main growth-promoting factor found in supernatants of PI-PLC treated cells. The basal growth-promoting activity released from control cells in the absence of PI-PLC was also inhibited by the anti-bFGF antibodies (not shown).

No mitogenic activity was released into the incubation medium of bFGF-containing cells treated with chondroitinase ABC. In contrast, exposure of cells (SMC) to bacterial heparitinase resulted in release of mitogenic activity toward EC, which was 70–80% higher than that released during treatment with PI-PLC (Fig. 5). This result suggests that bFGF is found on the cell surface as a complex with HS and heparin-like molecules and can be liberated by treatment with either HS-degrading enzymes (i.e., heparitinase), or by release of the entire HS proteoglycan from its anchorage to PI-glycan in the cell membrane.

For additional identification of the endogenous mitogenic factor released by treatment of cells with PI-PLC, supernatants of 3T3 fibroblasts and of the transfected 3T3/bFGF cells, treated or untreated with PI-PLC, were partially purified by heparin-Sepharose affinity chromatography, as described in Materials and Meth-

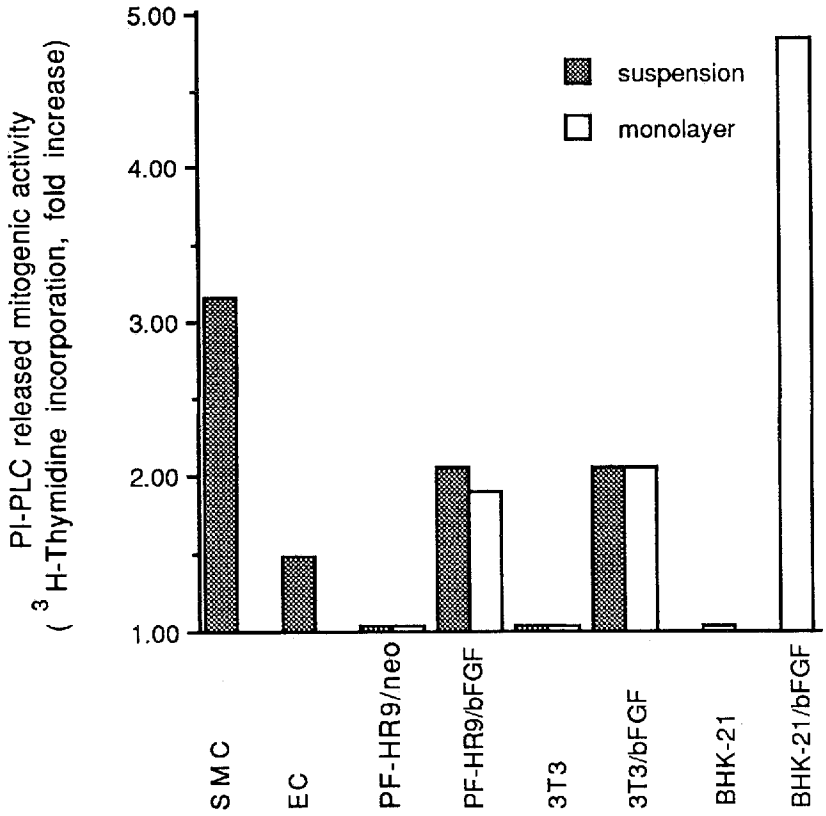


Fig. 4. Release of mitogenic activity from various cell types treated with PI-PLC. Cells were incubated (1 h, 37°C) in the absence or presence of PI-PLC (0.7 units/ml) either in suspension (6–8 × $10^6$/ml) following dissociation with 0.5 mM EDTA in PBS (shaded bars), or while adherent to ECM-coated 35 mm culture dishes (open bars). Incubation media were collected and aliquots (10–30 μl) were applied to growth-arrested 3T3 fibroblasts. DNA synthesis ($^3$H-thymidine incorporation) was measured as described in Materials and Methods. Results are expressed as fold increase of released mitogenic activity, relative to PI-PLC untreated cells. Each data point is a mean of six determinations and the variation between different determinations did not exceed 15% of the mean.

ods. The eluted fractions were examined for their ability to stimulate $H^3$-Thymidine incorporation by Balb/c fibroblasts. The unadsorbed material and the 0.6 M NaCl wash contained little, if any, mitogenic activity. Elution of the columns with 2 M NaCl, resulted in release of bioactive material in supernatants of the 3T3/bFGF cells but not of the parental 3T3 fibroblasts (results not shown), indicating release of bFGF-like material. Samples of these 2 M elution fractions and of nonconcentrated supernatants from BHK/bFGF cells were tested in a radioreceptor assay for inhibition of $^{125}$I/bFGF binding to BHK-21 cell membranes. Inhibition of $^{125}$I-bFGF binding was observed in the presence of supernatants from PI-PLC-treated bFGF-transfected cell lines (3T3/bFGF, BHK/bFGF), but was very low in supernatants of the same cells that were not treated with PI-PLC (Fig. 7). Basic FGF could not be detected in supernatants of PI-PLC treated or untreated 3T3 fibroblasts (Fig. 7). These results confirm that the endogenous bioactive material released by PI-PLC from the bFGF-transfected cells is in fact bFGF. As determined by the radioreceptor assay, the concentrations of bFGF released from PI-PLC-treated 3T3/bFGF and BHK/bFGF cells were 4.5 ng/ml and 11.5 ng/ml, respectively, as compared to 1.5 ng/ml and 0.5 ng/ml in untreated cells. Based on these determinations, we estimated that up to 12% of the total bFGF content of BHK/bFGF cells was released by treatment with PI-PLC.

## DISCUSSION

The data presented in this study indicate that PI-PLC induces release of mitogenic activity from cells that express bFGF, but not from cells that contain little or no bFGF. It is therefore conceivable that the mitogenic activity in supernatants of PI-PLC treated cells is due to bFGF and not to PI-PLC induced release of biologically active mediators, such as 1,2-diacylglycerol and phosphatidic acid. More conclusive evidence for release of bFGF was provided by almost complete inhibition of the PI-PLC released growth-promoting activity in the presence of neutralizing, affinity purified anti-bFGF antibodies. Supernatants of PI-PLC treated cells also efficiently competed with bFGF when tested in a radioreceptor assay. We have demonstrated that exposure to PI-PLC resulted in little or no cellular damage as indicated by very low or nondetectable LDH activity above the amount released from control cells. Cleavage of the PI-glycan linkage leaves the PI moiety anchored intact in the inner leaflet of the membrane bilayer. Treatment with PI-PLC is therefore not expected to cause leakage of cellular proteins, or to impair cell via-

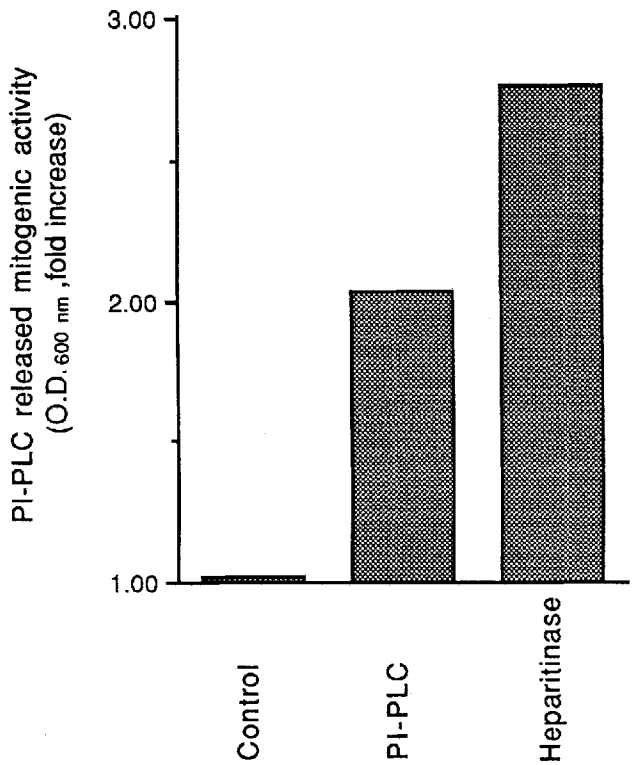


Fig. 5. Release of EC growth-promoting activity from vascular smooth muscle cells. Vascular SMC were dissociated with 0.5 mM EDTA in PBS, suspended ($3 \times 10^6$ cells/0.5 ml) in DMEM/0.05% BSA and incubated (1 h, 37°C) in the absence or presence of either PI-PLC (0.7 units/ml) or bacterial heparitinase (0.1 U/ml). The incubation media were collected and aliquots (10–30 μl) applied to vascular EC ($2 \times 10^2$ cells per well of a 96-well plate) for stimulation of cell proliferation, measured by the methylene blue uptake assay, as described in Materials and Methods. Results are expressed as fold increase in released mitogenic activity, relative to untreated control cells (0.072 $O.D._{600}$). Each data point is a mean of six determinations and the variation between different determinations did not exceed 15% of the mean.

bility, as also reported in other studies (Knudsen et al., 1989).

Our results indicate that 3–12% (depending on the cell type and assay system) of the total cellular content of bFGF is associated with glycosyl-PI anchored cell surface HSPG. The higher activity released by heparitinase as compared to PI-PLC indicates that a portion of the cell surface bFGF is associated with HSPG that is not anchored by the glycosyl-PI anchor. In fact, a membrane embedded HSPG that binds bFGF has recently been cloned and characterized (Kiefer et al., 1990). The presence of cell surface HSPG anchored through glycosyl-PI and accessible to release by PI-PLC has been demonstrated in various cell types (Ishihara et al., 1987; Low and Saltiel, 1988; Carey and Evans, 1989; Yanagishita and McQuillan, 1989; David et al., 1990). Apart from bFGF, PI-PLC releases HS-bound lipoprotein lipase from cultured heart cells (Chajek-Shaul et al., 1989). PI-PLC failed to release bFGF from the subendothelial ECM, indicating, as expected, that anchorage through glycosyl-PI is unique to the cell surface. In contrast, cleavage of HS and heparin-like molecules by heparitinase and heparinase resulted in release of bFGF from both the cell surface and ECM. Both the endogenous cell membrane associated bFGF and exogenously added $^{125}$I-bFGF were accessible to release from the cell surface by PI-PLC and heparitinase, and from ECM by heparitinase, and the released factor was biologically active.

Both HS-degrading enzymes and proteases release endogenous bFGF from ECM (Bashkin et al., 1989) as well as exogenously added bFGF from cell surface and ECM (Saksela and Rifkin, 1990). In the present study, cell-bound $^{125}$I-bFGF was released by PI-PLC, heparitinase, and heparinase, but a significant mitogenic effect was not exerted by supernate fractions of heparinase treated cells. This may be due to a difference in the size of the released bFGF-bound HS fragments. In fact, as demonstrated in Figure 2, more than 90% of the sulfate-labeled material released by heparinase was too small for precipitation with CPC, as compared to almost 100% and 55% precipitable material released by PI-PLC and heparitinase, respectively. In support of this explanation is a recent study showing that fibroblasts and muscle cells treated with heparinase lose their bFGF binding capacity and responsiveness. This effect was reversed by the addition of heparin (Rapraeger et al.,1991). Likewise, pretreatment of heparin with heparinase completely abolished its capacity to restore high affinity bFGF receptor binding to HS-deficient CHO mutant cells (Yayon et al., 1991). Our preliminary results indicate that a heparin derived oligosaccharide containing at least eight sugar units is needed for restoration of bFGF receptor binding in HS deficient mutant cells.

Interaction of bFGF-HS complexes with high affinity bFGF receptors on the cell surface has been shown to elicit cell proliferation and expression of PA activity in EC (Saksela et al., 1988; Flaumenhaft et al., 1989; Saksela and Rifkin, 1990). Our results indicate that bFGF associated with the cell surface may act in an autocrine manner, as evidenced by induction of cell proliferation upon addition of PI-PLC to sparsely seeded EC. There was no mitogenic effect on 3T3 fibroblasts containing little or no endogenous bFGF. We propose that bFGF bound to low affinity, presumably HS binding sites may not be active unless it is properly presented to high affinity bFGF receptors. This may be brought about by an enzyme such as PI-PLC. These high affinity receptors may, in contrast, not be accessible for bFGF binding and signal transduction in absence of the low affinity HS binding sites, as demonstrated in a recent study performed with HS deficient CHO cells (Yayon et al., 1991). Whereas it is likely that bFGF binds to a large variety of HS and heparin-like molecules on the cell surface (Nader et al., 1987), only a specific species of molecules may be involved in proper presentation of the bound bFGF to its high affinity receptors. This may be fulfilled by glycosyl-PI anchored HSPG and facilitated by exposure to PI-PLC. PI-PLC has been purified from rat liver plasma membranes (Fox et al., 1987) and its activity has been identified in the adrenal chromaffin granule (Fouchier et al., 1988). Anchor-specific phospholipase D's have been identified and purified from placenta (Malik and Low, 1986), from human plasma, bovine brain, and human mast cells (Low and Prasad,

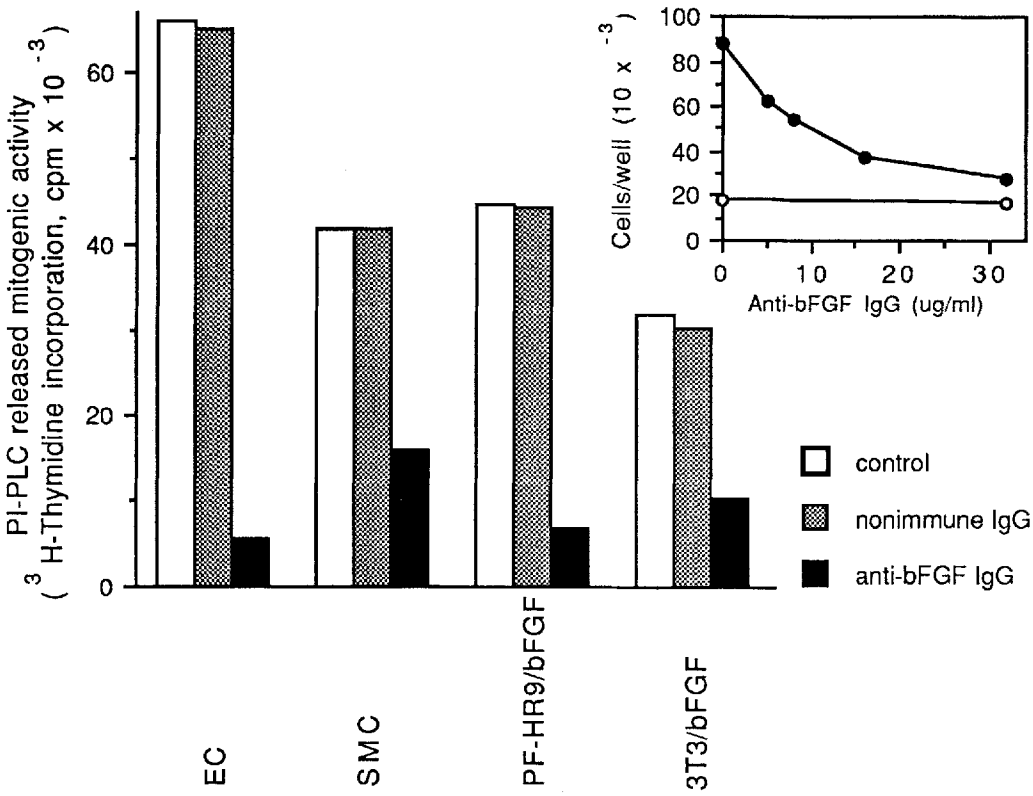


Fig. 6. Effect of anti-bFGF antibodies on mitogenic activity released from PI-PLC treated cells. Cells in suspension (EC, SMC, PF-HR9/bFGF, or 3T3/bFGF) ($3 \times 10^6$ cells/0.5 ml) were treated (1 h, 37°C) with PI-PLC (0.7 U/ml), centrifuged and aliquots of the supernatants applied to growth-arrested 3T3 fibroblasts. Incorporation of $^3$H-thymidine into DNA was measured in the absence or presence of neutralizing rabbit anti-bFGF IgG (75 μg/ml), or nonimmune rabbit IgG, as described in Materials and Methods. Recombinant bFGF (0.1 ng) yielded under the same conditions 83,000 cpm. This activity was inhibited by 85% in the presence of the anti-bFGF antibodies. Each data point is a mean of three determinations and the variation between the different values did not exceed 10% of the mean. Insert. Titration curve of anti-bFGF antibodies inhibition of bFGF induced proliferation of BHK-21 cells. BHK-21 cells were seeded on gelatin-coated, 24-well plates (15,000 cells/0.5 ml/well) in serum-free DF medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 10 μM ethanolamine. Cells were allowed to attach for 4 h. Increasing amounts of rabbit anti-bFGF IgG were then added to the wells, in the absence (○) or presence (●) of 1 ng/ml bFGF. The antibodies were added to the cells on day 3, with or without bFGF, and the cells were counted on day 4.

1988; Davitz et al., 1989; Hoener et al., 1990; Gleichauf et al., 1990), and may thus participate in release of membrane-bound bFGF.

Basic FGF is mostly an intracellular protein, consistent with its lack of a classical signal peptide. Since many cells that synthesize bFGF are capable of responding to bFGF through interaction with cell surface receptors (Folkman and Klagsbrun, 1987; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989), it is conceivable that cellular bFGF may reach an extracytoplasmic compartment where it can bind to its high affinity receptor and act as an autocrine growth and differentiation promoting factor. This possibility is supported by the findings that signal-peptide-bFGF (pIgbFGF) transfected cells no longer bind exogenous bFGF (Yayon and Klagsbrun, 1990) and that neutralizing anti-bFGF antibodies inhibit bFGF dependent functions, such as cell migration, cell proliferation, and repair of radiation induced damage, in the absence of exogenously added bFGF (Sakaguchi et al., 1988; Sato and Rifkin, 1988; Haimovitz-Friedman et al., 1991; Rifkin, 1991). Basic FGF, like other secretory proteins that lack a hydrophobic signal sequence, may follow a novel route(s) of membrane insertion and secretion (Muesch et al., 1990); however, no defined mechanism for release of intracellular bFGF has been identified. Other growth-promoting factors (i.e., IL-1, TGF-α, PDGF-B chain) are known to be associated with the cell surface and to induce both autocrine and paracrine responses (Kurt-Jones et al., 1985; Wong et al., 1989; Williams, 1988). Glycosyl-PI-anchored, HS-bound bFGF may function in a somewhat similar manner, as indicated by its ability to elicit EC proliferation once released by PI-PLC. It may also contribute, although to a lower extent, to the basal rate of EC proliferation observed in the absence of exogenously added PI-PLC or bFGF. HSPG released by PI-PLC can be internalized via a cell surface receptor that recognizes myo-inositol phosphate. The internalized HSPG is processed by a nonlysosomal pathway and a portion of the HS side chains may then be transported into the nucleus (Ishihara et al., 1986, 1987). PI-PLC may thus function also in translocation of bFGF from the cell surface into the nucleus (Bouche et al., 1987; Baldin et al., 1990; Tessler and Neufeld, 1990), where it may exert direct effects on genomic DNA.

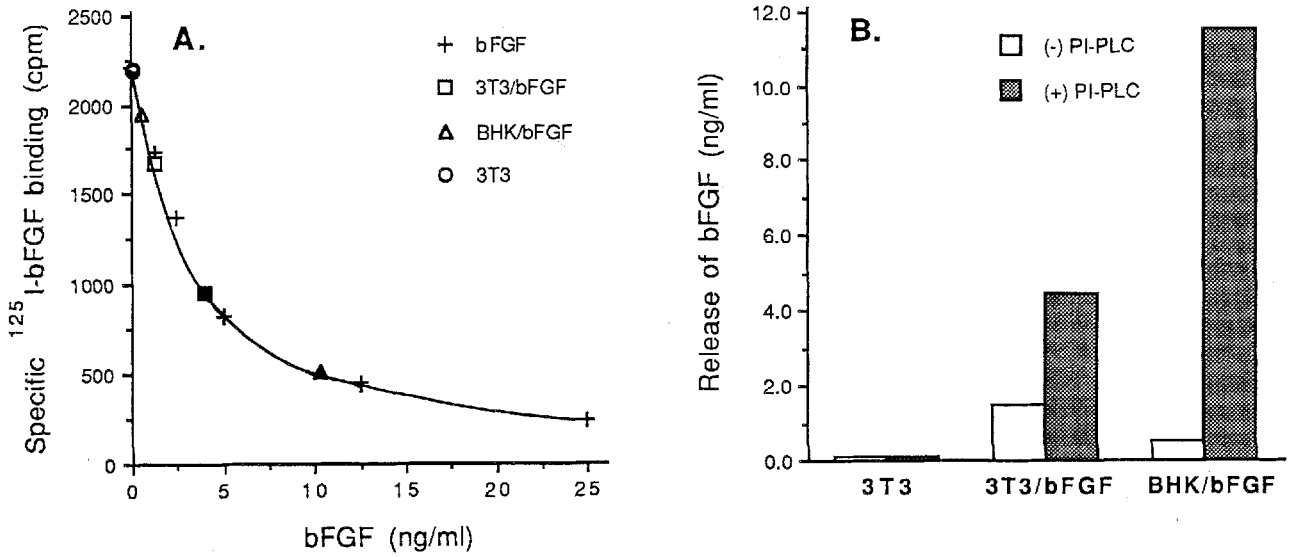


Fig. 7. **A.** Effect of supernatants of PI-PLC treated cells on specific $^{125}$I-bFGF binding to BHK-21 cell membranes. $^{125}$I-bFGF (80 pg) and BHK-21 cell membranes (5 μg protein) were incubated (1 h, 24°C) with aliquots of supernatants from cells (○ 3T3; □ 3T3/bFGF; △ BHK/bFGF) treated (closed symbols) or untreated (open symbols) with PI-PLC (0.7 U/ml, 1 h, 37°C). Supernatants were either concentrated (3T3; 3T3/bFGF) or not concentrated (BHK/bFGF) on heparin-Sepharose columns, as described in Materials and Methods. $^{125}$I-bFGF and BHK cell membranes were also incubated with increasing concentrations of unlabeled pituitary-derived bFGF (+). $^{125}$I-bFGF binding was then determined as described in Materials and Methods. In the presence of $^{125}$I-bFGF alone, $2.5 \times 10^3$ cpm were bound to the membranes. Nonspecific binding in the presence of 80 ng/tube pituitary bFGF was 310 cpm and this value was subtracted from the experimental values. Values are the means of triplicate determinations and the variation between replicates was <10%. **B.** Release of bFGF into supernatants of PI-PLC treated cells as determined by radioreceptor assay. Aliquots from supernatants of cells, treated (shaded bars) or untreated (open bars) with PI-PLC were applied to the radioreceptor assay, as described above. Results are expressed in ng/ml present in the applied sample, as determined by the radioreceptor assay and calculated from the titration curve (see A).

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## NOTE ADDED IN PROOF

A paper (Brunner et al., 1991) published after the submission of the present manuscript, reports that exogenously added bFGF is released by PI-PLC from human bone marrow cultures as a biologically active complex with phosphatidylinositol-anchored heparan sulfate proteoglycan.

*Abbreviations*

| | |
|---|---|
| bFGF | basic fibroblast growth factor |
| DMEM | Dulbecco's modified Eagle's medium |
| ECM | extracellular matrix |
| HSPG | heparan sulfate proteoglycans |
| PI-PLC | phosphatidylinositol-specific phospholipase C |

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