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Modulation of Mitogenic Activity and Cellular Binding of Basic Fibroblast Growth Factor by Basic Proteins

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Polycationic molecules were studied either for their ability to displace the binding of basic fibroblast growth factor (bFGF) to high- and low-affinity membrane interaction sites and/or to modulate bFGF-induced proliferation of fibroblasts. Heparin-binding polypeptides, such as polylysine, protamine, histones, and thrombin-displaced [^{125}I]bFGF bound to bovine brain membrane receptors. The most displacing polypeptides were those with the strongest affinity to heparin. Two of these polypeptides, protamine and polylysine, inhibited (at 5 μM) by more than 90% the mitogenic effect induced by bFGF on Chinese hamster lung fibroblast cells (CCL39). At the same dose, no effect was observed with basic proteins that do not bind to heparin, such as cytochrome C and lysozyme. An interesting observation was that protamine at 1 μM potentiated by 1.5-fold the mitogenic activity of bFGF, while it acted as an inhibitor at higher concentration.

Key words: protamine, heparin-binding proteins, cell proliferation, radioreceptor assay, growth inhibition

Protamine has been reported to inhibit mitogenic activity of several growth factors, such as acidic and basic fibroblast growth factors (a- and bFGF) [1,2] and platelet-derived growth factor (PDGF) [3], as well as to inhibit angiogenesis in vivo [4]. As protamine and these growth factors both have a strong affinity for heparin, they could interact with target cells with a heparin-like structure. Indeed, proteoglycans play a role in many different cellular activities such as cell adhesion and cellular growth, as well as regulation of receptor function [5].

A wide variety of cells are highly sensitive to the proliferation effect induced in vitro by factors belonging to the fibroblast growth factor family (a- and bFGF), eye-derived growth factor (EDGF), brain-derived growth factor (BDGF), and endothelial cell growth factor (ECGF) [6–10]. Moreover, these mitogenic polypeptides appear to play an important role in angiogenesis [11,12] and wound healing in vivo [13,14].

Biological activity of FGFs is mediated through specific high-affinity membrane receptors that have been characterized in vitro on cells grown in tissue culture, such as

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fibroblasts [15], muscle myoblasts [16], endothelial and epithelial cells [17,18], as well as on plasma membrane preparation from bovine brain [19].

Several molecules have been reported to affect the biological activity of a-and/a bFGF by either inducing a potentiation such as heparin [10,20,21] or an inhibition such as gamma-interferon (gamma-IFN) [22], beta-transforming growth factor (beta-TGF) [23], interleukin-1 (IL-1) [24], tumor necrosis factor (TNF) [25], suramine, and protamine [2,21,26]. Protamine inhibition is of particular interest, since protamine has been reported to be an inhibitor of angiogenesis in vivo [4].

In an attempt to study the specificity of this inhibitory effect, we tested the modulation of bFGF activity induced by various heparin-binding and other basic proteins on the proliferation of fibroblasts (CCL39) and the interference of protamine on the binding of bFGF to high- and low-affinity membrane interaction sites.

MATERIALS AND METHODS

Materials

Chinese hamster lung fibroblasts (CCL39) were obtained from the American Type Culture Collection (ATCC). Protamine (P4005), polylysine (P4408), cytochrome C (C7752), lysozyme (L7001), thrombin (T6759), and DNA type III (D1626) were obtained from Sigma Corporation. Protamine was further purified by reverse-phase high-performance liquid chromatography (HPLC) using a C_4 Vydac column eluted at 17% acetonitrile-O.1% TFA. Chicken erythrocyte histones were prepared from purified nuclei by salt extraction, ultracentrifugation, and chromatography [27]. Histones were free from DNA, and an electrophoretic profile indicated that H2a, H2b, H3, and H4 were present but not H1. In order to simplify the presentation of our results, we used 20,000 daltons as the average molecular weight for the whole histone preparation. Heparinase (50,000 U/ml) was a generous gift from J. Choay, Institut Choay (France). Bovine brain bFGF was purified as previously described using heparin-Sepharose chromatography [28]. Purity of bFGF was checked on overloaded silver-stained SDS-PAGE, on reverse-phase HPLC, and with biological assay.

Cell Culture and Mitogen Assay

CCL39 cells were grown in 24-well plates (Falcon, Oxnard, CA) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (Boehringer, Mannheim, FRG) and 1 mM glutamine, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (GIBCO) at 37°C in a humidified atmosphere containing 5% CO_2.

Cells were plated at 2×10^4 cells per cm^2 in 0.5 ml of culture medium. When the cells reached confluency, they were arrested for 24 h in serum-free medium and then incubated with 0.5 ml serum-free DMEM containing increasing concentrations of growth factors and/or reagents. After incubation at 37°C for 20 h, the cells were pulsed with 1 μCi methyl [^{3}H] thymidine (Oris, France) per well for 4 h, and the cell monolayers were treated with 0.5 ml 10% trichloracetic acid for 15 min at 4°C, washed with tap water, and dissolved in 0.5 ml of 0.1 M NaOH for counting in a scintillation counter. Assays were performed in triplicate.

The stimulation unit was defined as the dose of bFGF that induced 50% of the maximal methyl [^{3}H] thymidine incorporation (ED_{50}) and obtained for CCL39 cells

with 70 pM of bFGF; for other cells, such as endothelial cells, ED_{50} could be reached with 6 pM.

Radioiodination of bFGF

Briefly, 3 μg of bFGF were diluted in 0.1 M phosphate buffer (pH 7.4) containing 1% of polyethylene glycol (PEG) 1,000 and incubated with 1 mCi of Na[^{125}I] (Oris, France) and 20 μM of chloramide T at room temperature. After addition of 100 mM of N-acetyl-L-tyrosine, iodinated bFGF was purified by heparin-Sepharose chromatography. Biological activity was tested after iodination, and [^{125}I]bFGF was stored at −80°C. The specific activity of labeled bovine brain bFGF obtained was usually 80,000 cpm/ng.

Bovine Brain Membrane Preparation

Bovine brain membranes were prepared by a modification of the procedure described by Neville [29]. A batch of two freshly slaughtered bovine brains was dissected free of blood clots and meninges at 4°C and cut into small pieces. Homogenization was performed with 2 volumes of 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4) containing 5 mM EDTA, 1 μg/ml leupeptine, 1 μg/ml pepstatin, 5 UK/ml aprotinin, and 0.1 mM phenylmethane sulfonyl fluoride (PMSF) (buffer A) using a blender.

All procedures were performed at 4°C. The homogenized tissue was centrifuged (14,300*g* for 60 min), and the pellet was suspended with 2 volumes of buffer A using a Teflon-glass homogenizer. After centrifugation at 1,000*g* for 30 min, 2 volumes of buffer A containing 0.3 M saccharose were added to the pellet and centrifuged at 3,000*g* for 15 min. The resulting supernatant was centrifuged at 40,000*g* for 30 min. This pellet was resuspended with 10 volumes of buffer B (buffer A containing 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1 M NaCl, and no EDTA) and washed by three more centrifugations (40,000*g* for 30 min). The final resulting pellets, containing crude membrane preparation, were taken up in buffer B to give a final protein concentration of about 10 mg/ml. Membrane preparations were stored frozen at −80°C until use.

Radioreceptor Assays

Radioreceptor assays were performed as described [19]. Briefly, bovine brain membrane preparations containing 40 μg of proteins were incubated in a total volume of 0.5 ml of buffer B with 23 nM of [^{125}I]bFGF supplemented or not with competitors as indicated and incubated for 1 h at 4°C. These suspensions were centrifuged at 5,000*g* for 5 min. The clear supernatant was aspirated, and the radioactivity of the pellets was counted.

Nonspecific binding reached 20–30% of the specific binding. All assays were carried out in duplicate. The specificity of the binding of bFGF to its receptor using the conditions described was demonstrated by crosslinking experiments [19].

Heparin-Sepharose Chromatography

Proteins were loaded onto a heparin-Sepharose column in PBS buffer (6.5 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 3 mM KCl, 0.15 M NaCl, pH 7.4). After washing, retained proteins were eluted by a gradient of 0.15–3 M NaCl. Cytochrome C and lysozyme were eluted at 0.15 M, thrombin 0.30 M, histones 1.1 M, protamine 1.4 M, and polylysine 1.6 M NaCl.

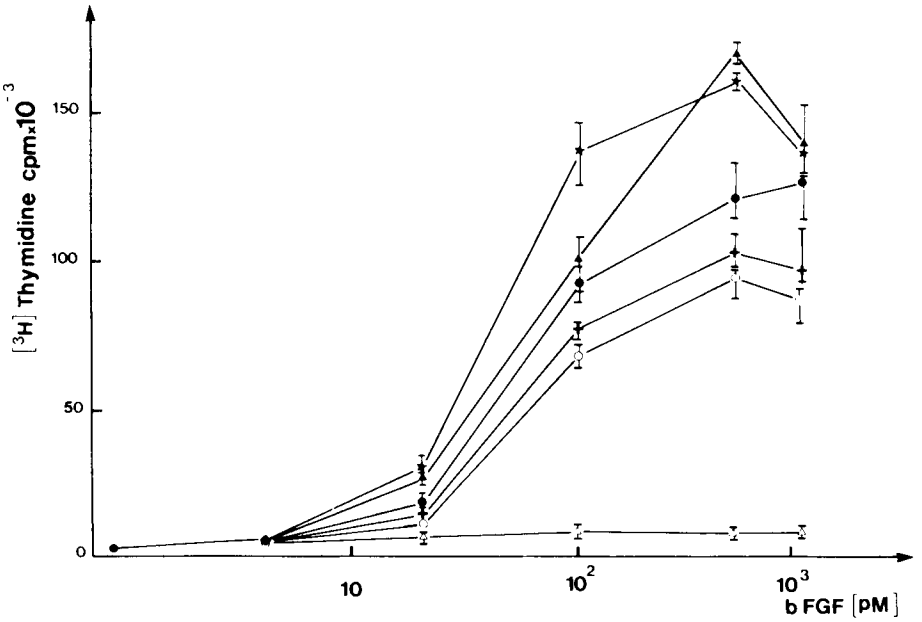

Fig. 1. Effect on biological activity of bFGF. Methyl [^{3}H]thymidine incorporation induced by bFGF at various doses in CC139 cells in the absence (+) and presence of protamine 0.5 μM (●), 1 μM (*), 1.5 μM (▲), 2.5 μM (O), and 5 μM (△).

RESULTS

Dose Effect of Protamine on Mitogenic Activity of bFGF

As previously described for in vitro proliferation of endothelial and 3T3 cells [21], the stimulation of CCL39 cells by bFGF was modulated by the addition of protamine to the culture medium (Fig. 1).

At low doses of protamine (0.5, 1, and 1.5 μM), a potentiation effect on the incorporation of [^{3}H]thymidine was observed for different concentrations of bFGF. Hence, in the presence of 1–1.5 μM of protamine, the maximum [^{3}H]thymidine incorporation obtained with bFGF alone was increased ranging from 1.5- to 2-fold. At higher doses, an inhibitory effect was observed. Almost complete inhibition of the bFGF stimulation was already obtained with 5 μM.

In order to study these effects further, bFGF was used at a dose inducing half of the maximum [^{3}H]thymidine incorporation (ED_{50} = 70 pM) with protamine. As shown in Figure 2, maximum potentiation was also obtained with 1 μM of protamine. For this concentration, a twofold increase of [^{3}H]thymidine incorporation was observed, and the value reached corresponded to the maximum incorporation induced by bFGF alone at higher doses (=10 ED_{50}). Complete inhibition was also obtained at 5 μM.

Dose Effect of Other Basic Proteins on Mitogenic Activity of bFGF

In order to analyse the specificity of the opposite effects induced by protamine, other basic proteins were tested. As shown in Figure 2, cytochrome C, histones, and

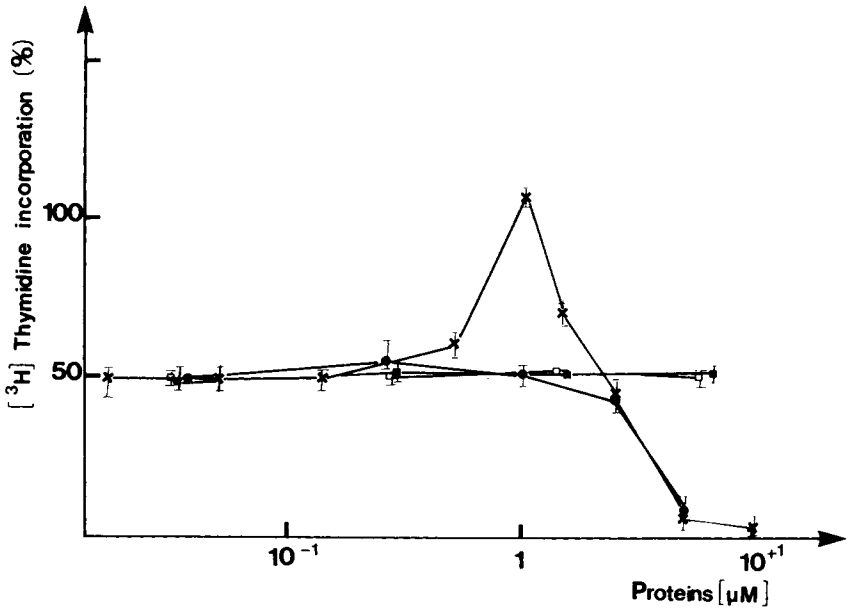

Fig. 2. Effect of various basic proteins of bFGF-stimulated cells. Methyl [^{3}H]thymidine incorporation stimulated by bFGF at a dose corresponding to ED_{50} (70 pM) in the presence of various concentrations of protamine (×), polylysine (●), cytochrome C (□), lysozyme (□), and histones (■).

lysozyme used in the same range of molarity as protamine did not affect the mitogenic activity of bFGF. In contrast, a synthetic polypeptide (polylysine) only acted as an inhibitor.

Reversibility of the Inhibitory Effect of Protamine by DNA

Protamine is a member of a family of proteins that binds strongly to DNA, giving soluble complexes [30]. When quiescent CCL39 cells were incubated with bFGF at doses corresponding to ED_{50} with 5 μM of protamine, increasing amounts of DNA added simultaneously to the culture medium reversed the inhibitory effect of protamine. This reversal was over 90% in the presence of 1 mg/ml of DNA, as shown in Figure 3. However, no effect was observed in the presence of DNA when CCL39 cells were stimulated by bFGF in the absence of protamine (results not shown).

The reversion of the inhibitory action of protamine by DNA suggested that protamine used at a dose of 5 μM was not cytotoxic for CCL39 cells, at least in these conditions.

Interference With [^{125}I]bFGF Receptor Binding by Protamine and Other Basic Proteins

The effect of protamine and other basic proteins on the binding of [^{125}I]bFGF to bovine brain membrane receptors was investigated. A constant amount of [^{125}I]bFGF 23 nM was incubated with bovine brain membranes (40 μg) in the presence of different basic proteins ranging from 10^{-4} to 10 μM. As shown in Figure 4, curve a, over 90% of displacement by unlabeled bFGF occurred at concentrations ranging from

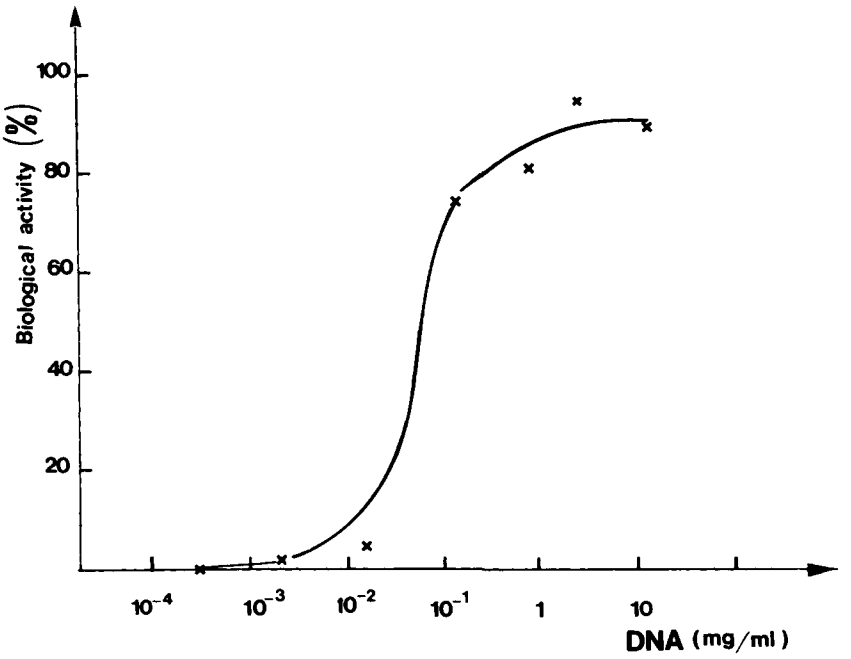

Fig. 3. Reversibility of protamine inhibition by DNA of bFGF-stimulated cells. Reversibility of the inhibitory effect of protamine at 5 μM on CC139 cells stimulated by bFGF at a dose corresponding to ED_{50} (70 pM) in the presence of various concentrations of DNA.

10^{-4} to 10^{-1} μM, suggesting that binding involved more than one type of site. Bound [^{125}I]bFGF was not displaced by cytochrome C or lysozyme used at concentrations up to 10 μM (Fig. 4).

However, protamine and polylysine displaced [^{125}I]bFGF with a half maximum displacement occurring respectively for 0.5 and 0.1 μM, while histones or thrombin induced a similar displacement at 1 μM.

To study if protamine or histones could interfere with the receptors and/or directly with bFGF, membrane preparations were preincubated with increasing concentrations of protamine or histones for 1 hr at 4°C and washed several times before adding [^{125}I]bFGF. Similar results were obtained whether preincubation was done or not, and a 90% displacement was achieved by adding 1 μM of protamine (not shown). These results indicated that protamine or histones, once they interacted with the membrane, could prevent the binding of [^{125}I]bFGF to its membrane receptor. They also suggested that protamine or histones did not interact with bFGF alone.

Heparinase Treatment of Membranes

In order to study the type of receptors interacting with [^{125}I]bFGF, a heparinase treatment was performed. [^{125}I]bFGF binding to receptors was measured after treatment with increasing concentrations of heparinase, as indicated in Figure 5 (insert). At 5 heparinase units, more than 75% of bound [^{125}I]bFGF was released from the membrane. For more reproductible results, the dose of 25 units was then chosen for

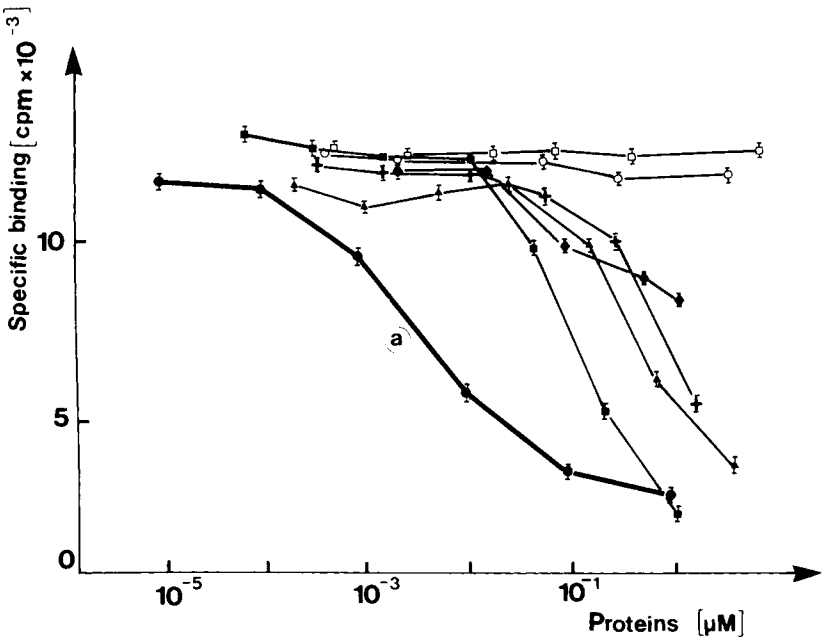

Fig. 4. Displacement of bFGF on bovine brain membranes by basic proteins. Competition binding curves of [^{125}I]bFGF (23 nM, 2,000 cpm/pmole) on bovine brain membranes, in the presence of increasing concentrations of bFGF (curve a) and basic proteins: (▲), protamine; (+), histones; (■), polylysine; (♦) thrombin; (○), cytochrome C; (□), lysozyme.

further experiments. Addition of increasing amounts of protamine displaced the remaining [^{125}I]bFGF still bound specifically to these membranes.

DISCUSSION

The data presented in this report suggest that the ability of proteins to bind to heparin could also reflect an ability to inhibit the mitogenic activity induced by bFGF. Protamine and polylysine strongly bound to heparin (elution from heparin Sepharose column at 1.4 M and 1.6 M, NaCl respectively) and were the most efficient inhibitors of [^{3}H]thymidine incorporation at 10 μM. However, histones that also bound to heparin (elution at 1.1 M NaCl) were not able to inhibit cell proliferation in the concentration range used (up to 100 μM). However, purified histone H2a at 75 μM has been described to completely inhibit bFGF-induced cell proliferation [1]. It is interesting to note that cytochrome C and lysozyme, which are basic proteins (with no affinity to heparin), did not inhibit the biological activity of bFGF, suggesting that the inhibitory effect may not be linked to ionic charges but to affinity to heparin or heparin-like structures.

Besides the presence of high-affinity specific receptors for bFGF, Scatchard analyses of binding data of radiolabeled bFGF on target cells revealed the presence of lower-affinity sites [31,32] whose interactions could be partially destroyed by hepa-

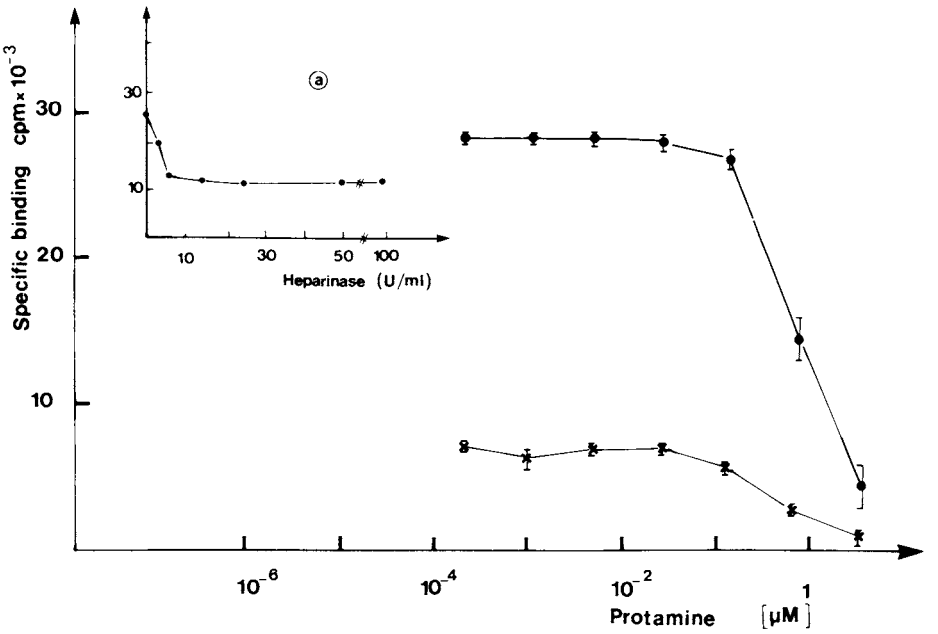

Fig. 5. Effect of heparinase treatment of bFGF binding on membranes in presence of proteins. Competition binding curves of [^{125}I]bFGF (0.023 μmole) on bovine brain membranes (●) or heparinase-treated bovine brain membranes 25 U (×) in the presence of various doses of protamine. Curve a: concentration effect of heparinase treatment on bovine brain membranes.

rinase treatment. This latter property suggests that this class of sites could be related to a heparin-like molecule whose biological significance remains unclear. Radioreceptor assays indicated that all the heparin-binding proteins tested could displace [^{125}I]bFGF bound to membrane receptors in a dose-dependent manner. The most efficient displacing polypeptides being those with the highest affinity to heparin, the results obtained suggest that heparin-binding proteins would compete for these low-affinity sites with bFGF. Indeed, heparinase treatment of bovine brain membranes reduced by 75% the total binding of bFGF.

In bovine brain membranes prepared as described here, our estimation of the ratio between the number of high- and low-affinity receptors is 1/500 [19]. One would therefore expect that heparinase treatment could reduce to a nondetectable level the total binding of bFGF if degradation by heparinase completely abolished low-affinity binding. The residual 25% of bFGF bound after heparinase treatments could simply indicate that heparinase does not degrade heparane sulfate molecules completely or that this degradation reveals other FGF binding sites. Since the residual bound bFGF could still be displaced by protamine, the first possibility seems more likely.

The most intriguing result is the potentiation of the bFGF-induced mitogenic activity in the presence of low concentrations of protamine (1 μM), as shown in Figures 1 and 2. When bFGF was used at ED_{50}, i.e., 70 pM for CCL39, 1 μM of protamine induced a 100% [^{3}H]thymidine incorporation (Fig. 2), as obtained with tenfold more bFGF alone. As already mentioned, this result cannot be explained. The potentiation of the mitogenic activity of aFGF by heparin has been previously described

[10]. It was explained by a direct interaction of aFGF with heparin [21], stabilizing aFGF and increasing its affinity to its cellular receptors.

However, the potentiation observed here with protamine and bFGF is unlikely to be due to a direct interaction of bFGF with protamine (Interference with [^{125}I]bFGF Receptor Binding by Protamine and Other Basic Proteins, in the Results section). Protamine has been shown to potentiate the biological activity of epidermal growth factor (EGF) [1]. Thus protamine might act by itself at cellular sites that are not associated with EGF or FGF receptors. When higher concentrations were used, this positive effect might be masked by its interactions with heparin-like molecules affecting directly or not FGF binding to specific cell surface receptors.

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