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Basic fibroblast growth factor requires a long-lasting activation of protein kinase C to induce cell proliferation in transformed fetal bovine aortic endothelial cells

Marco Presta, Laura Tiberio, Marco Rusnati, Patrizia Dell'Era, and Giovanni Ragnotti
Unit of General Pathology and Immunology
Department of Biomedical Sciences
and Biotechnologies
School of Medicine
University of Brescia
25123 Brescia, Italy

Basic fibroblast growth factor (bFGF) induces a protein kinase C (PKC)-dependent mitogenic response in transformed fetal bovine aortic endothelial GM 7373 cells. A long-lasting interaction of bFGF with the cell is required to induce cell proliferation. bFGF-treated cells are in fact committed to proliferate only after they have entered the phase S of the cell cycle, 12–14 h after the beginning of bFGF treatment. Before that time, the mitogenic response to bFGF is abolished by 1) removal of extracellular bFGF by suramin, 2) addition of neutralizing anti-bFGF antibodies to the culture medium, 3) inhibition of PKC activity by the protein kinase inhibitor H-7, and 4) down-regulation of PKC by cotreatment with phorbol ester. Thus the requirement for a prolonged interaction of bFGF with the cell reflects the requirement for a prolonged activation of PKC. Similar conclusions can be drawn for the PKC activators 12-O-tetradecanoyl phorbol 13-acetate and 1,2-dioctanoyl-sn-glycerol. The two molecules require 16 and 6 h, respectively, of activation of PKC to induce 50% of maximal cell proliferation. The requirement for a long-lasting activation of PKC appears to be a mechanism for the control of cell proliferation capable of discriminating among transient nonmitogenic stimuli and long-lasting mitogenic stimuli.

Introduction

Interaction of basic fibroblast growth factor (bFGF)[1] with its plasma membrane receptor induces a number of early intracellular events. They include activation of the tyrosine kinase activity of the bFGF receptor (Ruta *et al.*, 1989; Mansukhani *et al.*, 1990), increase of cytosolic pH (Magnaldo *et al.*, 1986; Moenner *et al.*, 1987; Nanberg *et al.*, 1990), calcium mobilization (Magnaldo *et al.*, 1986; Tsuda *et al.*, 1986; Pandiella *et al.*, 1989a), transcription of early genes (Tsuda *et al.*, 1986; Stumpo and Blackshear, 1986; Buchou and Mester, 1990), and activation of protein kinase C (PKC) (Tsuda *et al.*, 1986; Presta *et al.*, 1989a,b; Nanberg *et al.*, 1990). The complexity of the transmembrane signaling process is not unique to bFGF and also is shared by other growth factors, like epidermal growth factor (EGF) (Pandiella *et al.*, 1989b). For bFGF and EGF, these intracellular events occur very rapidly (within 60–90 min). Nevertheless, EGF must remain in the extracellular environment for 7–8 h before cell replication becomes committed (Carpenter and Cohen, 1976). This suggests that not only early intracellular signals but also late events are necessary to mediate the mitogenic activity of growth factors. In agreement with this hypothesis is the capacity of different mutants of acidic FGF to induce early responses (e.g., stimulation of receptor-mediated tyrosine phosphorylation and protooncogene expression) without stimulating cell proliferation (Burgess *et al.*, 1990; Imamura *et al.*, 1990).

bFGF induces activation of PKC in cultured fetal bovine aortic endothelial (FBAE) cells (Presta *et al.*, 1989a,b). Within 1–2 min from bFGF addition, PKC translocates from the cytosol to the cell membrane and phosphorylation of several proteins occurs, including a typical Mr 80 000 substrate. Protein kinase inhibitor 1-(5-isoquinolynsulfonyl)-2-methylpiperazine (H-7) or down-regulation of PKC after its prolonged

[1] Abbreviations used: bFGF, basic fibroblast growth factor; diC8, 1,2-dioctanoyl-sn-glycerol; EGF, epidermal growth factor; FBAE, fetal bovine aortic endothelial; FCS, fetal calf serum; H-7, 1-(5-isoquinolynsulfonyl)-2-methylpiperazine; PKC, protein kinase C; TPA, 12-O-tetradecanoyl phorbol 13-acetate.

stimulation by high doses of phorbol ester completely abolishes the mitogenic response to bFGF (Presta *et al.*, 1989a). Moreover, the extent of FBAE cell proliferation after treatment with bFGF is directly related to basal levels of PKC activity (Presta *et al.*, 1989b). These data indicate that bFGF induces cell proliferation in FBAE cells via activation of PKC.

Here we demonstrate that bFGF must interact with FBAE GM 7373 cells for at least 12–14 h to induce cell proliferation. The need for this long-lasting interaction arises from the requirement for a long-lasting activation of PKC.

Results

bFGF stimulates the proliferation of GM 7373 cells in a dose-dependent manner. GM 7373 cells undergo 0.8–1.0 cell population doublings during the first 24 h of treatment with 30 ng/ml bFGF (Figure 1A). Cells divide almost synchronously when stimulated by bFGF. Cells enter the phase S of the cell cycle at 12 h, and cell replication begins 4 h thereafter (Figure 1B).

To evaluate the shortest time interval required by bFGF to elicit a mitogenic response in GM 7373 cells, cultures were treated with the growth factor. Then, at different times after exposure to bFGF, cells were incubated for 20 min with 50 μg/ml suramin before extensive washing with serum-free medium. Suramin is known to remove bFGF from high-affinity plasma membrane receptors and from heparin-like low-affinity binding sites (Coffey *et al.*, 1987; Moscatelli and Quarto, 1989). Indeed, the washing procedure adopted here effectively removes free, receptor-bound, and extracellular matrix-bound ^{125}I-bFGF from GM 7373 cell cultures (data not shown). After washing, cells were incubated in fresh medium containing 0.4% fetal calf serum (FCS). All the cultures were trypsinized 24 h after the beginning of bFGF treatment. As shown in Figure 2, an early removal of extracellular bFGF inhibits cell proliferation and 16 h of exposure to bFGF are required to induce a mitogenic response equal to ~50% of the response elicited by a 24-h incubation with the growth factor. It must be noticed that a 24-h incubation of GM 7373 cells with 50 μg/ml suramin does not cause any significant modification of the morphology and of the cell number of bFGF-untreated cultures. Moreover, suramin does not inhibit the capacity of GM 7373 cells to proliferate when stimulated by 12-O-tetra-

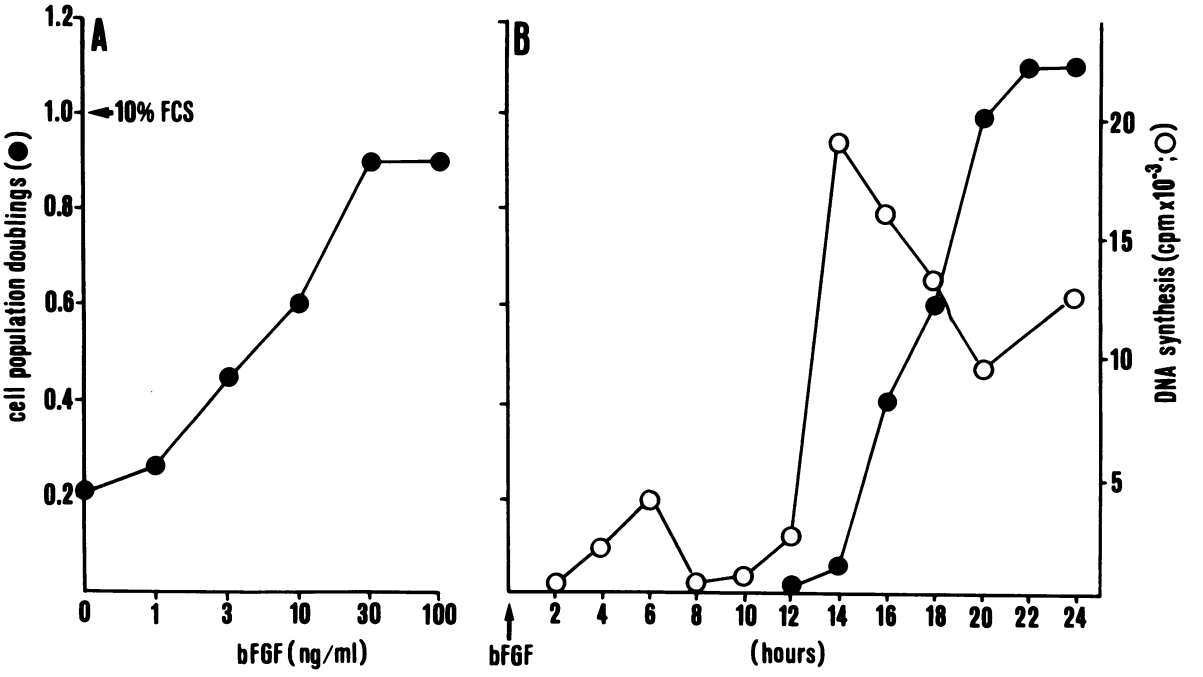

***Figure 1.* Mitogenic activity of bFGF in GM 7373 cell cultures.** (A) Cells were treated with increasing concentrations of bFGF in the presence of 0.4% FCS. After 24 h, cells were trypsinized and counted. Cultures grown in 10% FCS were used as a positive control. (B) Cells were treated with 30 ng/ml bFGF as in A. At different times cultures were pulse-labeled with ^{3}H-thymidine for 2 h (○) or trypsinized and counted (●). The values represent the difference between bFGF-treated cells and cultures treated with 0.4% FCS alone.

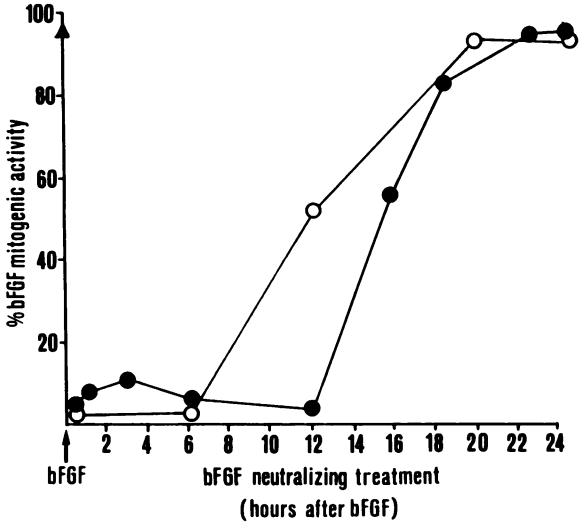

***Figure 2.* Effect of neutralizing anti-bFGF antibodies and of suramin on bFGF mitogenic activity.** GM 7373 cells were incubated with 10 ng/ml bFGF in 0.4% FCS. At different times cells were washed with 50 μg/ml suramin and maintained in fresh medium containing 0.4% FCS without bFGF (●) or were added with 5 μg/ml of neutralizing affinity-purified anti-bFGF antibodies without medium change (○). Some cultures were also cotreated with bFGF and 5 μg/ml of irrelevant rabbit IgG (▲). All cultures were trypsinized and counted 24 h after the beginning of bFGF treatment. The data are expressed as percent of the proliferation observed in cultures treated with bFGF alone.

decanoyl phorbol 13-acetate (TPA) or by 1,2-dioctanoyl-sn-glycerol (diC8) (data not shown).

Even though these findings demonstrate that the effect exerted by suramin on bFGF mitogenic activity does not depend on a cytotoxic effect of the molecule or on a direct inhibition of the PKC-dependent mitogenic pathway, they do not rule out the possibility that suramin might affect bFGF activity by also interacting intracellularly with the growth factor. On this basis, we have evaluated the extracellular effect of neutralizing affinity-purified anti-bFGF antibodies on the mitogenic activity of bFGF. Also in this case, an early neutralization of extracellular bFGF by addition of the antibodies to the culture medium fully abolishes the mitogenic activity of bFGF (Figure 2). The time course is similar, but not identical, to that observed for suramin, suggesting that part of the late action of suramin might indeed occur inside the cell. Nevertheless, the data obtained with the neutralizing antibodies indicate that bFGF must act extracellularly for 12 h to elicit 50% of the maximal mitogenic response.

The mitogenic activity exerted by bFGF in GM 7373 cells is mediated by the activation of PKC (Presta *et al.*, 1989a,b). Accordingly, the protein kinase inhibitor H-7 inhibits with the same potency the mitogenic response elicited by bFGF and by the PKC activators TPA and diC8 (Figure 3A). The ID_{50} of H-7 is equal to 6–6.5 μM, a value that is identical to the K_i of this molecule for purified PKC (Hidaka *et al.*, 1984). Also, H-7 does not affect the PKC-independent mitogenic activity exerted by FCS or EGF (Figure 3A). We are then confident that H-7 inhibits the mitogenic activity of bFGF by inhibiting PKC activity. On this basis, to evaluate whether the requirement for a long-lasting treatment with bFGF is related to the requirement for a long-lasting activation of PKC, cells were treated with bFGF and then added with 10 μM H-7 at different times. As shown in Figure 3B, H-7 fully inhibits the mitogenic activity of bFGF when added during the first 14 h of treatment. After this time, the addition of H-7 progressively looses its inhibitory effect. H-7 is completely unaffective when added during the last 4 h of treatment with bFGF. Similar results were obtained when cells were induced to proliferate by a low mitogenic dose (100 ng/ml) of the PKC activator TPA (Figure 3B). When cells were stimulated by a mitogenic dose (5 μg/ml) of the PKC activator diC8, the addition of H-7 was completely ineffective already 12 h after the beginning of diC8 treatment (Figure 3B). Thus the data indicate that a very long-lasting activation of PKC is required for inducing GM 7373 cells to proliferate when treated with bFGF or TPA. A shorter, but still prolonged, activation of the enzyme appears to be necessary when cells are stimulated by diC8.

Prolonged stimulation of PKC by high doses of phorbol ester causes the down-regulation of the enzyme, the half-life of PKC being reduced from ~24 to 2 h (Ballester and Rosen, 1985). A 24-h treatment with 3 μg/ml TPA, a dose 30 times higher than that utilized in the mitogenic assays described above, down-regulates PKC in GM 7373 cells (Presta *et al.*, 1989a). On this basis, we hypothesized that in bFGF-treated cells, where PKC is already activated by the growth factor (Presta *et al.*, 1989a), a lower dose of TPA should have been sufficient to cause down-regulation of the enzyme. To assess this hypothesis, we incubated GM 7373 cells with 30 ng/ml bFGF in the presence of low mitogenic doses of TPA, ranging from 0.1 to 100 ng/ml. After 24 h, cells were trypsinized and counted. As shown in Figure 4A, TPA causes a dose-dependent inhibition of bFGF-induced cell proliferation. TPA alone induces instead an increase of cell proliferation, confirming that at these doses TPA alone is not capable of down-regu-

lating PKC. In keeping with the incapacity of 1,2-diacylglycerols to down-regulate PKC (Issandou and Rozengurt, 1989), no inhibition of the activity of bFGF is induced by a mitogenic dose of diC8 (Figure 4A). Nevertheless, the inhibitory effect of TPA is specific. Indeed, no inhibition of the mitogenic activity of bFGF is caused by coincubation with phorbol, phorbol 12,13-diacetate, or 4 alfa-phorbol 12,13-didecanoate (Figure 4B), molecules unactive as PKC inducers (Castagna *et al.*, 1982; Noguchi *et al.*, 1985). Moreover, cells treated with bFGF plus TPA still proliferate when coincubated with the PKC-independent mitogens EGF or FCS, but not when coincubated with diC8 (Figure 4C). These data suggest that the inhibition of cell proliferation observed after treatment with bFGF plus TPA is due to down-regulation of PKC. To assess this hypothesis, PKC activity was evaluated in GM 7373 cells treated for 24 h with 30 ng/ml bFGF in the presence or in the absence of a mitogenic dose of TPA (30 ng/ml). As shown in Table 1, an 80% reduction of membrane-bound PKC activity was observed in cells treated with bFGF plus TPA in respect to untreated cells or cells treated with bFGF alone or with TPA alone. In agreement with previous observations, a complete down-regulation of PKC activity was detected in cells treated with 3 μg/ml TPA, a concentration of phorbol ester that fully inhibits the PKC-dependent mitogenic signaling pathway in GM 7373 cells (Presta *et al.*, 1989a; Maier *et al.*, 1989). Thus treatment of GM 7373 cells with bFGF plus low mitogenic doses of TPA inhibits cell proliferation possibly by causing an almost complete down-regulation of PKC.

On this basis, we have evaluated the effect of TPA on the mitogenic activity of bFGF when the phorbol ester was added at different times after the growth factor. As shown in Figure 5, TPA causes the inhibition of cell proliferation also when added 12 h after bFGF treatment. TPA was instead unaffective when added during the last 4 h of treatment. These data indicate that down-regulation of PKC inhibits the mitogenic activity of bFGF also when occurring as late as 12 h after the stimulation with the growth factor. Thus, in keeping with the results obtained with the PKC inhibitor H-7, a long-lasting stimulation of PKC activity is required by GM 7373 cells to proliferate after treatment with bFGF.

Discussion

In the present paper we demonstrate that a long-lasting interaction of bFGF with FBAE GM

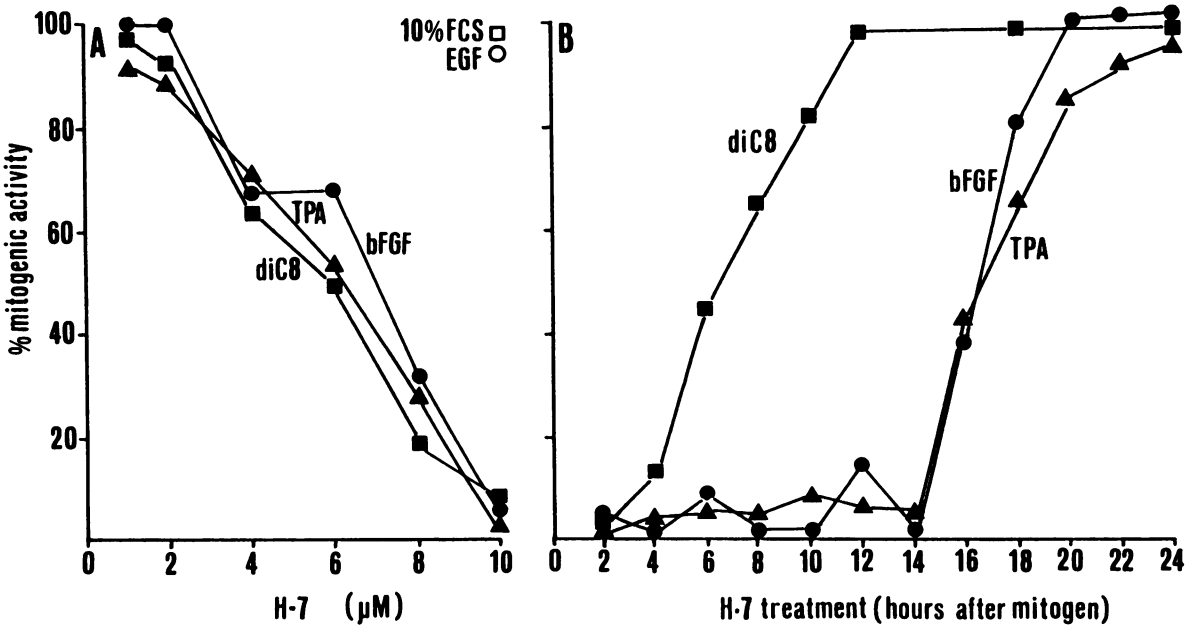

Figure 3. **Effect of H-7 on GM 7373 cell proliferation induced by the PKC-dependent mitogens bFGF, TPA, and diC8.** (A) Cultures were incubated in 0.4% FCS with 30 ng/ml bFGF (●), 100 ng/ml TPA (▲), 5 μg/ml diC8 (■), 30 ng/ml EGF (○), or in 10% FCS (□) in the presence of increasing concentrations of H-7. After 24 h cells were trypsinized and counted. (B) Cells were incubated with bFGF, TPA, or diC8 as in panel A. Ten micromolars of H-7 were added at different times after mitogen. All cultures were trypsinized and counted 24 h after the beginning of the treatment with the mitogen. Data are expressed as percent of the mitogenic activity exerted by the mitogen in the absence of H-7.

7373 cells is required to induce cell proliferation. Removal of extracellular bFGF with suramin or addition of anti-bFGF neutralizing antibodies to the culture medium completely abolishes the mitogenic activity of bFGF also when performed several hours after the beginning of the treatment with the growth factor. bFGF-treated cells appear to be committed to proliferate only after they have entered the phase S of the cell cycle. Previous reports had shown that EGF and platelet-derived growth factor must remain active in the extracellular environment for several hours before cell replication is committed (Carpenter and Cohen, 1976; Westermark and Heldin, 1985). Thus the requirement for a long-lasting interaction with the cell is a feature common to different growth factors. Recently, acidic FGF mutants capable of inducing early responses (receptor-mediated tyrosine phosphorylation and protooncogene expression) but lacking a mitogenic activity have been described (Burgess *et al.*, 1990; Imamura *et al.*, 1990). Our findings raise the possibility that these molecules may be unable to elicit the long-lasting events involved in the mitogenic response.

bFGF binds to high-affinity plasma membrane receptors and causes their rapid down-regulation (Presta *et al.*, 1989b). When GM 7373 cells (15 000 receptors/cell; Kd = 17 pM) (Presta *et al.*, 1989b) are continuously exposed to 10 ng/ml ^{125}I-bFGF at 37°C, 60–70% of the bFGF receptors are down-regulated within 1–2 h. Then, the number of receptors remains constant at 3500–5000 molecules per cell during the following 22 h of treatment (data not shown), indicating that extracellular bFGF immediately binds to newly exposed receptors and causes

Table 1. Effect of bFGF plus TPA on PKC activity in GM 7373 cells

Treatment	PKC activity[1]
Untreated cells	6.5 ± 1.6
bFGF (30 ng/ml)	7.5 ± 2.7
TPA (30 ng/ml)	14.1 ± 9.9
bFGF (30 ng/ml) plus TPA (30 ng/ml)	1.1 ± 1.1
TPA (3 μg/ml)	<0.1

[1] PKC activity is expressed as pmoles ^{32}P incorporated into PKC substrate/100 μg protein/15 min and it was calculated as the difference between the activities in the presence or in the absence of phosphatidylserine plus calcium acetate and phorbol myristate acetate. Values are the means ± SEM of three independent experiments in duplicate.

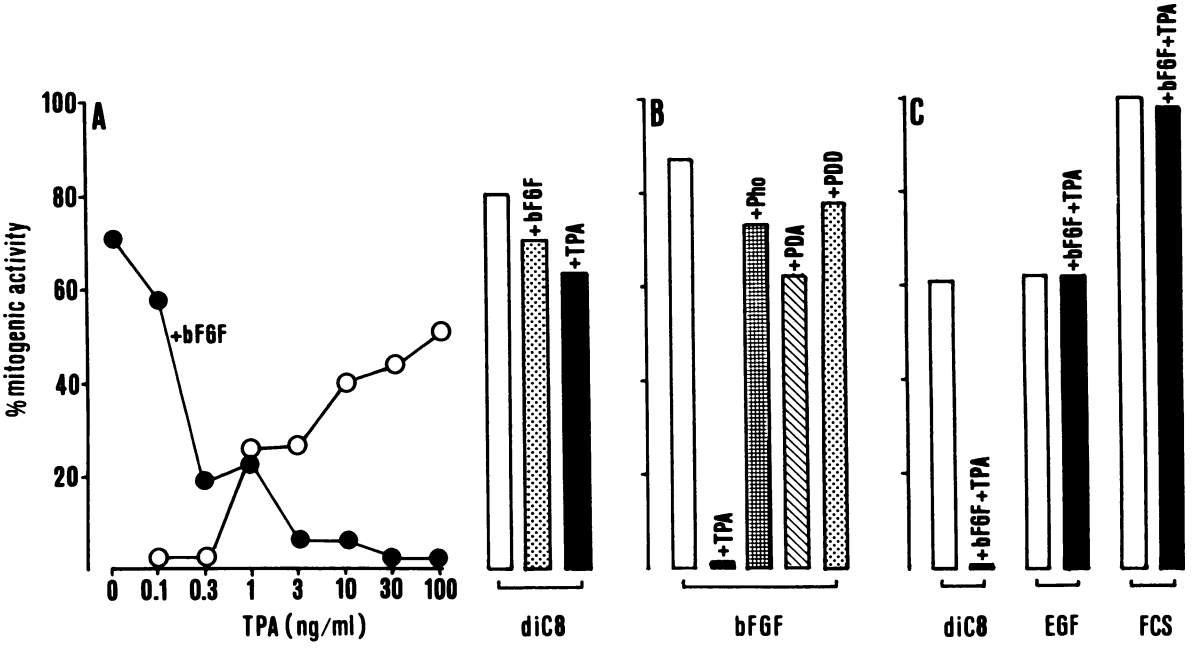

Figure 4. **Effect of TPA on bFGF mitogenic activity.** (A) GM 7373 cells were incubated with increasing concentrations of TPA in the absence (○) or in the presence (●) of 30 ng/ml bFGF. Some cultures were incubated instead with 5 μg/ml diC8 with no addition (□), 30 ng/ml bFGF (□), or 100 ng/ml TPA (■). (B) Cultures were incubated with 30 ng/ml bFGF with no addition or in the presence of TPA, phorbol (Pho), phorbol 12,13-diacetate (PDA), or 4 alfa-phorbol 12,13-didecanoate (PDD), all at 100 ng/ml. (C) Cultures were incubated with 5 μg/ml diC8, 100 ng/ml EGF, or 10% FCS with no addition (□) or with 30 ng/ml bFGF plus 100 ng/ml TPA (■). All cultures were trypsinized and counted after 24 h. Data are expressed as percent of the proliferation observed in cultures treated with 10% FCS with no addition.

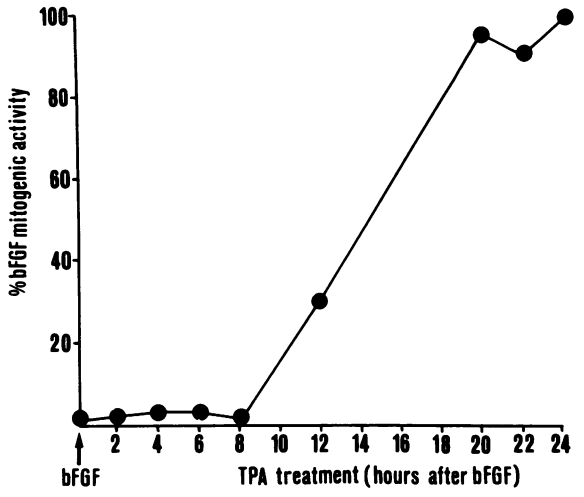

***Figure 5.* Effect of down-regulation of PKC on bFGF mitogenic activity.** GM 7373 cells were incubated with 30 ng/ml bFGF. At different times after treatment, cultures were added also with 100 ng/ml TPA to down-regulate PKC. Cell were trypsinized and counted 24 after bFGF treatment. Data are expressed as percent of cell proliferation observed in cultures treated with bFGF alone.

their internalization, thus maintaining receptor down-regulation. Our results demonstrate that the long-lasting interaction of bFGF with its receptors is necessary to induce cell proliferation.

The requirement for a prolonged bFGF receptor occupancy is in keeping with the requirement for a prolonged PKC activation. Indeed, inhibition of PKC activity by H-7 or by down-regulation of the enzyme fully inhibits the mitogenic activity of bFGF even when performed 12 h after the beginning of the treatment with the growth factor. Prolonged PKC activation also is required when cells are induced to proliferate by the phorbol ester TPA or by the synthetic 1,2-diacylglycerol diC8. Previous observations have indicated that prolonged PKC activation is required for interleuken-2 expression and secretion by T cells (Berry and Nishizuka, 1990). Also, a prolonged PKC activation is necessary to induce RNA and DNA synthesis, as well as protein N-glycosilation, in B cells activated by bacterial lipopolysaccharide (Rush and Waechter, 1987). Thus, even though PKC is rapidly activated by different stimuli, the full biological response mediated by PKC is achieved only after a prolonged activation of the enzyme. This appears to represent an important mechanism of control of cell proliferation. In fact, no mitogenic response will occur unless the rapid PKC activation is confirmed by a long-lasting signal.

In GM 7373 cells, diC8 induces a mitogenic response similar to that observed with TPA, and both PKC-activators cause a similar early translocation of the enzyme to cell membranes with a similar pattern and kinetic of protein phosphorylation (Maier *et al.*, 1989). Nevertheless, to induce cell proliferation, diC8 requires a PKC activation that is shorter (6 vs. 16 h) than that required by TPA. Even though phorbol esters and synthetic 1,2-diacylglycerols are usually thought to exert their function via a similar interaction with PKC (Nishizuka, 1986), several differences have been reported among the biological responses exerted by the two classes of compounds (Davis *et al.*, 1985; Kreutter *et al.*, 1985; Ramsdell *et al.*, 1986; Kolesnick and Paley, 1987; Bazzi and Nelsestuen, 1989; Issandou and Rozengurt, 1989; Maier *et al.*, 1989). These differences have been tentatively attributed to differences in the rate of metabolism, in the mechanism of activation and membrane-translocation of PKC, and in the activation of different PKC isoenzymes. Our results indicate that, to induce GM 7373 cell proliferation, bFGF triggers a PKC-dependent intracellular signaling pathway that is similar, if not identical, to that triggered by TPA but different from that triggered by diC8.

Early intracellular events induced by growth factors have been the focus of numerous researches aimed to define the mechanisms of regulation of cell growth. However, our results clearly indicate that the study of late events triggered by growth factors is of pivotal importance for a full understanding of the mechanisms that control cell proliferation.

Materials and methods

Chemicals

Human recombinant bFGF was obtained from Farmitalia-Carlo Erba, Milan, Italy. Phorbol esters, EGF, and diC8 were from Sigma Chemical (St. Louis, MO). Suramin was from F.B.A., Germany. H-7 was from Siekagaku America, FL. A neutralizing anti-human bFGF rabbit antiserum was obtained from D.B. Rifkin (New York University Medical Center, NY). Anti-bFGF antibodies were then affinity-purified on a recombinant bFGF-Affigel column, according to standard procedures.

Cell cultures and cell proliferation assay

Transformed FBAE GM 7373 cells were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). They correspond to the BFA-1c 1BPT multilayered clone described by Grinspan et al. (1983). GM 7373 cells were grown in Eagle's Minimal Essential Medium containing 10% FCS, vitamins, and essential and nonessential amino acids. Cell proliferation assay was performed as described (Presta *et al.*, 1989a). Briefly, cells were seeded at 70 000 cells/cm^2 in a 24-well dish. Plating efficiency was higher than 90%. After overnight in-

cubation, cells were incubated for 24 h in fresh medium containing 0.4% FCS and the mitogen to be tested. Then cells were trypsinized and counted in a Burker chamber. In these experimental conditions, GM 7373 cells proliferate when treated with bFGF, TPA, diC8, or EGF (Presta *et al.*, 1989a). To measure DNA synthesis, cells were seeded as above and pulse-labeled for 2 h with 0.5 μCi/ml ^{3}H-thymidine at different times after the beginning of bFGF treatment. Then, the amount of trichloroacetic acid-precipitable radioactivity was measured.

PKC assay

GM 7373 cell cultures were treated with 30 ng/ml bFGF, 30 ng/ml TPA, 3 μg/ml TPA, or with bFGF plus TPA, both at 30 ng/ml, in the presence of 0.4% FCS as described for the cell proliferation assay. At the end of the incubation, cells were scraped from the dish and sonicated in a lysis buffer (50 mM tris[hydroxymethyl]aminomethane-HCl, pH 7.5; 5 μg/ml phenylmethylsulfonylfluoride; 0.3% 2-mercaptoethanol; 5 mM EDTA; 10 mM ethylene glycol-bis[β-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid). Homogenates were centrifuged at 20 000 $\times$ *g* for 30 min. Particulate fractions were resuspended with lysis buffer containing 1% Nonidet P-40, held at 4°C for 1 h, and centrifuged at 20 000 $\times$ *g* for 30 min to separate detergent-solubilized material. PKC assay on 100-μg aliquots of solubilized membrane fractions was performed by using a PKC enzyme assay kit (Amersham, Arlington Heights, IL) according to manufacturer's instructions.

Acknowledgments

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