

# Lysophosphatidic Acid and bFGF Control Different Modes in Proliferating Myoblasts

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**Abstract.** Myogenic cells provide excellent in vitro models for studying the cell growth and differentiation. In this study we report that lysophosphatidic acid (LPA), a bioactive phospholipid contained in serum, stimulates the growth and inhibits the differentiation of mouse C2C12 myoblast cells, in a distinct manner from basic fibroblast growth factor (bFGF) whose mitotic and anti-differentiation actions have been well investigated. These actions of LPA were both blocked by pertussis toxin, suggesting the involvement of Gi class of G proteins, whereas bFGF acts through receptor tyrosine kinases.

Detailed analysis revealed that LPA and bFGF act differently in regulating the myogenic basic helix-loop-helix (bHLH) proteins, the key players in myogenic differentiation process. LPA stimulates the proliferation

of undifferentiated myoblasts allowing the continued expression of MyoD, but in contrast, bFGF does so with the MyoD expression suppressed at the mRNA level. Both compounds maintain the myf-5 expression, and suppress the myogenin expression. In addition, while LPA did not inhibit cell-cell contact-induced differentiation, bFGF strongly inhibited this process. Furthermore, LPA and bFGF act cooperatively in their mitogenic and anti-differentiation abilities.

These findings indicate that LPA and bFGF differently stimulate intracellular signaling pathways, resulting in proliferating myoblasts each bearing a distinct expression pattern of myogenic bHLH proteins and distinct differentiation potentials in response to cell-cell contact, and illustrate the biological significance of Gi-mediated and tyrosine kinase-mediated signals.

THE growth and differentiation of cells in organisms are subject to tightly concerted regulation that has long been a major focus of interest. Myogenic cell lines provide excellent in vitro models for studying the control of growth and differentiation mainly for two reasons. First, their growth and differentiation are easily controlled by manipulating the culture medium components (for review see Florini et al., 1991). Second, the MyoD family of basic helix-loop-helix (bHLH)<sup>1</sup> factors, which play essential roles in myogenic differentiation, have been cloned and well characterized (for reviews see Weintraub et al., 1991; Buckingham, 1992; Olson, 1992; Weintraub, 1993): these consist of MyoD (Davis et al., 1987), myoge-

nin (Wright et al., 1989), myf-5 (Braun et al., 1989), and MRF-4/myf-6/herculin (Rhodes and Konieczny, 1989; Braun et al., 1990; Miner and Wold, 1990).

Serum and certain fibroblast growth factors (FGFs) are able to stimulate the growth and prevent the terminal differentiation of a number of established myoblast cell lines (Clegg et al., 1987; Lathrop et al., 1985; Spizz et al., 1986). FGFs (mainly basic [b-] or acidic [a-] FGF) are the most investigated and the most characterized compounds that possess these activities (Florini et al., 1991). Other than FGFs, epidermal growth factor (EGF) and thrombin have been described to have these effects on a particular cell line, BC3H1, but detailed analyses have not yet been done (Wang and Rubenstein, 1988; Kelvin et al., 1989b).

Several findings indicate that serum regulates myoblast growth and differentiation by mechanisms independent of bFGF. A rat myoblast line, L6 (Yaffe, 1968), grows under high serum conditions, but does not respond to FGF. In addition, the serum activities are blocked by pertussis toxin (PT), which specifically inhibits the Gi class of G proteins (Kelvin et al., 1989a,b; Salminen et al., 1991), while FGF signals, which are transduced through receptor tyrosine kinases (RTK), are unaffected by PT (Fantl et al.,

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1. *Abbreviations used in this paper.* BrdU, bromodeoxyuridine; CK, creatine kinase; bHLH, basic helix-loop-helix; ITS, DMEM containing insulin, transferrin and sodium selenite; LPA, lysophosphatidic acid; MCK, muscle creatine kinase; PPP, platelet poor plasma-derived serum; PT, pertussis toxin.

1993; Jaye et al., 1992; Burgess and Maciag, 1989). Indeed, the FGF family of proteins have a strong affinity for the extracellular matrix and their contents in serum is very low (Klagsbrun and Baird, 1991; Burgess and Maciag, 1989).

Lysophosphatidic acid (LPA) is a phospholipid contained in serum (Gerrard and Robinson, 1989; Eichholtz et al., 1993), and has been revealed to elicit a variety of biological responses on a wide range of cells and organisms, including the stimulation of fibroblast proliferation (for reviews see Moolenaar, 1994, 1995). It functions by activating a number of intracellular signal transduction systems through putative cell surface receptor(s) coupled to G-proteins (van Corven et al., 1989, 1992; Jalink et al., 1990; van der Bend et al., 1992a; Thomson et al., 1994).

In this study, we report that LPA stimulates the growth and inhibits the differentiation of mouse C2C12 myoblast cells in a PT-sensitive manner. Detailed analyses in this study demonstrated that LPA and bFGF act on C2C12 cells by distinct mechanisms. In contrast to bFGF, which stimulated proliferation of undifferentiated myoblasts by repressing MyoD, as previously reported (Vaidya et al., 1989), LPA did so with MyoD expression maintained. In confluent cultures, while LPA could not inhibit the cell-cell contact-induced differentiation, bFGF strongly inhibited this process. In addition, LPA and bFGF acted cooperatively, suggesting that they stimulated different intracellular signaling pathways. This study illustrates distinct roles of Gi- and RTK-mediated signaling pathways in proliferating myoblasts.

## Materials and Methods

### Cells and Cultivations

The C2C12 mouse myoblast cell line (Yaffe and Saxel, 1977; Blau et al., 1983) was obtained from the Amer. Type Culture Collection (Rockville, MD). A subclone of C2C12 was obtained by limiting dilution for use in this study. C2C12 cells were maintained in DMEM supplemented with 10% FCS and kanamycin (60 µg/ml). To induce differentiation or to test the effects of the experimental reagents, the cells were cultured on collagen-coated tissue culture plates (Corning, NY/Iwaki, Tokyo, Japan), and then switched to a serum-free ITS medium, consisting of DMEM supplemented with insulin (10 µg/ml), transferrin (5 µg/ml), sodium selenite (10 nmol), and bovine serum albumin (BSA, 1 mg/ml). BSA was added to prevent the precipitation of LPA in the presence of calcium ion (Jalink et al., 1990).

### Reagents

FCS and platelet poor plasma-derived serum were purchased from Moregate Gr. Co. (Melbourne, Australia) and Biomedical Technologies Inc. (Stoughton, MA), respectively. Insulin (human), transferrin (human), and BSA were purchased from Sigma Chem. Co (St. Louis, MO). All lipids including LPA (synthesized L- $\alpha$ -monooleoyl phosphatidic acid) were also purchased from Sigma. bFGF (human, recombinant) were from GIBCO BRL (Gaithersburg, MD) and Pertussis Toxin was from Kaken Pharmacology Co (Chiba, Japan). Anti-MyoD (5.8A), anti-Myogenin (F5D), and anti-Troponin T (NT302) monoclonal antibodies were kindly provided by Drs. P. Houghton (Dias et al., 1992), W. Wright (Wright et al., 1991), and T. Obinata (Abe et al., 1986), respectively.

### Fusion Index/Nuclear Number Determination and BrdU Incorporation

C2C12 cell cultures were fixed and dehydrated with methanol at room temperature for 10 min, and then stained with Giemsa and Wright solutions (E. Merck, Darmstadt, Germany). The number of nuclei in mononucleated cells and in myotubes, defined as syncytia containing three or

more nuclei, were counted using an optical microscope at a magnification of 100 $\times$  or 200 $\times$ . Three independent fields of 1 mm<sup>2</sup> were counted to calculate the total number of nuclei per square millimeter and the fusion index (percent of total nuclei incorporated in myotubes). BrdU incorporation was assayed using a Cell Proliferation Kit (Amersham Corp., Arlington Heights, IL). Cells were labeled with BrdU for 4 h followed by immediate fixation with acid ethanol. BrdU incorporated into DNA was visualized by indirect enzymatic immunocytochemical staining and counterstaining with methyl green.

### Immunostaining

Cultured cells were fixed with 2% paraformaldehyde in phosphate-buffered saline at room temperature for 10 min. They were then permeabilized by 0.25% Triton X-100 (room temperature, 20 min) and reacted with the first antibody (4°C, overnight). After incubation with the second antibody (FITC-conjugated anti-mouse IgG), the cells were washed, DNA stained with 4, 6, -diamidino-2-phenylindole (DAPI), and observed under a fluorescent microscopy. In some cases, biotinylated anti-mouse IgG was used as the second antibody followed by visualization using a Vecstain ABC detection kit (Vector Labs., Burlingame, CA).

### RNA Analysis

Poly (A) RNA was isolated from the cells using a QuickPrep mRNA Purification Kit (Pharmacia LKB Biotechnology, Piscataway, NJ) according to the manufacturer's recommendation. Five micrograms of poly(A) RNA per lane were electrophoretically separated in a 1.5% agarose gel containing 7% formaldehyde, and then blotted onto Hybond N+ nylon membrane filter (Amersham) by microcapillary transfer. Phosphate-labeled double-stranded DNA probes specific for each transcript were generated using a Megaprime labeling kit (Amersham) and [ $\alpha$ -<sup>32</sup>P] deoxycytidine 5'-triphosphate (Dupont). Hybridization was performed at 42°C, overnight, in a hybridizing solution consisting of 5 $\times$  SSPE, 0.1% sodium dodecylsulfate and 50% formamide, containing 5 $\times$  Denhardt's solution and 100 µg/ml of denatured salmon sperm DNA as blocking reagents. Filters were then washed under stringent conditions (0.2 $\times$  SSPE, 65°C, 30 min). Imaging was performed by autoradiography or the BAS2000 Imaging System (Fuji Film, Tokyo, Japan). The filters were routinely deprobed and rehybridized with other radioprobes.

The probes used in this study were as follows: for MyoD and myogenin, 3' noncoding HindIII-EcoRI and EcoRI-EcoRI fragments of the mouse cDNAs were used, respectively (Fujisawa et al., 1990). In other cases, fragments were prepared by reverse transcriptase-PCR from cDNA prepared from C2C12 total RNA primed with random hexanucleotides, subsequently subcloned and sequenced. Primers used for PCR amplification were described in Hannon et al. (1992) for myf-5; G3PDH primers were purchased from Clontech Co. (Palo Alto, CA) (Arcari et al., 1984); MCK primers were CCACAGACAAGCATAAGACCGACC (position 377-401) and AACCTCCTTCATATTGCCTCCCTTC (position 819-795), (Buskin et al., 1985). Id primers were CACTCTGTTCTCAGCCTCCTC (position 39-59) and GGCTGGAGTCCATCTGGTCCCT (position 567-546) (Benezra et al., 1990).

### LPA Quantitation in Serum Samples

Total lipids were extracted from the samples by the method of Bligh and Dyer after acidification with hydrogen chloride to pH 2.5 (Bligh and Dyer, 1959; Tokumura et al., 1994), and subjected to a two-dimensional thin layer chromatography system using a silica gel 60 coated plate (Merck). The solvents used were chloroform/methanol/20% ammonium hydroxide (60:35:8, vol/vol/vol) for the first, and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, vol/vol/vol/vol) for the second dimensional development (Tokumura et al., 1994; Tigyi and Miledi, 1992). Phospholipids were made visible by spraying with Dittmer-Lester reagent, and color intensities of the LPA spots were measured against the standard LPA using Atto Densitograph (Atto, Tokyo, Japan).

## Results

### Mitogenic and Anti-Differentiation Activities of LPA

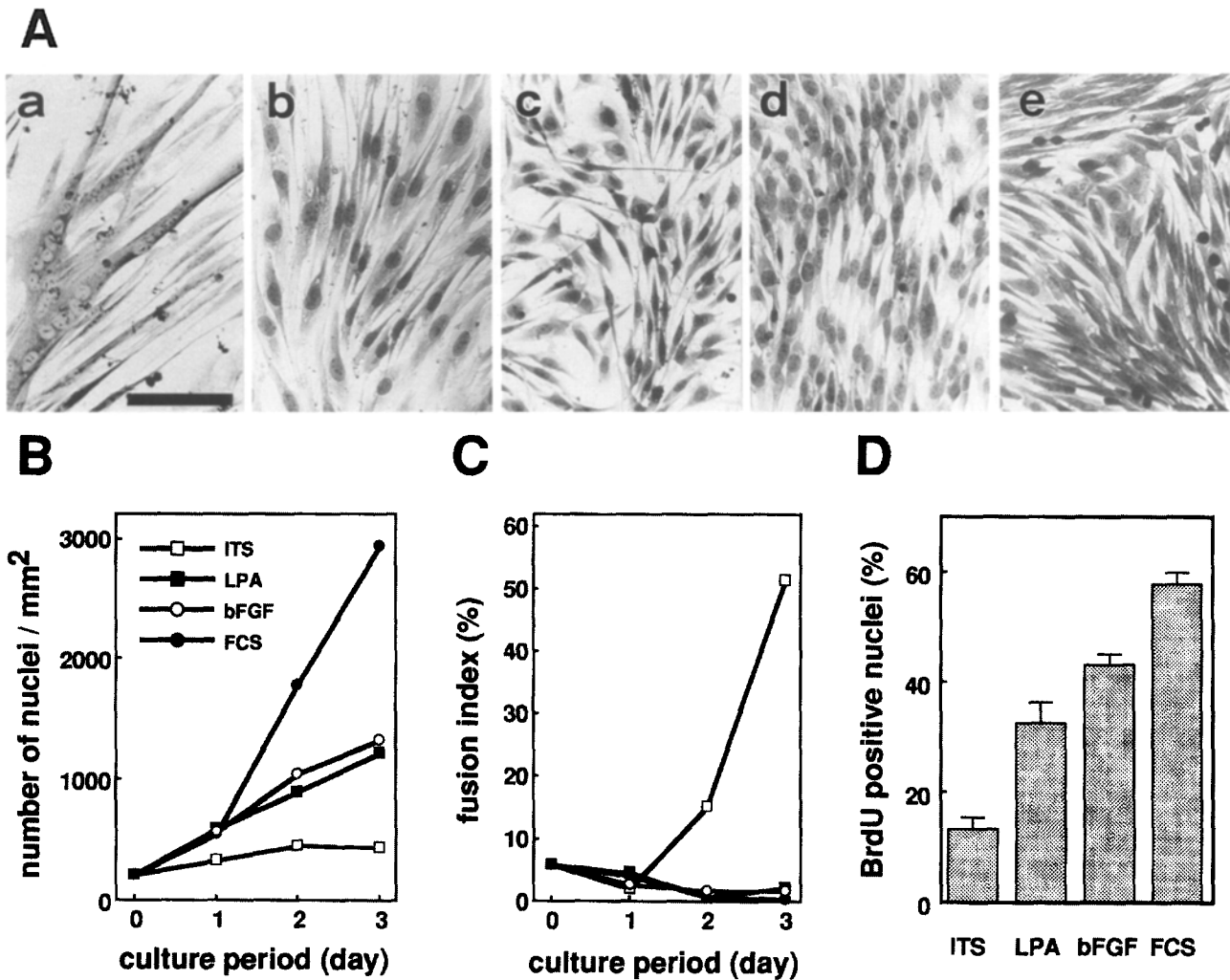
To assess the effects of LPA on myoblasts, we used a serum-free condition where C2C12 mouse myoblast cells

(Yaffe and Saxel, 1977; Blau et al., 1983), withdrew from the cell cycle and underwent differentiation. When C2C12 cells were cultured in serum free ITS medium (containing insulin, transferrin, and selenite, see Materials and Methods), they differentiated and fused to form multinucleated myotubes within three days (Fig. 1, A-a). Addition of LPA (10  $\mu\text{g/ml}$ ) to the medium markedly suppressed myotube formation (Fig. 1, A-b), creatine kinase (CK), and tropomyosin T expression (data not shown). The same results were obtained when bFGF (10 ng/ml) or FCS (10%) was added to the medium (Fig. 1, A-c and e). Time course of the proliferation (indexed by number of nuclei) and differentiation (indexed by fusion index) of C2C12 cells in the presence of LPA or bFGF was examined (Fig. 1, B and C, respectively). In ITS medium, cells ceased to proliferate

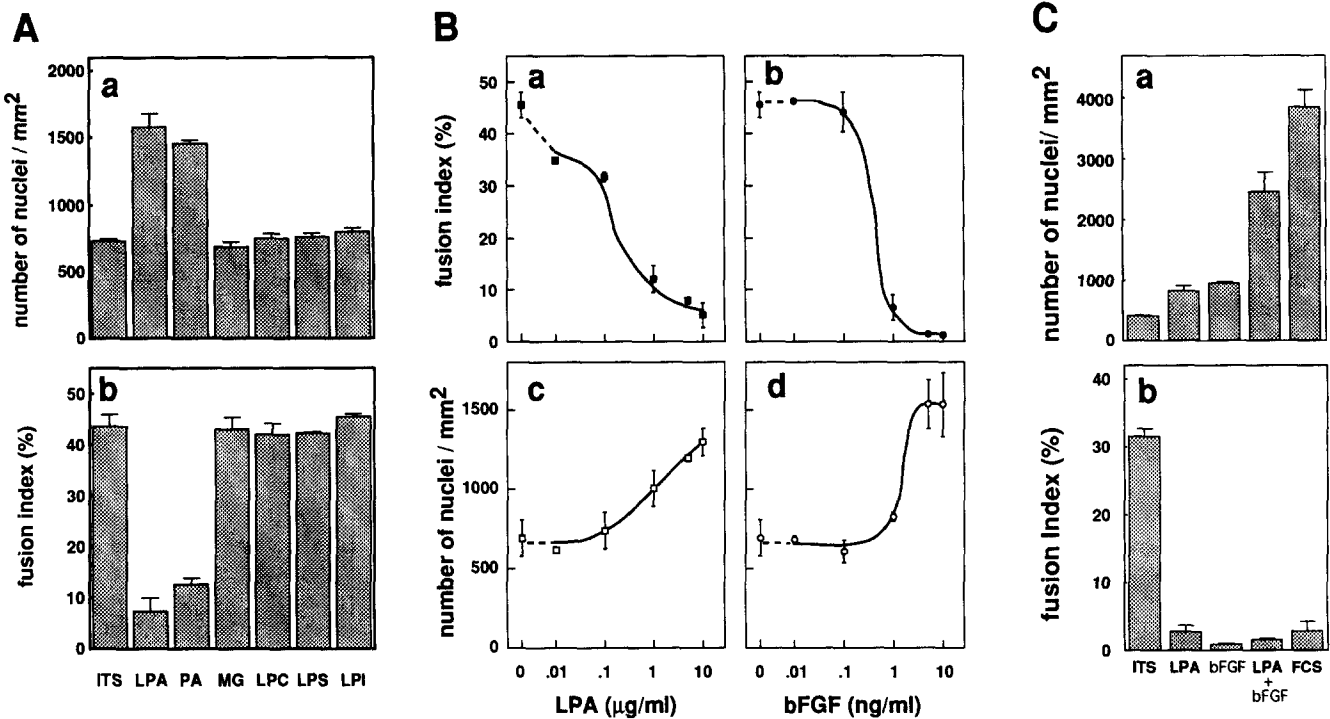
and differentiated to form myotubes by culture day three. In the presence of LPA or bFGF they continued to proliferate and did not form multinucleated myotubes during the same culture period. A bromodeoxyuridine (BrdU) incorporation experiment (Fig. 1 D) showed that LPA and bFGF stimulated DNA synthesis in a way that corresponds to the growth rates shown in Fig. 1 B. On the basis of these observations, we conclude that LPA, as well as bFGF, can stimulate the proliferation and inhibit the differentiation of C2C12 myoblasts.

### LPA Activities Are Specific among Structurally Related Lipids

The effects of lipids which are structurally related to LPA



**Figure 1.** Mitogenic and anti-differentiation effects of LPA and bFGF in C2C12 cells. (A) Morphological effects of LPA and bFGF. C2C12 cells cultured in DMEM containing 10% FCS were seeded onto collagen coated 24-well cell culture plates at the density of  $10^4$  cells/well. 24 h later the medium was switched to ITS medium (a) or ITS supplemented with LPA (10  $\mu\text{g/ml}$ , b), bFGF (10 ng/ml, c), LPA plus bFGF (10  $\mu\text{g/ml}$  and 10 ng/ml, respectively, d) or FCS (10%, e). After 3 d in culture with daily medium changes, the cells were fixed and Giemsa-stained. Bar represents 100  $\mu\text{m}$ . (B and C) Time course of proliferation and differentiation of C2C12 cells in ITS medium (open square), or ITS supplemented with LPA (closed square), bFGF (open circle), or FCS (closed circle). Culture conditions were described in the legend of A. Day 0 represents the day when the medium was switched to experimental medium. Cultures were fixed at 24-h intervals and the total nuclei number and fusion index were determined. (D) Effect of LPA and bFGF on DNA synthesis of C2C12 cells. Cells were cultured as in A. At 42 h, the cultures were labeled with BrdU for 4 h, and then fixed, stained with anti-BrdU antibody, and counted. Three independent fields of 1 mm<sup>2</sup> and at least 1,000 nuclei were counted for each data point. The error bars indicate SEM of the three determinations.



**Figure 2.** Specificity, dose response patterns and cooperative effects of LPA and bFGF. (A) Effect of LPA and structurally related lipids on proliferation and differentiation of C2C12 cells. Cells cultured as in Fig. 1 were switched to ITS media supplemented with LPA, L- $\alpha$ -dioleoyl phosphatidic acid (PA), 1-monooleoyl-rac-glycerol (monoacylglycerol, MG), L- $\alpha$ -oleoyl lysophosphatidyl-choline (LPC), L- $\alpha$ -lysophosphatidyl-L-serine (LPS), or L- $\alpha$ -lysophosphatidyl-inositol (LPI). After 3 d, with daily media changes, cultures were fixed and the total number of nuclei (a) and the fusion index (b) were determined. The concentration of the lipids used was 10  $\mu$ g/ml except MG which was 50  $\mu$ g/ml. (B) Dose-response curves of anti-differentiation and mitogenic effects of LPA and bFGF. After 3 d of culture as described in Fig. 1 in the presence of the indicated concentration of LPA (a and c) or bFGF (b and d), cultures were processed to their fusion index (a and b) and total number of nuclei (c and d) determination. (C) Effects of simultaneous addition of LPA and bFGF on mitogenic and anti-differentiation activities. The same procedures were performed as described in Fig. 1 in the presence of the factors indicated below each column. SEM of the three independent determinations is indicated by the error bars throughout this figure.

on C2C12 myoblasts were examined. As shown in Fig. 2 A, the growth promoting and anti-differentiation activities of the lipids examined were limited to LPA and phosphatidic acid (PA), suggesting that LPA functions as a bioactive ligand. This is compatible with the previously described functions of LPA (van Corven et al., 1989; Jalink et al., 1990; van Corven et al., 1993; Jalink et al., 1993). PA has been revealed to have similar effects to LPA in other systems (van Corven et al., 1989; Jalink et al., 1990), actually due to LPA which was present as a contaminant or a degradation product of PA. Indeed, we detected a significant amount (5–10%) of LPA contaminated in our PA samples (data not shown). Therefore, it may be that the PA activities revealed here are also due to the contaminated LPA.

### Dose-Response Profiles

As shown in Fig. 2 B, LPA stimulated growth and inhibited differentiation in a dose-dependent manner, and its effective concentration was higher than 1  $\mu$ g/ml (2.3  $\mu$ M). This is about the same dose required to evoke certain cellular responses including DNA synthesis in fibroblasts (van Corven et al., 1989, 1993). Throughout this study, we used 10  $\mu$ g/ml as the standard concentration of LPA, because higher concentrations (20–50  $\mu$ g/ml) sometimes caused cell lysis. Indeed, myogenic differentiation, indicated by fusion index (Fig. 2, B-a) and CK activity (data

not shown), was almost completely blocked at 10  $\mu$ g/ml, indicating that this concentration provides almost full LPA activity. In case of bFGF, the dose-response properties were essentially the same as previously reported (Fig. 2, B-b and d, Clegg et al., 1987). The concentration of bFGF was fixed at 10 ng/ml.

### Cooperative Effects of LPA and bFGF

We tested the proliferation and differentiation of C2C12 cells when both LPA and bFGF were added. Fig. 2, C-a shows that mitogenic effect of the simultaneous addition of the two agents is stronger than the separate effects of each agent. BrdU incorporation experiments provided essentially the same results (data not shown). Such a cooperative effect is also observed in the inhibition of differentiation indicated by the expression of the terminal differentiation markers (discussed later in Fig. 4). Figs. 1, A-d and 2, C-b did not demonstrate this combined effect on differentiation inhibition, because the fusion illustrated had already been repressed to below the basal level (<5%) by either treatment. Since the doses of LPA and bFGF used here can each cause a full effect, as discussed above, their combined effects suggested that LPA and bFGF activate distinct intracellular machineries that can act in cooperation with the control of growth and differentiation of C2C12 cells.

### LPA but Not bFGF Functions through Gi Proteins

In a number of systems, LPA evokes a wide variety of cellular responses through a putative cell surface receptor(s) coupled to G proteins (Moolenaar, 1994, 1995). Some of them are blocked by pertussis toxin (PT), indicating the involvement of Gi proteins, while others are mediated by other classes of G proteins and are resistant to PT (van Corven et al., 1989; Ridley and Hall, 1994). We, therefore, investigated the PT sensitivities of LPA in our system to test the possible involvement of Gi (Fig. 3).

The growth stimulatory and differentiation inhibitory effects of LPA (indexed by myogenin expression here) were canceled by the presence of PT in the culture at concentrations above 5–10 ng/ml (Fig. 3), indicating that LPA actions are mediated by Gi proteins. This data agrees with previous observations that the mitogenic activity of LPA in fibroblasts is PT-sensitive (van Corven et al., 1989, 1993). In contrast, the effects of bFGF were not affected by PT at any concentration tested; this result was expected because FGF receptor tyrosine kinases do not usually activate Gi proteins (Fantl et al., 1993; Jaye et al., 1992). The growth stimulation and differentiation inhibition by FCS were also repressed by PT as reported by others (Kelvin et al., 1989a,b; Salminen et al., 1991). Judging from these results, we conclude that LPA stimulates growth and suppresses differentiation of C2C12 myoblasts by a Gi-mediated mechanism(s), whereas bFGF's effects are independent of Gi proteins.

### Differential Regulation of Myogenic bHLH Factors and Id

To address the target of the anti-differentiation activity of LPA, we examined the expression of myogenic bHLH factors. Series of investigations have revealed the roles of these factors which control myogenic determination and differentiation (for reviews see Weintraub, 1993; Olson and Klein, 1994). MyoD and/or myf-5 are expressed in

proliferating myoblasts and are involved in their maintenance, while myogenin plays important roles when the cells decide to withdraw from the cell cycle and differentiate (Braun et al., 1992; Rudnicki et al., 1992, 1993; Nabeshima et al., 1993; Hasty et al., 1993). MRF4 may be important in the later steps of differentiation.

In culture medium containing 10% FCS, the majority of our C2C12 cells expressed MyoD protein and mRNA (identical to the results from ITS plus 10% FCS shown in Fig. 4, A-e, B, and C). When they were switched to bFGF medium, both MyoD protein and mRNA were dramatically reduced (Fig. 4, A-c, B, and C), as observed by Vaidya et al. (1989). In contrast, in LPA medium, the proliferating myoblasts expressed significant levels of MyoD protein and mRNA (Fig. 4, A-b, B, and C). Unlike MyoD, myf-5 mRNA was expressed at a relatively constant but low level under all the mitogenic conditions (Fig. 4 C). The expression of myogenin and MCK was strongly induced upon differentiation in ITS medium, but greatly suppressed under all the mitogenic conditions. The coexistence of LPA and bFGF caused a greater suppression of MCK and myogenin expression, suggesting some concerted effects of the two factors. MRF-4 mRNA could not be detected in any of the conditions tested (data not shown).

We conclude that LPA- and bFGF-stimulated growth and inhibition of differentiation of C2C12 cells occur in distinct manners that result in two modes of myogenic bHLH factor expression: LPA mainly results in MyoD positive, myf-5 positive, and myogenin negative cells, while bFGF mainly results in MyoD negative, myf-5 positive, and myogenin negative cells. These two modes in C2C12 cells can be reversed by switching the LPA medium to bFGF medium, and vice versa (data not shown).

We also examined the expression of the Id gene, which encodes an HLH factor which lacks the basic region and inhibits the MyoD family and other bHLH factors (Benzra et al., 1990). Myoblasts growing in a FCS-containing medium expressed a high level of Id mRNA, but, when in-

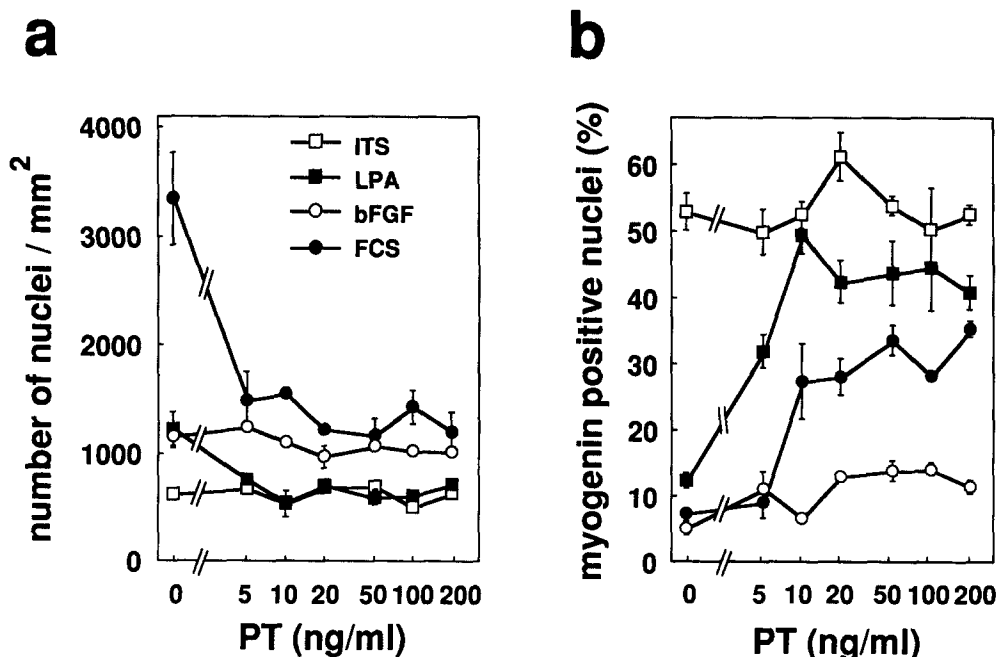
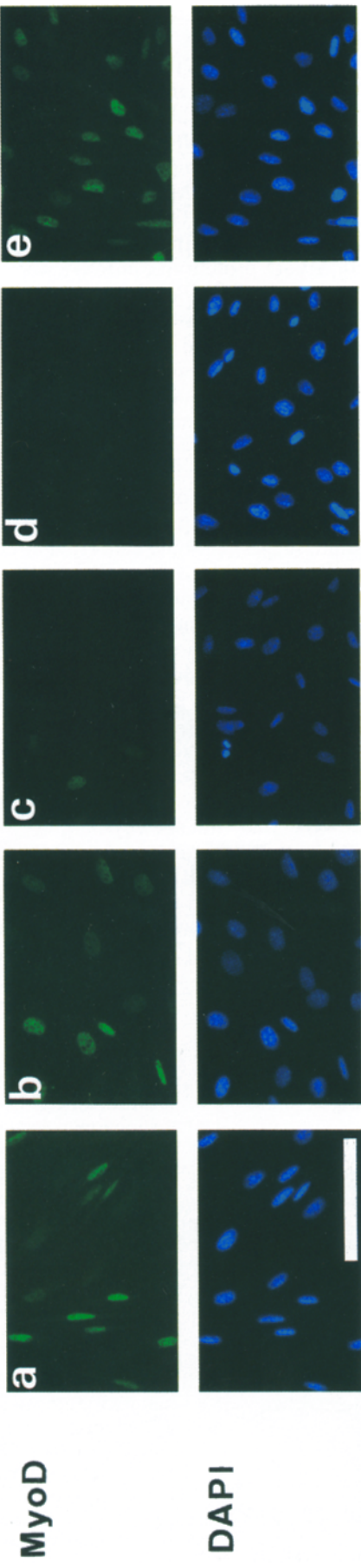
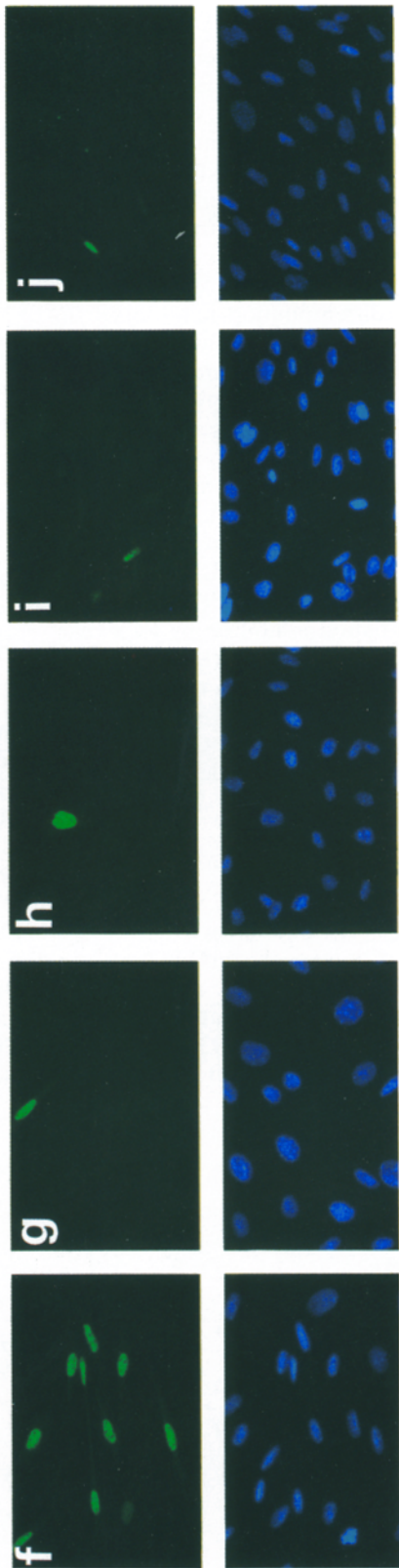


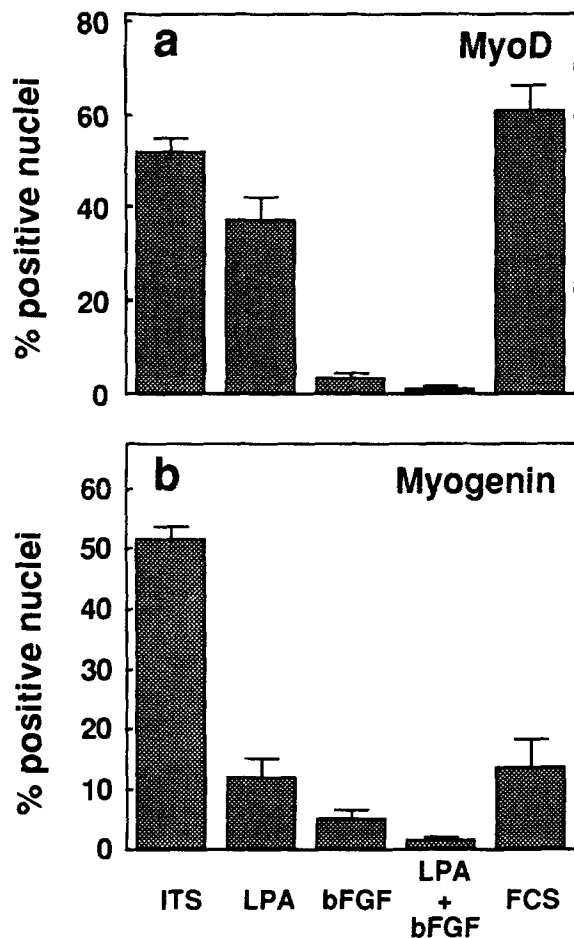
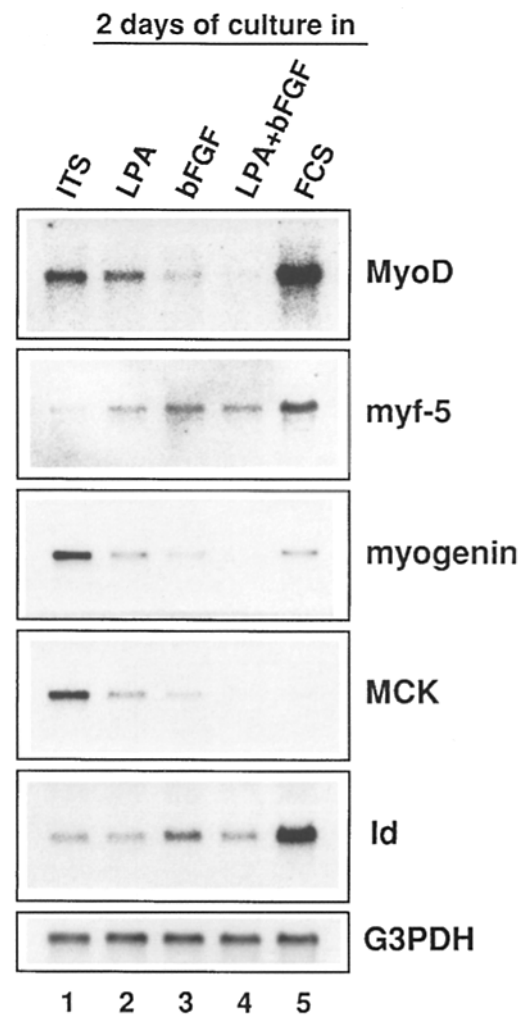
Figure 3. Effects of pertussis toxin on mitogenic and anti-differentiation activities of LPA and bFGF. The same protocol was used as in Fig. 1, except that the initial cell density was  $1.5 \times 10^4$  cells/well. 24 h after seeding in growth medium, cultures were switched to the experimental media containing mitogens and the titrated concentration of PT. After 48 h, cultures were fixed and stained for myogenin. Media were changed after 24 h. The total number of nuclei (a) and % myogenin positive nuclei (b) were determined for three independent fields. Bars indicate SEM.

**A**



**myogenin**



**B****C**

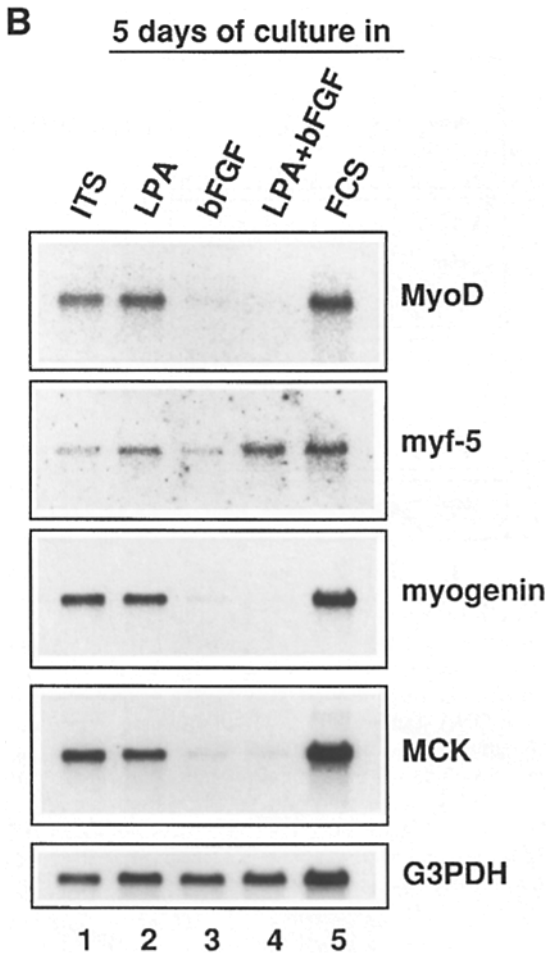
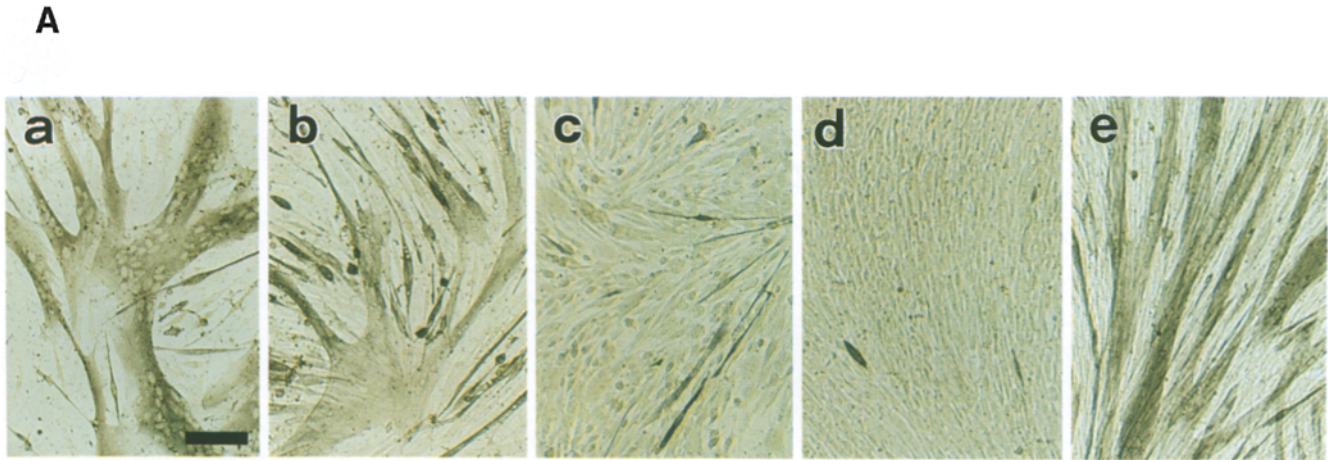
**Figure 4.** Expression of myogenic bHLH factors by proliferating C2C12 cells grown in LPA and bFGF. (A) Expression of MyoD and myogenin proteins. C2C12 cells were cultured for 2 d in ITS (a and f), ITS supplemented with LPA (b and g), bFGF (c and h), LPA and bFGF (d and i), or FCS (e and j). Cells were then fixed and subjected to indirect immunocytochemistry with anti-MyoD and anti-Myogenin antibodies, and then counterstained with DAPI. Photographs were taken under a fluorescent microscopy with a magnification of 200 $\times$ . Each upper and lower panel shows anti-MyoD or myogenin staining (FITC) or DNA staining with DAPI of the same representative field, respectively. Bar indicates 100  $\mu$ m. (B) Quantitation of MyoD (a) and Myogenin (b) positive nuclei. The same protocols as described in A was used and MyoD and myogenin positive nuclei were counted. Each data point was based on three independent determinations and shown as mean percent  $\pm$ SEM. (C) Northern blotting analysis. Poly(A) RNA were isolated from C2C12 cells grown for 2 d in ITS (lane 1) or ITS supplemented with LPA (lane 2), bFGF (lane 3), LPA and bFGF (lane 4), or FCS (lane 5). 5  $\mu$ g of poly(A) RNA was applied to each lane and Northern hybridizations were done as described in Materials and Methods. Identical filters were prepared and hybridizations were performed with the radioprobe indicated on the right. MCK, muscle creatine kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

duced to differentiate in ITS, Id level decreased dramatically (Fig. 4 C), a result consistent with previous observation (Benezra et al., 1990). When C2C12 cells were kept undifferentiated in the presence of LPA and/or bFGF, Id mRNA levels were significantly lower than in FCS and comparable to that in ITS (Fig. 4 C), indicating that Id is unlikely to play a major role in LPA- and bFGF-induced differentiation inhibition.

#### Distinct Differentiation Potentials in Response to Cell-Cell Contact

LPA and bFGF also differ in their effects on C2C12 cells

when the cells are cultured to confluence. It has been known that C2C12 myoblasts grown in a media containing high concentration of serum spontaneously differentiate to produce myotubes after reaching confluence. Similarly, C2C12 cells grown to confluence in LPA differentiated and formed large myotubes that expressed Troponin T (Fig. 5, A-b and e). Under these conditions, myogenin and MCK mRNAs were also expressed at high levels (Fig. 5 B). In contrast, confluence-induced differentiation was dramatically suppressed by bFGF; only small myotubes incorporating a few nuclei were formed (Fig. 5, A-c), and the expression of myogenin and MCK were very low (Fig. 5 B). The inhibition of differentiation caused by bFGF was



**Figure 5.** Differentiation of the confluent C2C12 cells in the presence of LPA and bFGF. (A) Troponin T staining of the confluent cultures. C2C12 cells were cultured as described in the legend of Fig. 1, in ITS medium (a), ITS supplemented with LPA (b), bFGF (c), LPA plus bFGF (d), or FCS (e). After 5 d with changing the media once a day, all cultures except for a reached confluence. Cultures were then fixed and stained with anti-Troponin T antibody by an enzymatic immunocytochemical technique. Bar indicates 100  $\mu$ m. (B) Northern blotting analysis. C2C12 cells were cultured as described in A. Isolation of poly(A) RNA and Northern blotting are described in the legend of Fig. 4 C.

augmented when LPA and bFGF were added simultaneously; only a small population of mononucleated cells (<0.1%) became positive for Troponin T (Fig. 5, A-d), and, myogenin and MCK mRNA expression were suppressed to an almost undetectable level (Fig. 5 B). In contrast to LPA or FCS, bFGF downregulated MyoD in confluent cultures. Unlike MyoD or myogenin, the expression of myf-5 transcripts was less influenced by these extracellular signals (Fig. 5 B).

These results suggest that LPA and bFGF each affect proliferating myoblasts differently in such a manner that

results in distinct modes in their expression pattern of myogenic bHLH factors and results in different competence for differentiation in response to cell-cell contact.

#### Contribution of LPA to Serum Activities

As described above, LPA shares a number of properties with serum, which lead us to evaluate its contribution to serum activities. We measured the LPA amount in our serum sample to be  $\sim 1.8 \mu$ g/ml (4.1  $\mu$ M). This is consistent with other groups' previous reports (Eichholtz et al., 1993;



Tokumura et al., 1994) and within the effective range for eliciting the growth-promoting and differentiation-inhibiting effects on C2C12 cells that we have demonstrated (Fig. 2 B).

We then examined the activities of platelet poor plasma-derived serum (PPP). To generate PPP, platelets, which release the majority of serum LPA when activated upon clotting (Eichholtz et al., 1993), were removed before clotting. Thus, PPP contains reduced quantities of LPA while retaining most other serum components. Indeed, we identified the concentration of LPA in PPP to be 0.19  $\mu\text{g/ml}$  (0.44  $\mu\text{M}$ ), one tenth that found in FCS. We found that PPP possesses some growth-promoting and differentiation-inhibiting activities, but these activities are weaker than those found in FCS (Fig. 6 A), and that addition of LPA to PPP strengthens both activities (Fig. 6 B). Moreover, we also observed that addition of suramin, an LPA antagonist (van Corven et al., 1992) inhibits these serum

activities (data not shown), although it must be noted that suramin can antagonize not only LPA but a broad spectrum of ligands (Coffey et al., 1987). Altogether, these results suggest that LPA is one of the components of serum that contribute to the control of growth and differentiation of myoblast cells.

## Discussion

### LPA Has Mitogenic and Anti-Differentiation Effects on Myoblasts

We have demonstrated the mitogenic and differentiation-inhibiting actions of LPA on C2C12 myoblasts. Among the wide variety of biological activities of LPA reported (for reviews see Moolenaar, 1994, 1995), this study is the first report to show that LPA controls both cell growth and dif-

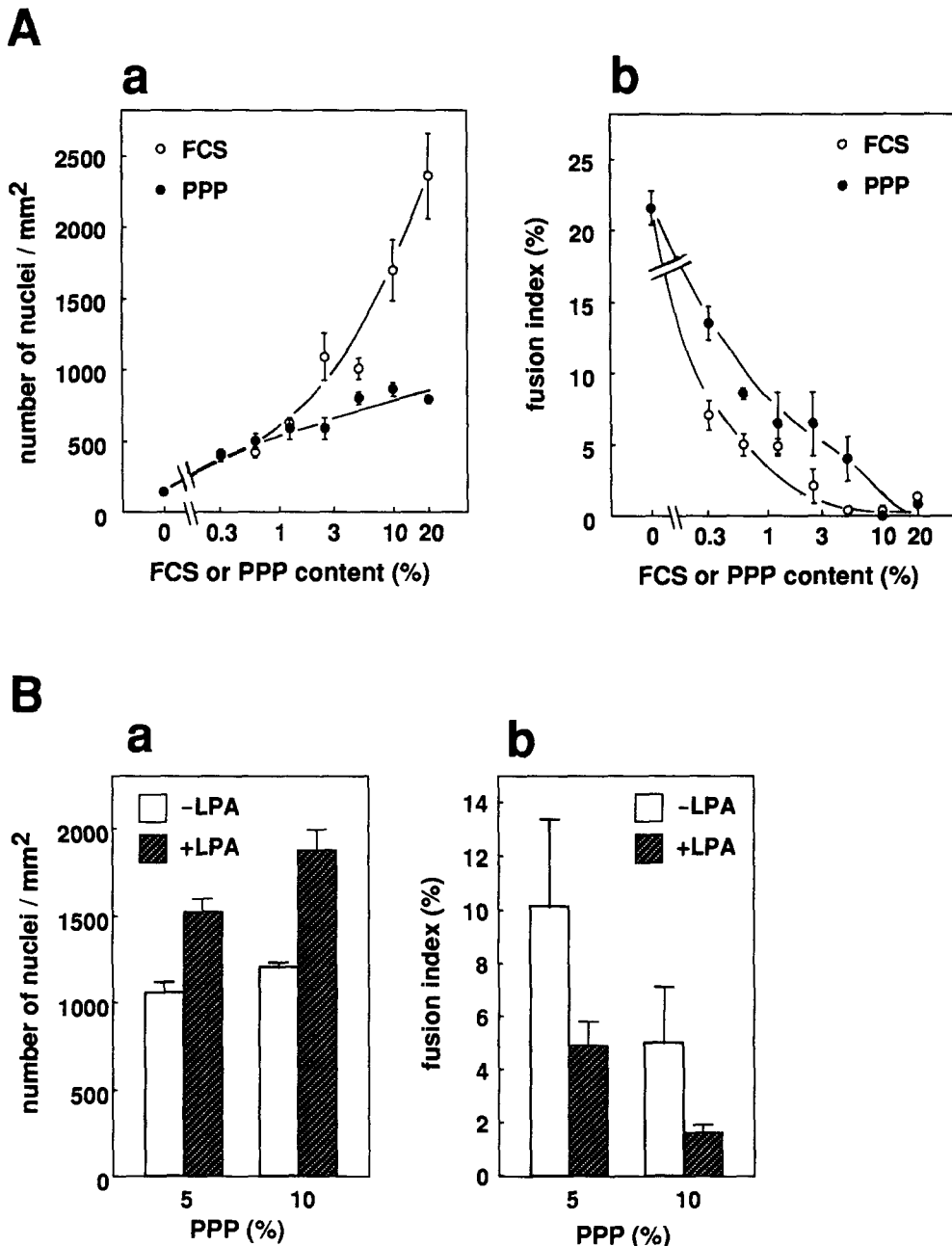


Figure 6. Effects of the platelet poor plasma-derived serum on C2C12 cells. (A) Growth-promoting (a) and differentiation-inhibiting (b) activities of FCS and platelet poor plasma-derived serum (PPP). After C2C12 cells were cultured as described in Fig. 1 in the ITS medium containing titrated concentrations of FCS or PPP for 3 d with the daily changes of medium, the resulting total number of nuclei (a) and fusion index (b) were determined. LPA contents in these FCS and PPP samples were 1.8  $\mu\text{g/ml}$  and 0.19  $\mu\text{g/ml}$ , respectively. (B) Addition of LPA to PPP raised its growth-promoting and differentiation-inhibiting activities. C2C12 cells were cultured and subjected to total nuclei number and fusion index determination, in the ITS medium containing indicated concentrations of PPP in the presence or absence of LPA (10  $\mu\text{g/ml}$ ). Each data point is represented as the mean  $\pm$  SEM of three determinations.

ferentiation. We also demonstrated that LPA actions were structure-specific and dose dependent. Both properties agree with previous studies indicating that LPA acts, through cell surface receptor(s), as a bioactive ligand (van Corven et al., 1989, 1992; Jalink et al., 1990).

LPA can elicit a variety of cellular responses, most of which are evoked by nanomolar concentrations of LPA, including  $Ca^{2+}$  mobilization (Jalink et al., 1990) and the formation of focal adhesions/actin stress fibers (Ridley and Hall, 1992, 1994). Because LPA-binding sites with kD on the nanomole order were detected in the membranes of Swiss 3T3 fibroblasts and rat brain (Thomson et al., 1994), it seems reasonable that LPA evokes these responses through activating this putative LPA receptor. But, in contrast, micromolar doses are required for the mitogenic action of LPA on fibroblasts (van Corven et al., 1989, 1993), and we figured out that mitogenic and anti-differentiation effects of LPA on myoblasts also require micromolar concentrations of LPA (Fig. 2 B). Intracellular signaling pathways leading to cell proliferation or differentiation inhibition might be activated only by higher concentrations of LPA through unidentified cell surface receptor(s). Alternatively, since these responses take long periods lasting for hours to days, LPA could be degraded and the actual concentration might be reduced as observed by van der Bend et al. (1992b). Further investigations are needed to clarify this issue.

#### *Gi-mediated LPA and RTK-mediated bFGF Inhibit the Differentiation by Distinct Mechanisms*

Both growth-promoting and differentiation-inhibiting signals activated by LPA are blocked by PT, indicating the involvement of Gi proteins. On the other hand, bFGF is known to act through cell surface RTKs (for reviews see Fantl et al., 1993; Jaye et al., 1992). Thus, C2C12 myoblasts have proved to be controlled by two distinct classes of mitogens; the Gi- and RTK-mediated mitogens. Such dual control of Gi- and RTK-mediated mitogens has been offered by others: thrombin and FGF on lung fibroblasts, and bombesin and PDGF on Swiss 3T3 fibroblasts (Chambard et al., 1987; Pouyssegur and Seuwen, 1992; Letterio et al., 1986). Those investigations demonstrated that both mitogens induce proliferation and early response gene expression in fibroblastic cells, but were unable to clarify a qualitative difference between these mitogens.

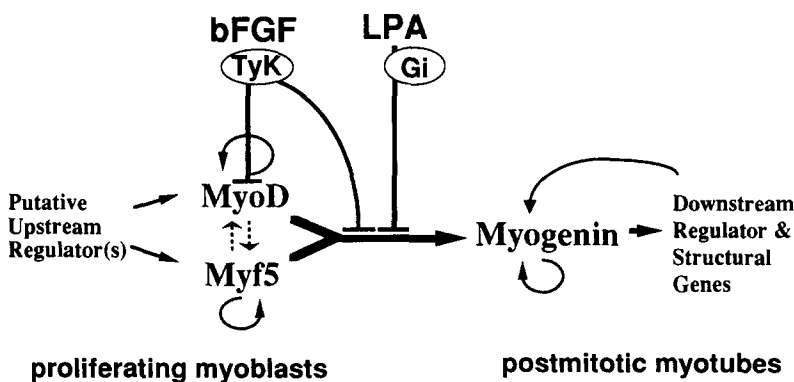
Our results provide an evidence that actions of these Gi-

and RTK-mediated mitogens are different in the control of the transcription network composed of the MyoD family of bHLH proteins; the MyoD gene is transcriptionally active in LPA-stimulated cells, but not in bFGF stimulated cells (Fig. 7). Upon terminal differentiation process, MyoD protein can transactivate the myogenin gene, without requiring de novo protein synthesis, which is considered to be among the earliest and the most important events in this process (Hollenberg et al., 1993). In the nuclei of the C2C12 cells proliferating in LPA, the MyoD protein is present but the myogenin gene is not activated (Fig. 4). Therefore, LPA must activate mechanisms that inhibit MyoD from activating the myogenin gene. The target of bFGF is different in that it inhibits the expression of MyoD at the transcriptional level (Fig. 5; and Vaidya et al., 1989). Coexistence of LPA and bFGF results in the suppression of MyoD, the bFGF-type phenotype (Fig. 4), suggesting that the action of bFGF dominates that of LPA, as shown in Fig. 7.

#### *LPA and bFGF Proliferate the C2C12 Cells as Myoblasts*

It should be mentioned that both LPA and bFGF proliferate the C2C12 cells with the myogenic potential maintained. Both the cells grown in LPA and/or bFGF were positive for Desmin, a marker for myogenic lineage; and they underwent differentiation to form myotubes when deprived of these factors (Yoshida, S., unpublished data). Considering that MyoD is expressed in LPA-stimulated cells but not in bFGF-stimulated cells, and that myf-5 is expressed in both cells (Fig. 4), we postulate that myf-5 plays an essential role to maintain the myogenic potential of the proliferating myoblasts, especially in the presence of bFGF. On the other hand, MyoD might play a role in maintaining the cells in a proliferating but ready-to-differentiate state to regulate the timing of terminal differentiation, in our in vitro system. Because myf-5 can activate the entire myogenic differentiation program in several non-muscle cells and MyoD knock-out mice (Braun et al., 1989, 1992), myf-5's activity to promote differentiation is inhibited in myoblasts growing in LPA or bFGF (Fig. 7).

We investigated the expression of Id, a negatively regulating HLH factor (Benezra et al., 1990; Jen et al., 1992), because it would throw light on the mechanism(s) with which LPA and bFGF keep the cells undifferentiated (Fig. 4 C). C2C12 cells growing in LPA and bFGF expressed



*Figure 7.* A schematic diagram of the probable target points of the anti-differentiation action of LPA and bFGF. LPA targets the transcriptional activation of the myogenin gene by the MyoD protein through a Gi protein dependent pathway. While bFGF, through receptor tyrosine kinases, inhibits not only activation of the myogenin gene by MyoD or myf-5, but also inhibits the expression of MyoD per se. The main transcriptional cascade of the MyoD family of bHLH proteins during differentiation is essentially the same as that proposed previously (Weintraub, 1993; Olson and Klein, 1994). *Gi*, Gi class of G protein; *TyK*, protein tyrosine kinase activities associate with FGF receptors.

only comparable levels of Id mRNA to that in differentiating cells in ITS, suggesting that Id is not always required for the inhibition of terminal differentiation. Other proposed mechanisms for differentiation inhibition, including induction of early response gene products, Jun, Fos or Myc (Rahm et al., 1989; Lassar et al., 1989; Bengal et al., 1992; Li et al., 1992a; Falcone et al., 1985; Denis et al., 1987), or activation of protein kinase C, which can phosphorylate and inhibit the function of myogenic bHLH factors (Li et al., 1992b), could be involved. Further investigations are needed to solve this issue.

### *Differentiation Induced by Cell-Cell Contact*

LPA and bFGF provide the C2C12 cells with distinct differentiation potentials in response to cell-cell contact; differentiation induced by cell-cell contact was inhibited by bFGF, but not by LPA. These findings suggest that intracellular signals activated by cell-cell contact, whose nature remains to be understood, can dominate the LPA signaling pathways that lead to growth stimulation and differentiation inhibition; but they do not affect, or are repressed by, signals activated by bFGF. Downregulation of the LPA receptors might be involved here since LPA binding ability has been shown to decrease in confluent cultures of Swiss 3T3 cells (Thomson et al., 1994). Investigations are still needed to reveal the mechanisms underlying these phenomena.

### *Contribution of LPA to Serum Activities*

LPA is contained in serum (Eichholtz et al., 1993; Tokumura et al., 1994) and was detected in a significant amount in our serum preparation. Then, we examined its contribution to the serum mitogenic and anti-differentiation activities, to determine if it can fully substitute for serum. We observed that PPP, which contained one tenth the LPA of FCS, had much lower activities than FCS in growth-promotion and differentiation-inhibition of myoblasts, and that addition of LPA to PPP increased these activities (Fig. 6). Moreover, suramin, an antagonist of LPA, repressed these effects of FCS (Yoshida, S., unpublished results). These findings support the idea that LPA is one of the serum components acting in its growth promotion and differentiation inhibition of myoblasts.

It is clear that LPA is not the only component responsible to the mitogenic and anti-differentiation actions of serum; the mitogenic activity of LPA, indexed by growth rate and BrdU incorporation, was significantly lower than that of serum in our study (Fig. 1). In addition, PT treatment was unable to completely inhibit the actions of serum (Fig. 3), and PPP promoted growth and inhibited differentiation to smaller extent than FCS (Fig. 6). Moreover, induction of Id requires serum component(s) other than LPA (Fig. 4). These indicate that a component(s) other than LPA is also involved in the growth-stimulating and differentiation-inhibiting effects of serum.

### *Possible Roles In Vivo*

LPA is thought to play an important role in wound healing and tissue regeneration because it is released by activated platelets upon clotting (Eichholtz et al., 1993; Tigy and

Miledi, 1992), and evokes a number of responses which seem to be important for wound repairing (discussed in Moolenaar, 1995). We and others (Eichholtz et al., 1993; Tokumura et al., 1994) have actually detected sufficient concentrations of LPA in serum to evoke mitotic response in myoblasts in vitro, indicating that platelets are capable of producing such high concentration of LPA upon clotting. Therefore, LPA might be involved in muscle regeneration; LPA is likely to be produced in the damaged muscle as a result of blood clotting and inflammation, and might stimulate the proliferation of resting myoblasts, the satellite cells, which is an initial and indispensable event for muscle regeneration (Allbrook, 1981). It is possible that LPA is also involved in embryonic myogenesis. Indeed, we have observed that LPA can stimulate the growth of L6 embryonic myoblast cells (Yoshida, S., unpublished results). The distribution of LPA and the LPA receptor, which has yet to be cloned during development, should be investigated to know whether LPA plays a role in embryonic myogenesis.

FGF has also been suggested to be involved in both muscle regeneration and embryonic myogenesis (Joseph-Silverstein et al., 1989; Gonzalez et al., 1990; Guthridge et al., 1992; Anderson et al., 1991). We speculate that if myoblasts are under the dual regulation of LPA and FGF, the distinct reactivities of LPA and bFGF to cell-cell contact would play an important role in the coordinated growth and differentiation of myoblasts; in the earlier stage of muscle formation, myoblasts might be required to proliferate to expand their population even in the differentiation promoting environment created by, for example, cell-cell contact. bFGF could provide myoblasts with this power. In later stages, on the other hand, myoblasts might have to proliferate with high differentiation potentials, preparing for timed growth arrest and terminal differentiation in response to extracellular cues. Myoblasts cultured in LPA might illustrate these properties in vitro.

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