

Lysophosphatidic Acid-Induced *c-fos* Up-Regulation Involves Cyclic AMP Response Element-Binding Protein Activated by Mitogen- and Stress-Activated Protein Kinase-1

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Abstract Lysophosphatidic acid (LPA) is a lipid growth factor that exerts diverse biological effects through its cognate receptor-mediated signaling cascades. Recently, we reported that LPA stimulates cAMP response element-binding protein (CREB) through mitogen- and stress-activated protein kinase-1 (MSK1). Previously, LPA has been shown to stimulate *c-fos* mRNA expression in Rat-2 fibroblast cells via a serum response element binding protein (SRF). However, involvement of CREB in LPA-stimulated *c-fos* gene expression is not elucidated yet. To investigate the CREB-mediated *c-fos* activation by LPA, various *c-fos* promoter-reporter constructs containing wild-type and mutated SRE and CRE were tested for their inducibility by LPA in transient transfection assays. LPA-stimulated *c-fos* promoter activation was markedly decreased when SRE and CRE were mutated. A dominant negative CREB significantly down-regulated the LPA-stimulated *c-fos* promoter activation. Chromatin immunoprecipitation assay revealed that LPA induced an increased binding of phosphorylated CREB and CREB-binding protein (CBP) to the CRE region of the endogenous *c-fos* promoter. Immunoblot analyses with various pharmacological inhibitors further showed that LPA induces up-regulation of *c-fos* mRNA level by activation of ERK, p38 MAPK, and MSK1. Taken together, our results suggest that CREB plays an important role in up-regulation of *c-fos* mRNA level in LPA-stimulated Rat-2 fibroblast cells. *J. Cell. Biochem.* 104: 785–794, 2008. © 2008 Wiley-Liss, Inc.

Key words: lysophosphatidic acid; *c-fos*; CREB; transcriptional regulation; protooncogene

Abbreviations used: LPA, lysophosphatidic acid; CRE, cAMP response element; CREB, cAMP response element-binding protein; MSK1, mitogen- and stress-activated protein kinase-1; SRE, serum response element; SRF, serum response factor; CBP, CREB-binding protein.

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Lysophosphatidic acid (LPA) is a lipid growth factor that plays important roles in many biological processes, such as oncogenesis, brain development, wound healing, and immune functions by regulating various cell functions, including cell proliferation, differentiation, migration, and survival [Moolenaar et al., 2004]. LPA signals transduce through at least five specific cell membrane-bound G protein-coupled receptors (GPCRs), designated as LPA1, LPA2, LPA3, LPA4, and LPA5 [Herr and Chun, 2007]. Binding of LPA to its cognate receptors elicits a variety of intracellular signaling cascades, including stimulation of phospholipase C and D, inhibition of adenylate cyclase, stimulation of small GTPase, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase [Herr

and Chun, 2007]. In addition to these changes in enzymatic activity within cytosol, LPA also has been shown to induce gene expression changes within nucleus, including up-regulation of protooncogene *c-fos* expression [Perkins et al., 1994; Hill and Treisman, 1995; Cook et al., 1999].

Activation of a protooncogene *c-fos* is one of the earliest known transcriptional responses to growth factors. When stimulated by mitogenic signals, the transcription rate of *c-fos* is dramatically increased within 5 min and reaches a maximal level by 10–15 min. Rapid increase in *c-fos* transcription rate causes sharp up-regulation of cytoplasmic *c-fos* mRNA level, followed by rapid degradation of the RNA, returning to pre-stimulation levels within 60–120 min [Greenberg and Ziff, 1984]. This rapid but transient expression of *c-fos* is a key event leading to alteration of various cell functions in response to growth factors and this cellular event reflects a sequence of growth-factor stimulation of many pre-existing cellular factors, including various MAPKs. Accumulation of *c-fos* protein out of constitutive concentration has been implicated in a wide variety of biological processes, including cell proliferation, differentiation, apoptosis, and oncogenesis [Treisman, 1985; Vadas and Pruzanski, 1986].

The serum response element (SRE) and the Sis-inducible element (SIE) on the *c-fos* promoter primarily mediate the response of *c-fos* gene expression to the growth factor induction [Treisman, 1995; Gonzales and Bowden, 2002]. A dimer of serum response factor (SRF), and one molecule of Elk-1 forms a ternary complex (TCF) and binds to the SRE located at –288 bp in the *c-fos* promoter [Norman et al., 1988; Shaw et al., 1989]. CRE-binding protein (CREB) binds to CRE at –60 bp in the promoter and also contributes to the response of *c-fos* to mitogenic stimuli [De Cesare et al., 1998]. Previous studies have shown that *c-fos* promoter elements can be activated by LPA through SRF binding to SRE [Hill and Treisman, 1995]. However, the specific contribution of CRE to the LPA-induced up-regulation of *c-fos* has not been fully evaluated yet.

Recently, we reported that LPA strongly stimulated CREB through mitogen- and stress-activated protein kinase-1 (MSK1) and that this MSK1-dependent CREB phosphorylation is mediated by ERK1/2 and p38 MAPK pathways in Rat-2 fibroblast cells [Lee et al., 2003]. Similar observation was reported in embryonic stem

cells [Schuck et al., 2003]. Since LPA activated not only *c-fos* promoter through SRE [Hill and Treisman, 1995], but also stimulated CREB phosphorylation [Lee et al., 2003], we reasoned that CREB might contribute to LPA-stimulated *c-fos* expression. This study was undertaken to test this possibility and to determine signaling pathways responsible for the activation of CREB. Herein, we provide evidence that CREB activation is necessary for maximal activation of *c-fos* by LPA and that activation of ERK, p38 MAPK, and MSK1 are responsible for the CREB activation.

RESULTS

LPA Increases the *c-fos* mRNA Level in a Dose- and Time-Dependent Manner

Dose–response relationships for LPA effects on the level of *c-fos* mRNA are shown in Figure 1A. The level of *c-fos* mRNA reached maximum at 5 μ M LPA. In addition, the exposure of Rat-2 fibroblast cells to 5 μ M LPA caused a time-dependent augmentation of *c-fos* mRNA, with a maximal increase at 30 min, as shown by Northern blot analysis (Fig. 1B). The level of *c-fos* mRNA returned to near basal level by 90 min, whereas the mRNA level of cyclophilin, a housekeeping gene, did not change for 2 h in the presence of 5 μ M LPA. The *c-fos* protein level paralleled the *c-fos*

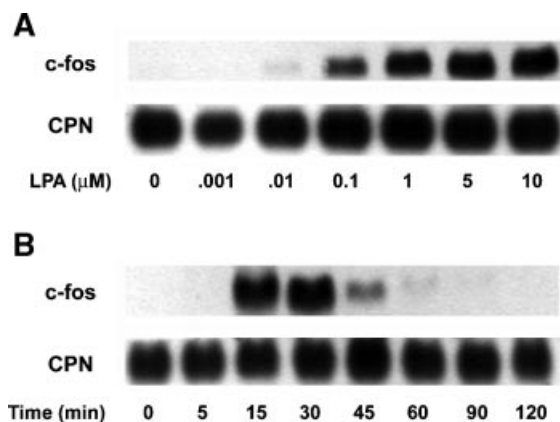


Fig. 1. Induction of the *c-fos* mRNA by LPA. Rat-2 cells were cultured in 60 mm culture dishes to near confluence and then starved in DMEM containing 0.5% FBS for 24 h. **A:** Serum starved cells were exposed to increasing concentrations of LPA for 30 min and Northern blot analysis was performed with a digoxigenin-labeled *c-fos* probe. **B:** Cells were treated with 5 μ M of LPA for indicated time. The level of the *c-fos* mRNA was detected by Northern blot analysis. Cyclophilin (CPN) was used as internal RNA standard.

mRNA level in both time- and dose-dependent manner (data not shown).

Full Activation of *c-fos* Promoter Requires CRE and SRE in LPA-Stimulated *c-fos* Gene Expression

Our previous report on the LPA-dependent activation of CREB phosphorylation [Lee et al., 2003] and current data on the time- and dose-response relationship of the *c-fos* mRNA (Fig. 1), prompted us to test involvement of CRE/CREB in the LPA-stimulated *c-fos* mRNA up-regulation in Rat-2 fibroblast cells. Past studies have focused mostly on the role of SRE in LPA-stimulated activation of *c-fos* gene expression in NIH3T3 cells [Hill and Treisman, 1995]. However, the role of CRE in LPA-stimulated activation of *c-fos* gene expression has not been assessed in detail. Thus, in order to resolve this issue, we investigated the effects of mutations on SRE- and CRE- in the *c-fos* promoter in LPA stimulated *c-fos* gene expression. Using PCR-based mutagenesis, we constructed promoter mutations that have been

tested previously [Hardingham et al., 1997]; pFos-mSRE (CCATATTAGG → CCCGATTGGG), pFos-mCRE (TGACGTTT → TGTGGTTT) (Fig. 2A). Promoter activity was tested with mutated promoter-luciferase constructs. LPA increased *c-fos* promoter activity in the cells transfected with wild-type construct (pFos-WT) by 3.4-fold. However, a point mutation in CRE site attenuated LPA-induced *c-fos* promoter activation by 25% (Fig. 2B). Furthermore, when cells were transfected with both pFos-mSRE and pFos-mCRE/mSRE, LPA-induced transcriptional activation was decreased by 80% and 92%, respectively. Taken together, these data demonstrate that in addition to SRE, CRE is required for maximal *c-fos* promoter activation triggered by LPA.

CRE and SRE Play Independent Roles in LPA-Stimulated *c-fos* Promoter Activity

Our data on the effect of mutated *c-fos* promoter (see Fig. 2A) suggested that CRE is required for full activation of *c-fos* promoter

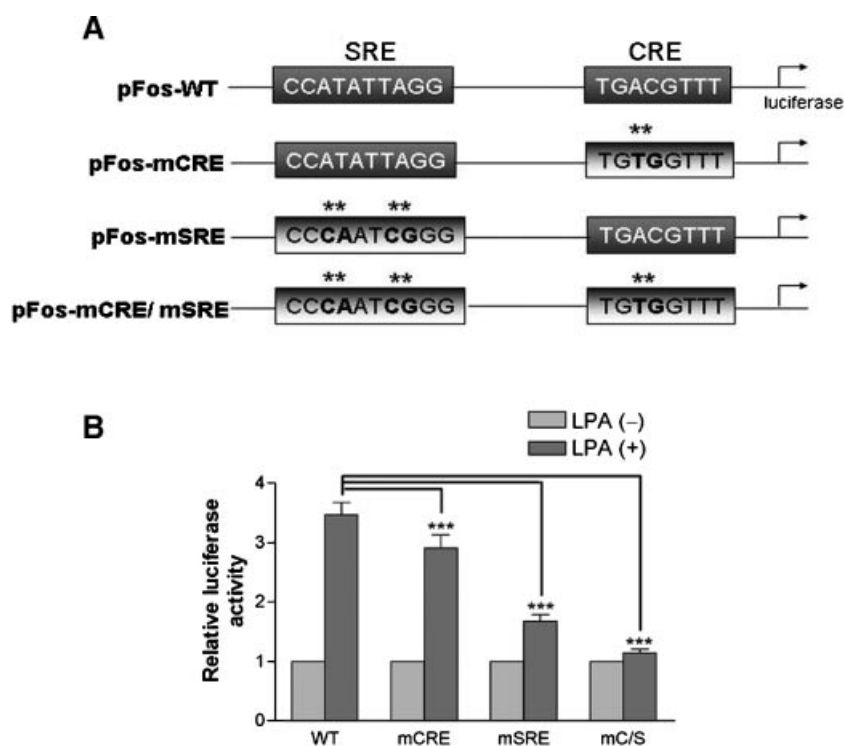


Fig. 2. Involvement of SRE and CRE sites in *c-fos* transcriptional induction by LPA. **A:** A schematic diagram of the *c-fos* promoter illustrating the in-context mutations. **B:** Luciferase assay was used to measure activation of the *c-fos* promoter after transfection of wild-type (pFos-WT) or one of the following plasmids containing in-context mutations: mutation of CRE (pFos-mCRE), SRE (pFos-mSRE), or both (pFos-mCRE/mSRE). Cell lysates were isolated from unstimulated cells or cells stimulated with LPA (5 μ M). Results are the means \pm SE of six independent experiments and each are carried out in triplicate ($P < 0.001$).

activity in LPA-stimulated Rat-2 cells. However, the extent of CRE contribution was much less than that of SRE (see Fig. 2B). Therefore, to further verify if CRE indeed contributes to the LPA-stimulated *c-fos* promoter activation, we performed transient transfection experiment with a chimeric promoter constructs which consisted of four-times tandemly repeated CRE elements (4×CRE) fused to a heterologous minimal promoter from thymidine kinase of herpes simplex viral origin. When this 4×CRE-thymidine kinase minimal promoter construct (4×CRE-Luc) was transfected and stimulated by various concentrations of LPA, the promoter activity exhibited up to 4.9-fold induction by 5 μ M LPA treatment (Fig. 3A). Similarly, 5 μ M LPA also increased the activity of SRE-containing minimal promoter, SRE-Luc by 9.2-fold. This result further suggests that CRE is involved in LPA-stimulated up-regulation of *c-fos* promoter activity.

A Dominant Negative CRE Corroborates Involvement of CRE in LPA-Induced *c-fos* Promoter Activation

Since we showed that CRE is required for LPA-stimulated activation of *c-fos* promoter, we next attempted to test whether CREB is also involved. To address this, we adopted a dominant negative CREB, A-CREB, whose basic residues within the bZIP domain have been mutated to acidic residues and thereby preventing its binding to the CRE [Ahn et al.,

1998]. A-CREB partially but significantly attenuated *c-fos* promoter activity stimulated by 5 μ M LPA (Fig. 4). This result suggests that the binding of CREB to the CRE is necessary for LPA-induced *c-fos* promoter activation in Rat-2 fibroblast cells.

Chromatin Immunoprecipitation Confirms LPA-Stimulated Binding of Phospho-CREB to CRE Region of the *c-fos* Promoter

Having shown the involvement of CREB, we next questioned if the LPA-stimulated CREB physically binds to the CRE in the chromatin-associated *c-fos* promoter region in vivo. To test this possibility, we employed the chromatin immunoprecipitation (ChIP) analysis with antibodies against the CREB, p-CREB, and CBP. As shown in Figure 5, the extent of CRE bound with phospho-CREB within the *c-fos* promoter sequences spanning -167 bp to $+41$ bp from the transcription initiation site, was significantly increased by treatment of 5 μ M LPA (lanes 5 and 6). Furthermore, LPA treatment also increased the binding of CREB binding protein (CBP), a coactivator protein that are known to be recruited upon binding of CREB on CRE element (lanes 7 and 8).

LPA-Induced Up-Regulation of *c-fos* mRNA Involves CREB Phosphorylation Through MSK-Dependent Signaling Pathway

In order to elucidate whether MSK1-activated CREB that was previously observed in our

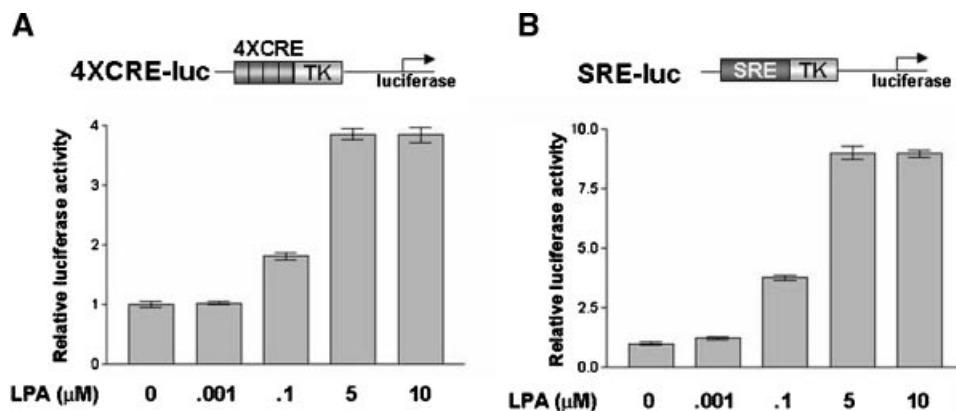


Fig. 3. Confirmation of CRE or SRE activity using heterologous thymidine kinase minimal promoter harboring 4×CRE or SRE sequences. Rat-2 cells were cultured in 24-well plates to 80–90% confluence. **A:** Cells were transiently transfected with the 4×CRE-luciferase construct. To measure the efficiency of transfection, pRSV β -galactosidase was used. Cells were incubated for 48 h after transfection, and then starved for 24 h. Cells were treated with indicated concentrations of LPA for 4 h.

Luciferase activity was measured and was normalized with β -galactosidase activity as described in "Materials and Methods Section." **B:** Cells were transfected with the SRE-luciferase construct. After starvation, cells were stimulated with various concentrations of LPA for 4 h and relative luciferase activities were measured. Results are the means \pm SE of four independent experiments, each carried out in triplicate ($P < 0.001$).

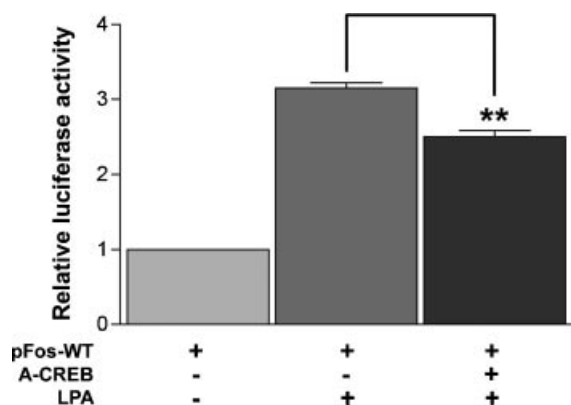


Fig. 4. The effect of a dominant negative CREB mutant (A-CREB) on the LPA-induced activation of *c-fos* promoter. Cells were transiently co-transfected with the reporter plasmid containing wild-type *c-fos* promoter (pFos-WT) and empty vector or A-CREB. After serum starvation, cells were exposed to 5 μ M LPA for 4 h. The luciferase activities of pFos-WT construct were measured. The results were obtained from four independent experiments, each carried out in triplicate ($P < 0.01$).

laboratory [Lee et al., 2003] is actually involved in LPA-stimulated up-regulation of endogenous *c-fos* mRNA, the effect of H89, inhibitor of MSK-1, was tested. As expected, the induction of endogenous *c-fos* mRNA by LPA was down-regulated by H89, suggesting a role of MSK1/CREB pathway for induction of *c-fos* mRNA. Involvement of immediate upstream regulators of MSK1 was further verified. To further test whether chromosome-bound phospho-CREB, which correlates with *c-fos* up-regulation by LPA (see Fig. 5), is activated through MSK-1

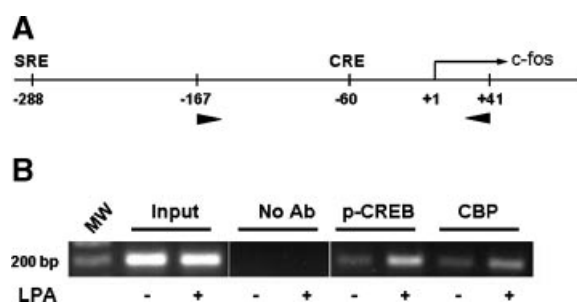


Fig. 5. Verification of p-CREB and CBP binding on *c-fos* promoter by Chromatin immunoprecipitation (ChIP) assay. **A:** A schematic diagram represents the location of primers used for ChIP assay. The arrow heads indicates primers located between -288 bp and +41 bp from the transcription-starting site in *c-fos* promoter region. **B:** ChIP assays were performed as described in Materials and Methods Section. Cells were treated for 5 min with 5 μ M LPA or without LPA, prior to ChIP assay. "Input" indicates chromatin samples analyzed prior to immunoprecipitation to assure the equal amount of starting material.

signaling pathway, effects of various pharmacological inhibitors of protein kinases on the phosphorylation of CREB was examined. LPA-induced CREB phosphorylation was reduced by PD98059, SB203580, and H89, inhibitors of protein kinases, MEK, p38 MAPK, and MSK1, respectively (Fig. 6B). This result indicates that LPA activates transcription factor CREB through ERK/MSK1 and p38 MAPK/MSK1 pathways, and that LPA-stimulated phosphorylation of CREB eventually participates in the *c-fos* gene expression in Rat-2 fibroblast cells (Fig. 7).

DISCUSSION

The question addressed in this study was whether CREB is involved in regulation of *c-fos* mRNA in Rat-2 fibroblast cells treated with LPA. The main finding of this study is that CREB is necessary for up-regulation of *c-fos* mRNA in LPA-stimulated Rat-2 fibroblast cells. In addition, we demonstrated that phospho-CREB activated by MSK1 signaling pathway was involved in up-regulation of *c-fos* mRNA level. Our results incorporate previous studies

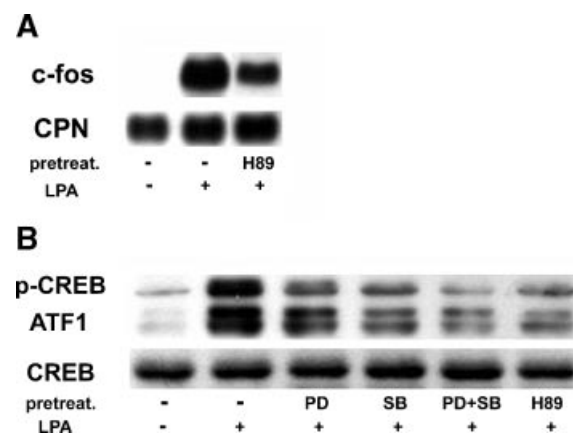


Fig. 6. Involvement of MSK-dependent signaling pathway in the LPA-induced CREB phosphorylation and *c-fos* mRNA induction. **A:** Confluent Rat-2 fibroblast cells were starved for 24 h, cells were pretreated with vehicle (DMSO) or 10 μ M H89 for 1 h, and then incubated with 5 μ M LPA for 30 min. Total RNAs were isolated and analyzed by Northern blot analysis with DIG-labeled *c-fos* probe or cyclophilin probe as an internal loading control. **B:** Cells were starved for 24 h and then pretreated in the absence (-) or presence (+) of 50 μ M PD98059, 10 μ M SB203580 or 10 μ M H89 for 1 h followed by incubation with 5 μ M LPA for 5 min. Whole cell lysates were subjected to 10% SDS-PAGE and immunoblotted with phospho-specific CREB antibody or anti-CREB antibody as described in "Materials and Methods Section."

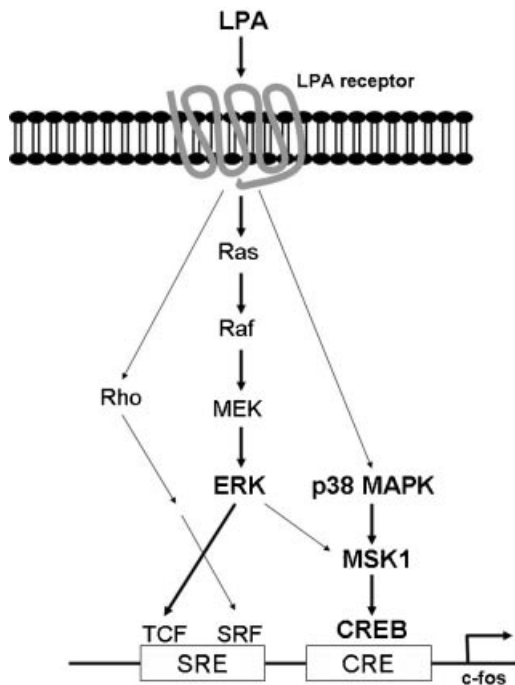


Fig. 7. A proposed model for the CREB signaling cascade in LPA-induced *c-fos* up-regulation in Rat-2 cells. LPA stimulates rapid activation of ERK and p38 MAPK, followed by activation of MSK1. The activated MSK1 mediates the phosphorylation of CREB, subsequently leading to transcriptional activation of the *c-fos* gene.

[Perkins et al., 1994; Hill and Treisman, 1995; Gineitis and Treisman, 2001], and add new observations that lead to more detailed understandings on the role of CREB in LPA-induced signaling pathway.

Investigations from other laboratories have shown that LPA can up-regulate *c-fos* gene expression mostly by the activation of SRE [Perkins et al., 1994; Hill and Treisman, 1995; Gineitis and Treisman, 2001]. Recently, involvement of CRE in LPA-induced *Egr-1* gene regulation were reported [Cui et al., 2006]. However, the involvement of CRE in LPA-stimulated *c-fos* mRNA regulation has not been clearly defined yet. Previous study from our laboratory has shown that LPA stimulates CREB through kinase MSK-1 in Rat-2 fibroblast cells [Lee et al., 2003], but we did not evaluate the functional significance of LPA-stimulation of CREB. Thus, in this study we tested whether the LPA-stimulated CREB can up-regulate *c-fos* mRNA level, since the *c-fos* gene harbors well-characterized functional CRE on its proximal 5'-flanking region of the transcription initiation site.

Evidences that CRE is involved in LPA-stimulated up-regulation of *c-fos* mRNA are supported by our experimental results. First, the *c-fos* promoter activity stimulated by LPA was markedly decreased when CRE sequences of the native *c-fos* promoter were mutated (Fig. 2). Second, a chimeric promoter construct consisted of four-times tandemly repeated CRE fused to a heterologous minimal promoter from thymidine kinase of herpes simplex virus was also induced by LPA, indicating that LPA can activate CRE that would result in up-regulation of *c-fos* mRNA (Fig. 3). Third, LPA-stimulated activation of *c-fos* promoter activity was effectively attenuated by a dominant negative CREB, A-CREB, suggesting that the binding of CREB to CRE is an important requirement for normal *c-fos* gene expression in LPA-stimulated Rat-2 cells (Fig. 4). Finally, the ChIP analysis further revealed that LPA enhanced binding phospho-CREB to the CRE of the innate *c-fos* promoter, indicating an important role of CRE in LPA stimulated cells (Fig. 5).

The *c-fos* gene is the prototypical signal-regulated immediate early response gene that has been used in many studies as a paradigm for understanding how signals generated by growth factors are transduced to the nucleus for regulation of gene expression [Herschman, 1991; Robertson et al., 1995]. Lipid growth factors, including LPA, has been known to activate the Ras-dependent Raf-MEK-ERK cascade [Cook et al., 1993; Howe and Marshall, 1993; Hordijk et al., 1994]. When activated, ERK accumulates in the nucleus, binds to Elk-1 and forms a TCF with SRF [Marais et al., 1993; Treisman, 1994; Gille et al., 1995], thereby stimulating *c-fos* expression. In addition, SRF responds to growth factor signaling pathways and plays a critical role in mediating the effects of serum and LPA on *c-fos* expression [Hill and Treisman, 1995]. Previous studies have mostly dealt with the involvement of SRF on the activation of *c-fos* gene expression, but the role of CREB in LPA-stimulated cells has not been studied in detail. Thus, we compared the relative contribution of SRE-regulated and CRE-regulated pathways in *c-fos* expression using the reporter plasmids containing point mutations of the promoter region as readout of the endogenous *c-fos* expression by LPA (Fig. 2). Mutation of SRE on the *c-fos* promoter attenuated LPA-induced activation of *c-fos* promoter, which is consistent with previous

studies [Hill and Treisman, 1995]. Interestingly, by introducing plasmids containing in-context mutation of CRE site on the promoter region of *c-fos*, we found that the activation of *c-fos* promoter is partially reduced by LPA, but the reduction was significant ($P < 0.0001$). These data indicate that CRE as well as SRE is important in the *c-fos* gene expression by LPA (Figs. 2 and 3).

The level of *c-fos* mRNA is dramatically up-regulated by LPA more than ten fold (Fig. 1), while transcriptional activation of *c-fos* promoter is only induced about 3.4-fold by LPA (Fig. 2B). Although we have not addressed this difference in our study, there are several previous observations that could explain this difference. One is the difference in chromatin structure and gene activation mechanism between endogenous *c-fos* chromatin and the reporter plasmids [Georgel, 2002; McCall et al., 2003]. The other is existence of distinct machineries accounted for mRNA stability or other unidentified *cis*-acting elements that may cause this difference [Chen et al., 1994, 2002].

LPA-induced up-regulation of *c-fos* mRNA level along with LPA-induced phosphorylation of CREB was blocked by H89, one of pharmacological inhibitors that block the function of CREB kinase MSK-1 (Fig. 6A) [Chijiwa et al., 1990], which suggests that CREB phosphorylation by MSK-1 might be involved in LPA-stimulated up-regulation of *c-fos* mRNA level.

As shown in Figure 6B, LPA-induced CREB phosphorylation was markedly reduced by protein kinase inhibitors H89, an MSK1 inhibitor. High concentration of H89 is also reported to inhibit protein kinase A (PKA) [Chijiwa et al., 1990]. In order to exclude PKA induced CREB phosphorylation in our study, we used Rp-cAMP, a selective inhibitor of PKA. As the result, we found that Rp-cAMP did not block phosphorylation of CREB by LPA (data not shown [Lee et al., 2003]). Taken together, we conclude that PKA is not involved in LPA-stimulated phosphorylation of CREB. Although it appears that MSK-1 plays a major role in phosphorylation of CREB in LPA-stimulated Rat-2 fibroblast cells, we do not exclude possible involvement of other kinases known to phosphorylate CREB.

In previous studies, it has been shown that CREB ser-133 phosphorylation do not change the affinity of CREB for the somatostatin CRE, but greatly increase the affinity of CREB for

the tyrosine aminotransferase (TAT) CRE [Weih et al., 1990]. Several other studies have also failed to detect phosphorylation-induced changes in the CREB/DNA interaction, with respect to both the somatostatin CRE and other CREB-binding elements, such as the Tax-response element [Hagiwara et al., 1993; Anderson and Dynan, 1994]. Likewise, as shown in our ChIP experiment result (Fig. 5), CREB/CRE interaction at the *c-fos* promoter also was not altered by stimulation of LPA (data not shown), whereas the level of phospho-CREB bound to CRE site was clearly increased by LPA. Additionally, the ChIP experiment also demonstrated that there is an increase in recruitment of CBP molecules to the *c-fos* promoter in LPA-stimulated Rat-2 cells, thus corroborating our notion that *c-fos* activation by LPA involves the functional interaction of CREB and its associated proteins with CRE on the *c-fos* promoter.

In summary, we have found that CREB is an important component of transcriptional regulatory elements necessary for activation of *c-fos* in LPA-stimulated Rat-2 fibroblast cells, and that a phosphorylation of CREB induced by MSK1 is associated with up-regulation of *c-fos* gene expression by LPA.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade if not stated otherwise. LPA (1-oleoyl-sn-glycerol 3-phosphate) was purchased from Sigma (St. Louis, MO). Antibodies to phospho-CREB/ATF1 and CREB were obtained from Cell Signaling Technology (Beverly, MA). PD98059 and SB203580 were from Tocris (Bristol, UK), and H89 from Biomol (Plymouth Meeting, PA). Luciferase assay system and pGL3-Basic plasmid were purchased from Promega (Madison, WI). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and Lipofectamine 2000 were obtained from Gibco/BRL (Grand Island, NY). Rat-2 cells, SRE-luciferase and human -711 *c-fos*-luciferase plasmids were a gift from Dr. J-H. Kim (Korea University, Seoul).

Cell Culture

Rat-2 fibroblast cells were grown at 37°C under a humidified atmosphere of 5% CO₂. Cells were cultured in DMEM, 10% FBS, penicillin (50 U/ml), and streptomycin (50 µg/ml). For LPA

stimulation, cells were starved with DMEM containing 0.5% FBS. After 24 h, the cells were treated with various inhibitors as described in the figure legends, prior to LPA stimulation.

Northern Blot Analysis

Total RNA was isolated using a rapid guanidine thiocyanate–water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate [Chomczynski and Sacchi, 1987]. Total RNA (5 µg) was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham, Buckinghamshire, UK). After UV cross-linking, the membranes were pre-hybridized at 68°C for at least 1 h in prehybridization buffer containing 50% formamide. The *c-fos* probe [Curran et al., 1987] and cyclophilin probe [Danielson et al., 1988] were synthesized by *in vitro* transcription. Digoxigenin (DIG)-labeled RNA probes were synthesized with a DIG RNA Labeling Kit (Roche) according to the instruction manual. The DIG-labeled probes were added to prehybridization buffer containing 50% formamide. The membrane was incubated overnight at 68°C in a rolling hybridization oven. After washing the membrane, the phosphatase complex on the nylon membrane was visualized with chemiluminescent substrate CSPD (Roche).

Isolation of Total Protein and Western Blot Analysis

Rat-2 cells were starved on 60 mm culture dishes in DMEM with 0.5% FBS for 24 h. Cells were pretreated with various compounds as indicated in each figure legend, stimulated with LPA, and then washed twice with ice-cold PBS. Cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM Na₃VO₄, 5 mM NaF and protease inhibitor cocktail). After incubation on ice for 30 min with vortexing every 10 min, the lysates were centrifuged (15,000g, 15 min). Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of protein were separated by SDS–PAGE (8% or 10% reducing gels), transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 5% non-fat milk. Membranes were incubated in primary antibody overnight at 4°C. Membranes were then washed in TBST (10 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.6),

incubated with appropriate secondary antibody, and washed again in TBST. Bands were visualized by enhanced chemiluminescence and exposed to X-ray film.

Site-Specific Mutagenesis

All *c-fos* mutants of promoter regions were derivatives of the wild-type *c-fos* plasmid that contains –711 bp of 5' flanking sequences. The following mutants were generated by PCR-based mutagenesis, and plasmids were verified by sequencing. Forward primer and reverse primer were designed to create *Xho*I and *Hind*III sites, respectively. Oligonucleotides containing the following wild-type and mutated sequences where changes from wild-type are underlined: Wild-type *c-fos* SRE, 5'-TTACA-CAGGATGCCATATTAGGACATCTGCGT-CAG-3'; mutation of the SRE site (mSRE-*Fos*), 5'-TTACACAGGATGTCCCAATCGGGACATCTGCGTCAG-3', wild-type *c-fos* CRE, 5'-GGT-TGAGCCCGTGACGTTTACTCATTC-3'; mutation of the CRE site (mCRE), 5'-GGT-TGA-GCCCGTGTGGTTTACTCATTC-3'. To study transcriptional activation, the oligonucleotides were inserted into the *Xho*I and *Hind*III sites of pGL-3 vector (Promega) to generate plasmids mSRE-*Fos*-Luc, mCRE-*Fos*-Luc and mCRE/mSRE-*Fos*-Luc.

Transient Transfection and Luciferase Assay

Rat-2 cells were cultured to 80–90% confluence for transfection experiments in 24-well plates. For each well, Lipofectamine 2000 reagent was used according to the manufacturer's instructions. Plasmids with four consecutive CRE sequences (5'-TGACGTCC-3'), fused to luciferase expression vector (CRE-Luc), were kindly provided by Dr. Hiroshi Ishiguro (Organon, Japan). A dominant negative CREB, A-CREB, was a gift from Dr. David D. Ginty (Johns Hopkins University). β-Galactosidase gene driven by the RSV promoter was cotransfected to normalize the transfection efficiency. DNA and the Lipofectamine 2000 reagent were diluted separately in 50 µl of serum-free medium without antibiotics, mixed together, and incubated at room temperature for 30 min. The culture plates were washed with phosphate-buffered saline (PBS), and 400 µl of 1% serum and antibiotic-free medium was added. The 100 µl of the plasmid/Lipofectamine 2000 mixture was then gently added to each well, and the plates were incubated at 37°C for

6 h. Then the cells were washed with PBS and added DMEM containing 0.5% FBS for 42 h. Thereafter, cell extracts were prepared by rinsing each plate twice with PBS and lysing the cells in 150 μ l of Reporter Assay Lysis Buffer (Promega). The lysed cells were collected and the supernatants were assayed for luciferase and β -galactosidase activities. They were measured with a Promega luciferase assay system using an Autolumat Lumino-meter. Statistical analysis was carried out by Student's *t*-test.

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP analyses were performed according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). Cells (one 150-mm dish) were cross-linked with 1% formaldehyde for 10 min at room temperature. After washing twice with ice-cold PBS containing protease inhibitors, scraping and centrifugation. Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, 1 mM Na₃VO₄, and protease inhibitor cocktail). After incubation for 10 min at 4°C, cell lysates were sonicated with six times for 15 s on ice. After centrifugation, the supernatant was precleared by incubation with 40 μ l of 50% (v/v) protein A/G agarose beads for 2 h at 4°C. Precleared supernatant was incubated overnight at 4°C with anti-CREB, anti-p-CREB, or anti-CBP (Santa Cruz Biotechnology) on a rocking platform. Immune complexes were recovered by the addition of 40 μ l of 50% (v/v) protein A/G agarose beads and incubation for 2 h at 4°C. The agarose beads were sequentially washed 5 times with lysis buffer, once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl) and high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% SDC, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)) and finally twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immune complexes were eluted by incubation with 500 μ l elution buffer (1% SDS, 0.1 M NaHCO₃) for 15 min at room temperature. In order to reverse the cross-linking of immune complexes, the samples were incubated for overnight at 65°C. After treatment with proteinase K for 1 h at 55°C, DNA was recovered by phenol-chloroform extraction

and isopropanol precipitation with 20 μ l of 5 M NaCl. DNA was detected by PCR with pair of primers specific to the *c-fos* promoter region containing CRE, forward primer 5'-ACAGACT-GAGACGGGGGTT-3' and reverse primer 5'-GGCTCTATCCAGTCTTCTCA-3' PCR reaction was carried out for 30 cycles.

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