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Induction of Novel CREB Kinase during Neuronal Differentiation

## **Basic Fibroblast Growth Factor-induced Activation of Novel CREB Kinase During the Differentiation of Immortalized Hippocampal Cells\***

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**Abstract:** Growth factors bind to their specific receptors on the responsive cell surface and thereby initiate dramatic changes in the proliferation, differentiation, and survival of their target cells. In the present study we have examined the mechanism by which growth factor-induced signals are propagated to the nucleus, leading to the activation of transcription factor, *cis*-acting cAMP response element (CRE)-binding protein (CREB), in immortalized hippocampal progenitor cells (H19-7). During the differentiation of H19-7 cells by basic fibroblast growth factor (bFGF) a critical regulatory Ser-133 residue of CREB was phosphorylated followed by an increase of CRE-mediated gene transcription. Expression of S133A CREB mutants blocked the differentiation of H19-7 cells by bFGF. While the kinetics of CREB phosphorylation by EGF was transient, bFGF induced a prolonged pattern of CREB phosphorylation. Interestingly, bFGF-induced CREB phosphorylation and subsequent CRE-mediated gene transcription is not likely to be mediated by any of previously known-signaling pathways that lead to phosphorylation of CREB, such as MAP kinases, protein kinase A, protein kinase C, phosphatidylinositol 3-kinase-p70<sup>S6K</sup>, calcium/calmodulin-dependent protein kinase, and casein kinase 2. By using *in vitro* in-gel kinase assay the presence of a novel 120-kDa bFGF-inducible CREB kinase was identified. These findings identify a new growth factor-activated signaling pathway that regulates gene expression at the CRE.

**Key Words:** CRE, CREB, H19-7, bFGF, EGF, Neuronal Differentiation

**Running title:** Induction of Novel CREB Kinase during Neuronal Differentiation

## INTRODUCTION

Growth factors act by binding to cell surface receptors to elicit the regulation of cell growth and differentiation. This, in turn, triggers a variety of intracellular signaling pathways that ultimately control cell physiology. Activation of signaling cascades results in the pattern of gene expression through the functional modulation of various transcription factors.

Basic fibroblast growth factor (bFGF)<sup>1</sup> is a potent mitogenic factor that is also known to initiate changes important for neural differentiation, survival, and plasticity (1). Mechanisms underlying these diverse actions of bFGF are not well understood but may result, at least in part, from distinct signaling pathways controlling gene expression. The bFGF receptor belongs to the tyrosine kinase class of membrane receptors. The binding of bFGF to its receptor results in the activation of Ras-dependent mitogen-activated protein kinase (MAPK) cascade (2). Coupled phosphorylation events induce the sequential activation of Raf-1 kinase, MAPK kinase (MEK), and MAPK (or extracellular signal-regulated protein kinase, ERK) (3).

Multiple signaling pathways converge at the level of the cyclic AMP response-element (CRE)-binding protein (CREB), a transcriptional factor that regulates expression of CRE-containing genes (4). CREB mediates cellular responses to a variety of physiological signals, including neurotransmitters, depolarization, synaptic activity, mitogenic and differentiating factors, and stressors (5-9). Upon phosphorylation at Ser-133, CREB can facilitate transcriptional activation of the genes containing the CRE motif (10). The activity of CREB is regulated after various kinds of stimulation by

multiple kinases, including protein kinase A (PKA), protein kinase C (PKC), isozymes of calcium/calmodulin-dependent kinase (CaM kinase) (11-13), p90<sup>RSK</sup> (14), p70<sup>S6K</sup> (15) and MAPK-activated protein (MAPKAP) kinase-2 (7). Moreover, phosphorylation of CREB at Ser-133 regulates expression of the *c-fos*, somatostatin, and tyrosine hydroxylase genes in PC12 cells (10,16).

To identify signaling pathways transmitting extracellular FGF signal to nucleus, the induction mechanism of immediate early gene *pip92* by bFGF was previously studied in rat hippocampal progenitor H19-7 cells. While signaling through Raf-1 occurs exclusively through the SRE, bFGF can also activate a region of the *pip92* promoter that contains a CREB binding site near the site of transcription initiation (17). Given the role for CREB in regulating genes which mediate a multitude of cellular responses, and to investigate the signaling mechanisms by which bFGF regulates gene expression, we examined the effect of bFGF-induced neuronal differentiation on the activation of CREB and subsequent CRE-mediated gene transcription in neuronal H19-7 cells. The present study suggests that CRE-mediated gene transcription appears to be important during the differentiation of neuronal H19-7 cells induced by bFGF and the activation of novel protein kinase-signaling pathway is required for the bFGF-responsiveness.

## **EXPERIMENTAL PROCEDURES**

*Materials*-Fetal bovine serum, Dulbecco's modified Eagles medium, hygromycin and

geneticin were purchased from Life Technologies (Grand Island, NY, USA). PD98059, SB203580, Ro-31-8220, LY294002, rapamycin, KT5720, and KN-62 were purchased from Calbiochem (La Jolla, CA, USA). Rp-cAMPS, 2-hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid, herbimycin A and tyrohostin 47 were purchased from Biomol (Plymouth Meeting, PA, USA). EGF and bFGF were purchased from Bachem (Bubendorf, Switzerland). The assay kits of PKC and CaM kinase 2 were purchased from Upstate Biotechnology, and a colorimetric PKA assay kit (Spinzyme format) was purchased from Pierce. Anti-phosphoCREB and CREB antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and anti-phosphop70<sup>S6K</sup> was from New England Biolabs (Beverly, MA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA). Plasmids encoding wild type and mutant GST-CREB were provided by M. Comb. Mammalian wild type and mutant CREB expression plasmids (pCG-CREB, PCG-L, and pCG-ΔCREB S119A), pCRE-TK-Luc, and parental pTK-Luc vectors were kindly provided by K. Saeki. A plasmid encoding mutant CREB with S133 phosphorylation site mutated to A133 (pRSV-CREB S133A) was provided by J. Eberwine. Dominant-negative SEK mutant, pSEK1(AL)/EE-CMV, and pEE-CMV DNAs were kindly provided by D. Templeton, and dominant inhibitory Ras mutant was provided by C. Marshall.

*Cell culture*-The rat neuronal hippocampal progenitor cell line (H19-7) was generated by transduction with the retroviral vectors containing temperature-sensitive simian virus 40 large T antigen that is functionally active at 33°C and inactive at 39°C as described elsewhere (18). ΔRaf-1:ER cells were made by stable transfection of H19-7

cells with estradiol-regulated Raf-1 generated by fusing a constitutively active, oncogenic portion of human Raf-1 to the hormone-binding domain of human estrogen receptor as described elsewhere (19). The proliferating cells were cultured at 33°C in medium containing 10% fetal bovine serum and 200 µg/ml of geneticin to maintain selection on the transduced immortalization vector. ΔRaf-1:ER cells were also grown in hygromycin. Prior to differentiation, cells were shifted to 39°C in N2 medium for 2 days (H19-7 cells) or 1 day (ΔRaf-1:ER cells). The H19-7 cells were differentiated with 10 ng/ml bFGF. ΔRaf-1:ER cells were differentiated with 1 µM estradiol. As a measure of neuronal differentiation, morphological changes were quantitated by measuring the length of processes in differentiated cells and the expression of neuronal markers were measured by immunocytochemistry and immunoblotting. Differentiated cells are defined as those cells containing at least one neurite longer than the diameter of cell body. When specified, cells were pretreated with 30 µM of p38 kinase inhibitor, SB203580, or MEK inhibitor, PD98059, 30 min prior to bFGF stimulation to block the activation of p38 or MEK. When indicated, the cells were pretreated with LY294002, rapamycin, KT5720, or KN62 for 30 min to block the activation of PI-3 kinase, pp70<sup>S6K</sup>, PKA or CaM kinase 2, respectively.

*Transient transfection and luciferase assay-pCRE-TK-Luc*, A reporter plasmid was transiently transfected by using a LipofectAMINE reagent (GIBCO/BRL) either alone or with kinase-inactive SEK or MEKK mutant plasmid, as indicated. Plasmid pCMV-EGFP (Clontech), which contains jellyfish green fluorescent protein gene driven by the cytomegalovirus promoter, was used as an internal control to determine transfection

efficiency. In every transfection experiment, the CRE-lacking thymidine kinase (TK) promoter construct (pTK-Luc) was used as a negative control. Luciferase activity was measured by using a luciferase assay kit (Promega) and luminometer (EG & G Berhold, Germany). When specified, the cells were stimulated with 10  $\mu$ M forskolin as a positive control for CRE-mediated gene transcription.

*Western blot analysis*-Cells were solubilized with lysis buffer A containing 20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM Na<sub>2</sub>EDTA, 10% glycerol, 1.0% Triton X-100, 0.2 mM phenylmethylsulfonylfluoride, 1  $\mu$ g/ml aprotinin, 20  $\mu$ M leupeptin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 10 mM NaF, 1mM tetrasodium pyrophosphate, and 1mM  $\beta$ <sup>+</sup> glycerophosphate. Then cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with a suitable antibody, according to its manufacturers protocol. The membrane was then incubated with a peroxidase-conjugated secondary antibody, and the bands visualized by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, United Kingdom).

*Measurement of PKC, PKA, and CaM kinase activity*-PKA activity was measured using the colorimetric PKA assay kit. The treated cells were washed with ice-cold PBS twice, resuspended in lysis buffer (50 mM Tris-HCl, 2.5 mM EDTA, 1 mM MgCl<sub>2</sub>, 10 mM NaF, 10% glycerol, pH 7.2), sonicated, and centrifuged. Supernatants were subsequently taken as lysates. Thirty micrograms of cell lysate was used to measure PKA activity. The activities of PKC and CaM kinase 2 were measured by using PKC and CaM kinase 2 assay kit (Upstate Biotechnology) as described in the manufacturer's



protocol. Final volume of each incubation sample was 60  $\mu$ l and reaction was terminated by transfer of 25  $\mu$ l onto phosphocellulose filter. The radioactive phosphopeptide bound to the filter was quantitated after 1 % phosphoric acid wash.

**CK2 Assay**-The assay for phosphotransferase activity of CK2 was conducted in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM MgCl<sub>2</sub>, and 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP in the presence of 5 mg/ml  $\beta$ -casein in a total volume of 30  $\mu$ l. The reaction was started by the addition of cell lysate including enzyme and incubated at 37°C for 1 hr. The reaction was terminated by spotting 10  $\mu$ l of the reaction mixture on to P-81 phosphocellulose paper. The paper was washed in 100 mM phosphoric acid, and the radioactivity was measured by scintillation counting.

*In vitro in-gel kinase assay*-A 12.5 % gel for SDS-PAGE was prepared by using 50  $\mu$ g of bacterially expressed wild type or mutant GST-CREB per ml as the substrate for phosphorylation. The cell extracts stimulated with bFGF in the absence or presence of various protein kinase inhibitors were applied to the gel. All gel renaturation and phosphorylation protocols were performed as described elsewhere (20).

## RESULTS

### *CREB activates CRE-mediated gene transcription in response to bFGF in H19-7 cells-*

In order to assess whether bFGF exerts its stimulatory effect on the activation of transcription factor CREB and subsequent CRE-mediated gene transcription, we assayed the gene expression of CRE-containing thymidine kinase (TK) promoter-

reporter construct. Treatment of H19-7 cells with bFGF resulted in the increase of CRE-mediated gene transcription in a time-dependent manner, and it reached a plateau after 2 hr (Fig. 1). In order to test the role of CREB phosphorylation on CRE-mediated gene transcription, cells were transfected transiently with pCRE-TK-Luc reporter plasmid plus mutant CREB expression vector, such as pCG-L, in which RRPSY from 130 to 134-th amino acid of CREB is replaced by RRSLY, or pCG- $\Delta$ CREB S119A, in which 88 to 101-th amino acids of CREB are spliced out and Ser-119, a target of protein kinase A that corresponds to Ser-133 of CREB is replaced to Ala-119. Expression of CREB proteins with the mutation of critically regulatory Ser-133 residue significantly inhibited the activation of luciferase activity by bFGF (Fig. 1). These results imply that the stimulation of H19-7 cells with bFGF caused CREB activation, possibly through the phosphorylation of its Ser-133 residue followed by the activation of CRE-mediated gene transcription.

*Expression of S133A CREB mutant blocks bFGF-induced neurite outgrowth in H19-7 cells*-The functional role of CREB activation during FGF-induced differentiation of H19-7 cells was further examined. Treatment of H19-7 cells with bFGF induces differentiation at 39°C at which large T-antigen is inactivated (21). Differentiated cells are resistant to mitogenic stimulation by serum, and express neuronal markers, such as neurofilament and brain type II sodium channel. A dominant-negative construct encoding CREB protein with S133 phosphorylation site mutated to A133 (pRSV-CREB S133A) was used to block the activation of CREB and a jellyfish green fluorescent protein gene (GFP) (pCMV-EGFP) as a marker for the transfected cells,

respectively. Either empty or mutant CREB expression vector (pRSV-CREB S133A) was cotransfected along with pCMV-EGFP and subsequently the formation of neurite outgrowth in GFP-positive cells was analyzed in H19-7 cells. Previously, it was determined that the efficacy for expressing cotransfected plasmids in the same cell is about 80% in H19-7 cells (22). As shown in Fig 2, untransfected control cells not expressing GFP had a similar percentage of differentiated cells (~63%) in two separate transfection experiments. However, cells that express GFP in the mutant CREB-transfected population had only 27% differentiated cells, in contrast to 58% differentiated cells in the pCMV-transfected population. Taken together, these results suggest that a relatively stable CREB phosphorylation by bFGF is likely to play a role in the differentiation of neuronal H19-7 cells.

*EGF and bFGF stimulates CREB phosphorylation at Ser-133 residue in a distinct kinetic pattern in H19-7 cells*-To confirm the previous finding that bFGF exerts its stimulatory effect on the activation of CREB during CRE-mediated gene transcription, Western blot analysis was performed by using an antibody specific for Ser133-phosphorylated form of CREB. During the differentiation of H19-7 cells by bFGF, Ser-133 residue in the CREB protein was phosphorylated rapidly and sustained for 1-2 hrs after growth factor stimulation (Fig. 3). Differences in CREB phosphorylation are known to be critical in the determination and regulation of EGF-mediated proliferation and NGF-induced differentiation of neuronal PC12 cells (5). Like PC12, H19-7 cells respond differentially to EGF and bFGF. While EGF treatment induces a proliferation at the permissive temperature (33°C), the addition of bFGF, but not EGF, induces

differentiation at the nonpermissive temperature (39°C) (21). Based on this finding, it was examined whether CREB phosphorylation occurred differentially by EGF and bFGF in H19-7 cells. In contrast to prolonged CREB phosphorylation by bFGF, EGF treatment, leading to proliferation but not differentiation, induced transient CREB phosphorylation and it was declined to basal levels within 30 min (Fig. 3). Taken together, these results suggest that stable CREB activation by bFGF is important to make a decision on its fate of hippocampal progenitor cell to terminally differentiate to neuronal cells.

*FGF-dependent CREB phosphorylation does not require the activation of extracellular signal-regulated kinases in H19-7 cells*-Upon binding to bFGF, its receptor dimerizes and activates an intrinsic tyrosine kinase activity leading to the increase in intracellular calcium, phosphoinositide turnover, phosphorylation of intracellular proteins, and activation of immediate early genes, such as *c-fos* and *myc* (23). To clarify the downstream signaling pathways of bFGF, the effect of receptor tyrosine kinase inhibitors on bFGF-induced CREB phosphorylation was examined. As shown in Fig. 4A, herbimycin A, a benzoquinoid ansamycin antibiotic, which irreversibly and selectively inhibits receptor tyrosine kinases by reacting with thiol groups, completely blocked the CREB phosphorylation by bFGF in H19-7 cells. Pretreatment of the cells with tyrphostin 47, another selective inhibitor of receptor tyrosine kinases also decreased CREB phosphorylation to the basal levels, compared to that of bFGF alone (Fig. 4A). Stimulation of bFGF receptors is known to activate Ras and a subsequent kinase cascade culminating in the activation of p42 and p44 ERKs (2). To identify early

events in the signaling pathway leading to CREB phosphorylation, the role of Ras activation was examined. Transient transfection of dominant-negative Ras mutant (pMT3RasN17) significantly inhibited the activation of ERK, but not CREB phosphorylation by bFGF (Fig 4B), suggesting that CREB is phosphorylated via Ras-independent signaling pathway during the differentiation of H19-7 cells by bFGF.

In neuronal PC12 cells, neurogenic NGF activates the extracellular signal-regulated kinases (ERKs), which in turn activate pp90 ribosomal S6 kinase family of Ser/Thr kinases (p90<sup>RSK</sup>), all three members of which were found to catalyze CREB Ser-133 phosphorylation (14). A major pathway by which p90<sup>RSK</sup> is activated by growth factor receptors involves sequential activation of Raf, MEK, and ERK. It was previously shown that the stimulation of H19-7 cells with bFGF induced the activation of the Raf-MEK-ERK pathway, resulting in the differentiation (22). To analyze the initial signals generated by Raf-1, H19-7 cells were transfected with a vector expressing  $\Delta$ Raf-1:ER (24). Upon exposure to estradiol (E2),  $\Delta$ Raf-1:ER is activated within minutes, enabling one to monitor downstream signaling events after Raf-1 activation. As shown in Fig. 5A, stimulation of  $\Delta$ Raf-1:ER cells with bFGF caused a rapid and prolonged CREB phosphorylation, which was similar to that in H19-7 cells. In order to test whether CREB was phosphorylated by the Raf-1-MEK-ERK-p90<sup>RSK</sup> signaling pathway,  $\Delta$ Raf-1:ER cells were pretreated with 30  $\mu$ M MEK inhibitor PD98059 for 30 min before bFGF stimulation. Although PD98059 completely blocked the activation of ERK by bFGF in  $\Delta$ Raf-1:ER cell (19), there was no significant inhibition of CREB phosphorylation (Fig. 5B), interestingly with a delay of initial CREB phosphorylation by approximately 15 min. When  $\Delta$ Raf-1:ER cells were stimulated with 1  $\mu$ M estradiol

leading to the activation of Raf-1 kinase followed by the sequential activation of ERK pathway and the differentiation of the cells (19), CREB was not phosphorylated at all (Fig. 4). The ERK induction by estradiol is due to selective Raf-1 activation, since no induction of ERK by estradiol was observed in the parent H19-7 cells lacking the  $\Delta$ Raf-1:ER fusion protein (17). Taken together, these data indicated that bFGF-induced signals leading to the CREB phosphorylation are not transmitted through Raf-MEK-ERK-p90<sup>RSK</sup> pathway in H19-7 cells.

*Activation of stress-activated protein kinases, such as JNK, and p38 kinase is not involved in the bFGF-induced CREB phosphorylation and the differentiation of H19-7 cells*-In addition, MAPK-activated protein kinase-2 (MAPKAP kinase-2), an enzyme that lies immediately downstream of p38 kinase, was recently shown to mediate CREB Ser-133 phosphorylation in neuroblastoma SK-N-BE cells exposed to bFGF (7). In order to investigate the effect of stress-activated protein kinases, such as JNK and p38 kinase, on bFGF-induced CREB phosphorylation, H19-7 cells were pretreated with chemical p38 kinase inhibitor, SB203580 or transiently transfected with kinase-deficient SAPK kinase (SEK) mutant cDNA, and subsequently bFGF-dependent CREB phosphorylation and subsequent induction of luciferase activity by CRE-thymidine kinase promoter were measured. As shown in Fig. 6, 30  $\mu$ M SB203580 or kinase-deficient SEK mutant had no effect on the ability of bFGF to induce CREB phosphorylation (Fig. 6A) and to stimulate CRE-mediated luciferase activity (Fig. 6B), suggesting that CREB phosphorylation is not mediated by the activation of stress-activated JNK or p38-MAPKAP kinase-2 pathway. As a control, we previously

observed that pretreatment of SB203580 or transient transfection of kinase-inactive SEK results in a significant inhibition of p38 induced by anisomycin (25) or JNK activity by NMDA in H19-7 cells (26). In addition, neither JNK nor p38 was shown to be significantly activated by activated Raf-1 (17) or bFGF<sup>2</sup> within the first few hours of stimulation in  $\Delta$ Raf-1:ER and H19-7 cells.

Consistent with the result in Fig. 5, pretreatment of the cells with 30  $\mu$ M MEK inhibitor PD98059 for 30 min did not inhibit the activation of reporter luciferase by bFGF (Fig. 6). As a positive control, the addition of adenylate cyclase activator, 10  $\mu$ M forskolin, increased the luciferase activity approximately 5-fold greater than that of FGF. Taken together, these results indicated that bFGF-induced CREB phosphorylation is not mediated via the activation of stress-activated JNK or p38 signaling pathways.

*bFGF-dependent CREB phosphorylation is not likely to be mediated by PKA, PKC, CaM kinase, PI-3K/p70S6K, and CK 2 in H19-7 cells*-The binding of bFGF to its receptor is known to induce receptor dimerization, autophosphorylation at Tyr-766, and activation of phospholipase C, which in turn activates PKC. As Ser-133 residue of CREB is contained within a consensus sequence of PKC phosphorylation and is phosphorylated by PKC (27), we examined whether TPA-sensitive isoforms of PKC affect the levels of CREB phosphorylation during the FGF-induced differentiation of H19-7 cells. Pretreatment of the cells with competitive PKC inhibitor, Ro-31-8220 or chelerythrine chloride, failed to inhibit bFGF-induced phosphorylation of CREB (Fig. 7A), suggesting that bFGF stimulates CREB phosphorylation via a pathway distinct from that activated by PKC. In addition, bFGF-induced CREB phosphorylation was not

changed significantly with other PKC inhibitors, such as calphostin C and hypericine (data not shown). As a control, the PKC activation by phorbol-12-myristate-13-acetate (PMA) was inhibited to the basal level by Ro-31-8220 and chelerythrine at a similar concentration (Figure 7B).

Many growth factors activate p70<sup>S6K</sup>, a protein kinase that is activated by a Ras-independent pathway (28), and which appears to be triggered by the activation of PI-3K (29,30). Both CREB and CRE modulator (CREM), another member of the CREB family, were efficiently phosphorylated *in vitro* by p70<sup>S6K</sup> (15). The macrolide rapamycin is an efficient and specific inhibitor of the mitogen-induced activation of p70<sup>S6K</sup> (31). LY294002, a selective PI-3K inhibitor, inhibits mitogenesis, glucose transport, and activation of p70<sup>S6K</sup> (30,32). When the H19-7 cells were pretreated with 50 ng/ml rapamycin or 10  $\mu$ M LY294002, the levels of CREB phosphorylation induced by bFGF were not changed remarkably (Fig. 7C). As a control, addition of LY294002 or rapamycin at the same concentration blocked the activation of p70<sup>S6K</sup> by serum in H19-7 cells (Fig. 7D).

Ser-133 residue of CREB is phosphorylated in response to an increase in intracellular cAMP and Ca<sup>2+</sup> concentration by PKA (10,11) and/or by CaM kinase (12,13,33). When H19-7 cells were pretreated with 0.5  $\mu$ M KT5720, specific PKA inhibitor, or 0.1  $\mu$ M KN-62, CaM kinase antagonist (34,35), serum-induced PKA activation (Fig. 8A) and the activation of CaM kinase 2 by ionomycin (Fig. 8C) were remarkably blocked, respectively. However, pretreatment of the cells with KT5720 or KN62 did not change the levels of FGF-induced CREB phosphorylation significantly,



compared to that of bFGF alone (Fig. 8B and D). In addition, pretreatment of the cells with other specific inhibitors for PKA and CaM kinase 2, such as K-252a and 2-hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid (CaM kinase), did not cause a remarkable decrease of CREB phosphorylation by bFGF (data not shown).

CREB is known to be a substrate of CK2 (36). Recently, CREB was reported to be phosphorylated in a cell cycle dependent manner and the pDE-1 domain (Ala<sup>106</sup>-Gln<sup>122</sup>) of CREB is phosphorylated by CK2 (37). While the addition of specific CK2 inhibitors, 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) or heparin (av. mol. wt. 3000) completely blocked the induction of CK2 activity by serum (Fig. 8E), it did not attenuate bFGF-induced CREB phosphorylation, respectively (Fig. 8F).

Furthermore, when the cells were pretreated with rapamycin, KT5720, DRB, and/or KN-62 together for 30 min prior to bFGF stimulation, the levels of CREB phosphorylation were not changed noticeably (data not shown). Overall, these results suggested that bFGF-dependent CREB phosphorylation does not require the activation of previously-known CREB kinase pathways, such as PKA, PKC, PI-3K/p70S6K, CaM kinase, or CK2.

*Identification of novel CREB kinase(s) activated by bFGF during the differentiation of H19-7 cells*-To identify bFGF-inducible CREB kinase(s), *in vitro* in-gel kinase assays were performed by using either wild type or mutant GST-CREB proteins as the substrates. Extracts containing equal protein from H19-7 cells that had been stimulated with 10 ng/ml bFGF in the absence or presence of combined various protein kinase inhibitors were resolved by SDS-PAGE, renatured, and assayed for CREB

phosphorylation in the gel. As shown in Fig. 9, a slight- and constitutive-active kinase of ca. 36-kDa was observed. In addition, the results showed two previously unreported CREB kinases of approximately 76- and 120-kDa are activated markedly by bFGF (Fig. 9A). Although pretreatment of the cells with the inhibitors of PI-3K, PKC, PKA, and CaM kinase does not affect the activity of 76-kDa kinase, it has a high basal activity without bFGF. In contrast, the 120 kDa kinase is greatly inducible by bFGF treatment. When the protein kinase inhibitors for PKA and PKC were added during the kinase assay as well as the treatment of H19-7 cells, the activation pattern of two novel kinases was not affected (data not shown), implying that the novel CREB kinases are not regulated by protein dissociation. Furthermore, no significant kinase activity was detected when the mutant GST-CREB S133A, in which Ser-133 residue of CREB had been mutated to Ala-133 (Fig. 9B), or GST protein (data not shown) was used as a substrate. These results suggested that a novel bFGF-inducible CREB kinase of 120 kDa phosphorylates the Ser-133 residue and is likely to play an important role in the differentiation of immortalized hippocampal neuronal cells.

## DISCUSSION

The present study demonstrated that bFGF stimulates the phosphorylation of CREB at Ser-133 residue, which plays an important role during the differentiation of neuronal H19-7 cells. This post-translational modification leads to an increase in CREB's transcriptional activity, as shown by using a CRE-TK-Luc reporter system. CREB was shown to play an important role in neuronal differentiation. NGF induces CREB

phosphorylation (38), and a dominant-negative ATF1 blocks neurite outgrowth in a subcell line of PC12 (39). In F11 neuroblastoma cells, cAMP induced neurite outgrowth and activated CREB (40).

A number of different kinases may be capable of mediating CREB phosphorylation under different circumstances, although the relative contributions of particular kinases in cells have not been clearly determined. PKA, PKC, CaM kinase, CK2, MAPKAP kinase-2, p60<sup>S6K</sup>, and p90<sup>RSK</sup> have been reported to phosphorylate CREB. By using specific inhibitors or kinase-deficient mutant cDNA, we demonstrated that bFGF-mediated CREB activation appears not to require any of the signaling pathways leading to the phosphorylation of CREB in H19-7 cells. We do not rule out the possibility that a redundant combination of two known pathways is responsible for CREB phosphorylation and the use of inhibitors are not sufficiently exhaustive and detailed to allow definite conclusion regarding the identity of the isoforms involved. For example, particular p38 isoform, such as p38 $\gamma$ , is not sensitive to SB203580 inhibition.

In addition, cAMP activated the transcription factor Elk-1 and induced neuronal differentiation of PC12 cells via its activation of the MAP kinase cascade (41). These cell type-specific actions of cAMP require the expression of the serine/threonine kinase, B-Raf, and activation of the small G protein, Rap1. Rap1, activated by mutation or by PKA, is a selective activator of B-Raf and an inhibitor of Raf-1. Thus it is possible that the differentiating signal of bFGF is transmitted through the activation of B-Raf, but not by Raf-1, which will also be important for the differentiation of H19-7 cells. However, base on the finding that combined treatment of the cells with various protein kinase inhibitors altogether did not attenuated the levels of phosphorylated

CREB by FGF (Fig 8 & 9), and the size of novel CREB kinase obtained from an in-gel kinase assay does not correspond to the reported sizes of those protein kinases, our current study shows the presence of a new growth factor-activated signaling pathway that regulates gene expression at the CRE.

The 15 min delay of initial CREB phosphorylation peak in the presence of PD980509 initially made us to think that bFGF signals are transmitted through two pathways, which was implicated in FGF-induced induction of *pip92*; a transient one for Raf-1-MEK-ERK that parallels the time-dependent curve of EGF, and the other one for novel kinase (17). A loss in the signal during initial time points when ERK is suppressed could be explained with this interpretation. However the results in Fig. 5 & 6, and from the previous *pip92* deletion analysis (17) indicated that the activation of Raf-1-MEK-ERK pathway is not required for the CREB phosphorylation by bFGF. In consistent with these findings, transient CREB phosphorylation by EGF is not suppressed considerably in the presence of PD98059<sup>3</sup>.

Recently, Akt was shown to promote phosphorylation of CREB, and activate cellular gene expression via a CRE-dependent mechanism (42). Akt is rapidly and specifically activated by diverse ligands, such as PDGF, EGF, and FGF, and promotes cell survival (43). Several lines of evidence support that ligand-induced activation of Akt is mediated through PI-3K signaling and Akt may represent novel PI-3K targets. Based on the findings, it was tested whether the activation of Akt is involved in the activation of CREB by bFGF in H19-7 cells. The finding that PI-3K inhibitor, LY294002, was not blocking bFGF-induced CREB phosphorylation by bFGF, and the molecular weight of novel CREB kinase did not correspond to that of Akt (60 kDa)

suggests that the activation of Akt is not necessary for CRE-mediated gene induction by bFGF. In a similar to our finding, exposure of PC12 cells to physiological levels of hypoxia rapidly induced a phosphorylation of CREB and this effect was not mediated by any of the previously known CREB activation pathways (44).

Although EGF and bFGF initially induce very similar intracellular signaling pathways, these two growth factors ultimately elicit very different cellular responses in H19-7 cells. Likewise, PC12 cells, when exposed to NGF, traverse the cell cycle several times and then differentiate into postmitotic cells that in many ways resemble sympathetic neurons (45). In contrast to NGF, EGF is a mitogen for PC12 cells.

By controlling the phosphorylation of transcription factors such as Elk-1 and CREB, and thereby regulating the expression of immediate early gene and possibly delayed response gene (DRG), MAPK pathway was suggested to transmit the signals of divergent cell fates, including proliferation and differentiation. FGF, NGF, or EGF induces the MAPK pathway with different time courses and the difference in kinetics accounts for the differential response of PC12 and H19-7 cells to these two agents (17,22,46). In both cells exposed to NGF (PC12) or bFGF (H19-7), sustained activation of the MAPK lasts for several hours. In contrast, treatment with the mitogenic EGF leads to transient activation of the MAPK signaling pathways, lasting only minutes after the initial EGF stimulus (47,48). In support of this finding, when EGF receptors are overexpressed in PC12 cells, the time course of EGF-induced Ras-MAPK activation is prolonged, and the overexpressing PC12 cells differentiate along a neuronal pathway in response to EGF (49).

Segal and Greenberg proposed that sustained activation of the Ras signaling pathway in NGF-treated PC12 cells can result in the sustained phosphorylation of

transcription factors such as CREB (50). This may allow CREB to selectively activate DRGs that have CREB-binding sites within their regulatory regions. Such DRGs would be activated in response to NGF, but not EGF, and might encode proteins that contribute to the acquisition of a neuronal phenotype (5). Consistent with this possibility, CREB-binding sites have been found within the promoters of genes that respond to NGF with delayed kinetics, such as tyrosine hydroxylase (51), transin (52), and neuronal secretory protein, VGF (53).

The current model for neuronal differentiation based on PC12 cells is that prolonged activation of ERK is both necessary and sufficient for differentiation. However, recent reports have shown that this model is not generally applicable in other neuronal cells. For example, prolonged MAP kinase is not sufficient for the differentiation of H19-7 cells, even though prolonged activation of Raf-1 is sufficient (22), indicating that under physiological conditions MAP kinase is neither necessary nor sufficient. Furthermore, our current finding that Raf-MAPK pathways are not required for CREB phosphorylation by bFGF in H19-7 cells, suggests that mitogenic and differentiating signals transmit through distinct pathways within PC12 and H19-7 cells, respectively.

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## Footnotes

<sup>1</sup>Abbreviations used are: bFGF, basic fibroblast growth factor; CaM kinase, calcium/calmodulin-dependent kinase; CC, chelerythrine chloride; CK2, casein kinase 2; CRE, cAMP response element; CREB, CRE binding protein; CREM, CRE modulator; DRB, 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole; DRG, delayed response gene; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP kinase 2, MAPK activated protein kinase 2; MEK, MAPK kinase; MI, MEK inhibitor PD98059; NGF, nerve growth factor; PI-3K, phosphatidylinositol 3'-kinase; PMA, phorbol-12-myristate-13-acetate; SAPK stress-activated protein kinase; TK, thymidine kinase

<sup>2</sup>Chung et al., unpublished observation

<sup>3</sup>Chung et al., unpublished observation

## Figure Legends

**Fig. 1 bFGF-induced activation of CRE-mediated gene transcription via *cis*-regulatory CRE motif.** 1  $\mu$ g DNA of pCRE-TK-Luciferase reporter plasmid was transiently transfected into immortalized hippocampal H19-7 cells with 5  $\mu$ g of either parental vector, pCG or mutant CREB expression vector pCG-L, or pCG- $\Delta$ CREB S119A. Then the cells were stimulated with 10 ng/ml bFGF for the indicated time, and the luciferase activity of reporter plasmid was measured. Data are plotted as the percent of maximum luciferase activity and represent the mean plus the range of the samples from three independent experiments in triplicates.

**Fig. 2 Effect of dominant-negative CREB on bFGF-induced neurite outgrowth in H19-7 cells.** (A) The cells were either untreated (Control) or cotransfected with empty parental control vector (P) or construct encoding dominant-negative CREB (mCREB), plus pCMV-EGFP vectors (GFP). The cells were subsequently stimulated with bFGF under differentiation condition, and the GFP-expressing cells were scored for differentiation as judged by morphological changes. The differentiation percentage of transfected cells was obtained by dividing the number of differentiated and GFP-expressing cells by total number of GFP expressing cells. The total numbers of GFP-expressing cells counted were 200 to 250 for empty and mutant CREB vectors, respectively, and the numbers of untransfected cells counted were approximately 200. (B) Micrographs of the H19-7 cells from panel A which were co-transfected with S133A mutant CREB and pCMV-EGFP vectors and subsequently treated with bFGF.

The GFP-positive cells were observed by fluorescence microscopy (GFP) and the same field of the cells was visualized by phase-contrast microscopy (Morphology). The GFP-positive cells are indicated by arrows.

**Fig. 3 Differential kinetic pattern of CREB phosphorylation by either bFGF or EGF and its functional role during the differentiation of H19-7 cells.** The cells were stimulated with 10 ng/ml bFGF or 10 nM EGF for the indicated time. The phosphorylated and endogenous CREB proteins were identified by Western blot analysis (A). These results are representative of two independent experiments. In lower panel B, phosphorylated CREB bands were quantified with phosphoimage analyses.

**Fig. 4 Inhibition of bFGF-inducible CREB phosphorylation by the receptor tyrosine kinase inhibitors, but not by dominant-negative Ras in H19-7 cells.** H19-7 cells were stimulated with 10 ng/ml bFGF for 30 min in the absence or presence of 1  $\mu$ mol/ml of herbimycin A (Her), or 10  $\mu$ mol/ml of tyrphostin 47 (Tyr), respectively (A). When specified, cells were transiently transfected with 5  $\mu$ g of dominant-negative Ras expression plasmid, pMT3RasN17 (Ras N17) (B). Phosphorylated ERK and CREB proteins were identified by Western analysis. These results are representative of two independent experiments. As a control for equal protein loading, the amounts of non-activated CREB were measured.

**Fig. 5 bFGF-inducible CREB phosphorylation is independent of the activation of Raf-1 kinase in neuronal H19-7 cells.**  $\Delta$ Raf-1:ER cells were untreated (NoT) or stimulated with (A,B) 10 ng/ml bFGF (FGF) or (C,D) 1  $\mu$ M estradiol (E2) for the indicated time,

respectively. When specified, cells were pretreated with 30  $\mu$ M MEK inhibitor, PD98059 (MI) for 30 min before bFGF or E2 stimulation (B). Phosphorylated CREB and ERK proteins were measured by using Western analysis. These results are representative of three independent experiments.

**Fig. 6 Effect of JNK and p38 kinase activity on bFGF-induced CREB phosphorylation and subsequent CRE-mediated gene transcription in H19-7 cells.** A. Where indicated, 5  $\mu$ g kinase-inactive SEK mutant DNA was transiently transfected into the cells. The cells were stimulated with bFGF for 1 hr in the absence or presence of 30  $\mu$ M SB203580, and subsequently CREB phosphorylation was measured. B. 1  $\mu$ g DNA of pCRE-TK-Luciferase reporter plasmid was transiently transfected into H19-7 cells either alone or with 5  $\mu$ g of a kinase-inactive mutant SEK (mSEK\*). Where indicated, cells were pretreated with 30  $\mu$ M PD98059 or 50  $\mu$ M SB203580 for 30 min. Then the cells were stimulated with 10 ng/ml bFGF for 1 hr, and the luciferase activity of reporter plasmid was measured. Data are plotted as the percent of maximum luciferase activity and represent the mean plus the range of the samples from two independent experiments in triplicates.

**Fig. 7 Effect of specific protein kinase inhibitors, such as PKC, PI 3-kinase, or S6 kinase on bFGF-induced CREB phosphorylation in H19-7 cells.** In panel A and C, H19-7 cells were either untreated (NT) or pretreated with 20  $\mu$ M chelerythrine chloride (CC) or 100 nM Ro-31-8220 (Ro), 50 ng/ml rapamycin (R), or 10  $\mu$ M LY294002 (LY), for 30 min to block the activation of PKC or PI-3 kinase-p70<sup>S6K</sup>, respectively.

The cells were either untreated (C) or stimulated with 10 ng/ml bFGF (FGF), and Western blot analysis was performed to identify phosphorylated CREB bands. In panel B, after addition of 10  $\mu$ M of phorbol-12-myristate-13-acetate (PMA) in the absence or presence of 20  $\mu$ M chelerythrine chloride (C) or 100 nM Ro-31-8220 (Ro) for 30 min, the PKC activity was measured. In panel D, the activation of p70<sup>S6K</sup> by 10% serum in the absence or presence of 50 ng/ml rapamycin (R) or 10  $\mu$ M LY294002 (LY) was measured. These results are representative of two or three independent experiments.

**Fig. 8 Effect of the specific inhibitors of PKA, CaM kinase or CK2 on bFGF-induced CREB phosphorylation.** In panel A and D, H19-7 cells were untreated (NT or C) or pretreated with 0.5  $\mu$ M KT5720 (KT), 0.1  $\mu$ M KN-62 (KN), 100  $\mu$ M 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) or 300  $\mu$ M heparin (Hep) for 30 min to block the activation of PKA, CaM kinase 2 or CK2, respectively. The cells were then stimulated with 10 ng/ml FGF (F), and Western blot analysis was performed to identify the phosphorylated CREB bands. These results are representative of two or three independent experiments. In panel B, C, and E, the cells were grown on DMEM containing 1% FBS at 33°C for 24 hr. Where indicated, the cells were pretreated with KT5720 (panel B), KN62 (panel C), DRB, or heparine (Hep) for 30 min, and 10% serum (PKA and CK2) or 500 nM ionomycin (CaM kinase 2, Iono) was added directly to the culture medium. The treated cells were harvested, and then PKA, CaM kinase 2 or CK2 activities was measured. Values are means  $\pm$  SEM of three independent experiments.

**Fig. 9 Identification of novel bFGF-inducible CREB kinases in H19-7 cells.** H19-7 cells were untreated (NT) or pretreated with various protein kinase inhibitors (PKIs), including LY294002, KT5720, KN-62, and Ro-31-8220 for 30 min, and then stimulated with 10 ng/ml bFGF for 30 min. Cell extracts containing 40-50  $\mu$ g of proteins were resolved by SDS-PAGE gel containing 50  $\mu$ g per ml of bacterially expressed wild type GST-CREB (A) or mutant GST-CREB S133A (B) as a substrate. The in-gel renaturation assay was performed and the positions of protein molecular mass markers are shown on the left hand side of figure. Two novel CREB kinases that are activated by bFGF and are distinct from other already-known CREB kinases are indicated by filled arrows, and a constitutive- and slight-active CREB kinase is depicted by an open arrow on the left hand side. These results are representative of three independent experiments.



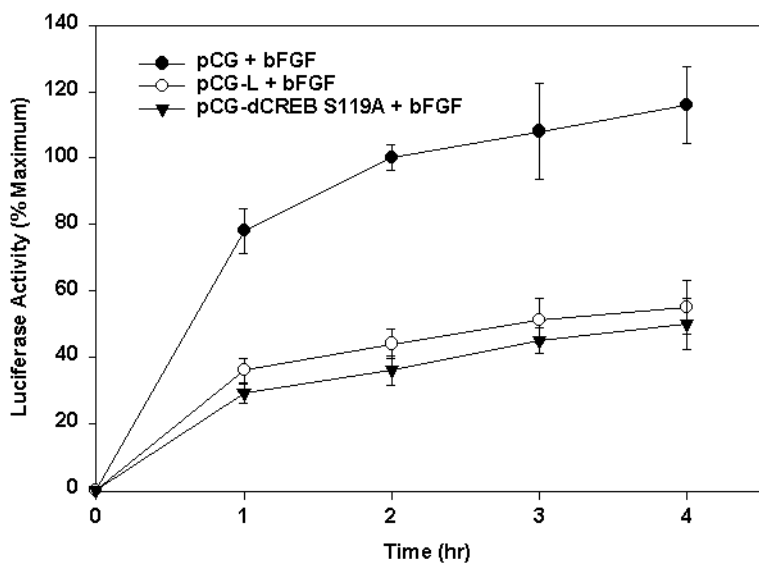


Fig. 1

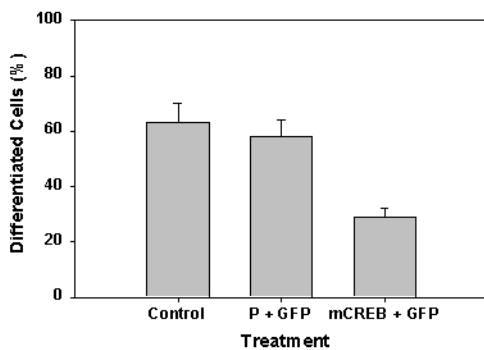
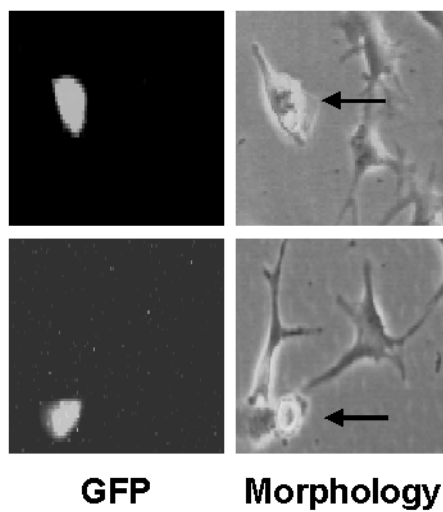
**A****B**

Fig. 2

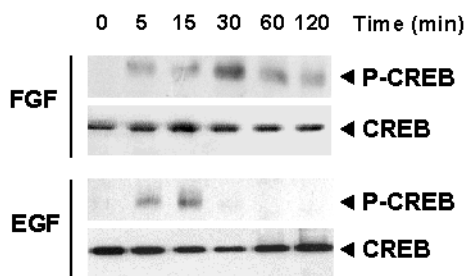
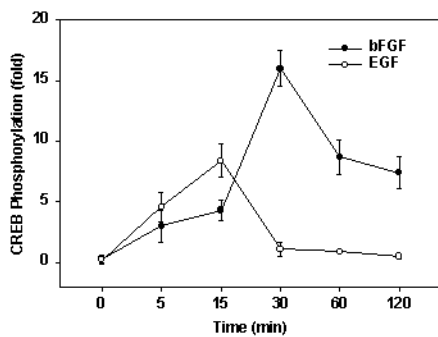
**A****B**

Fig. 3

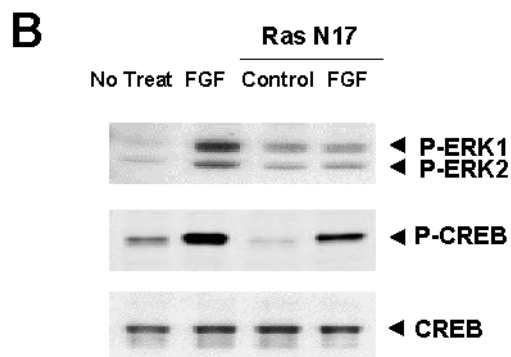
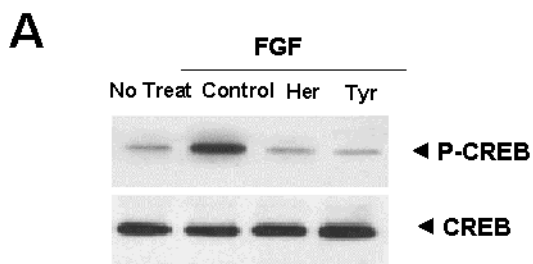


Fig. 4

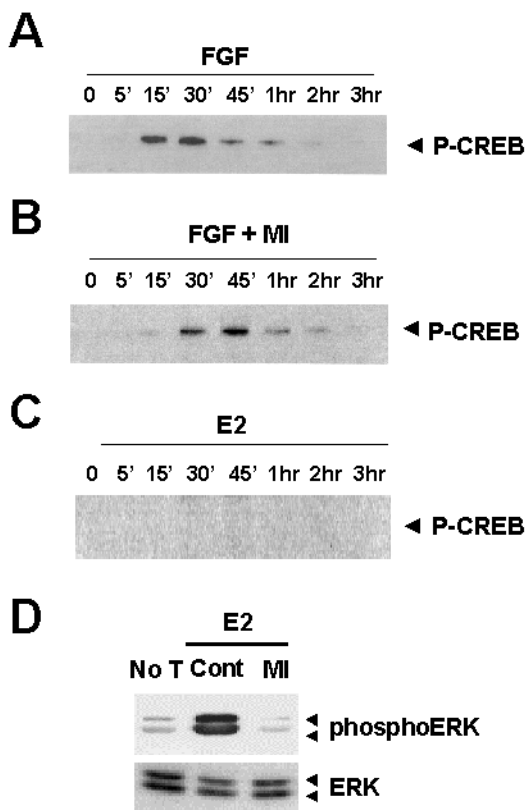


Fig. 5

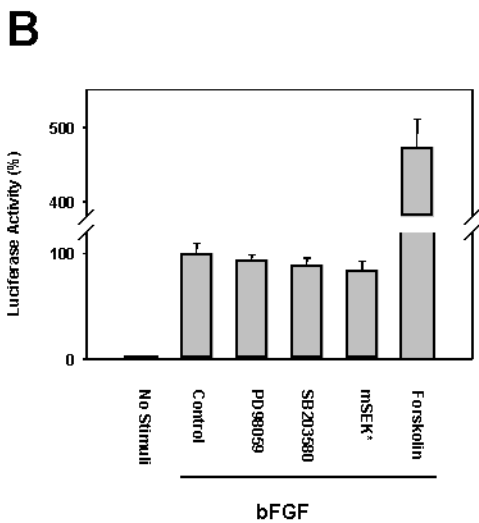
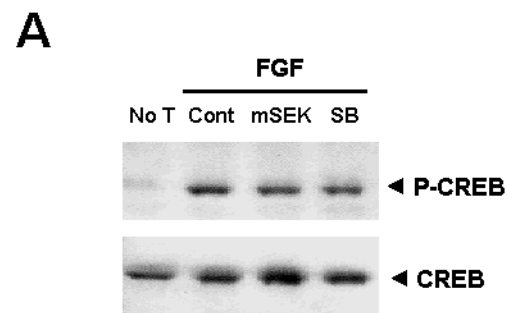


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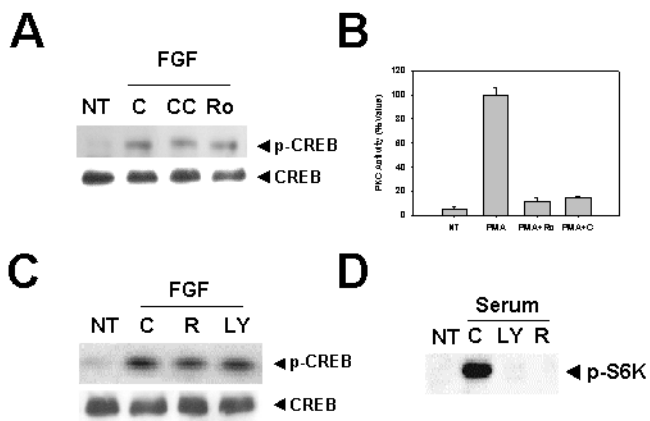


Fig. 7

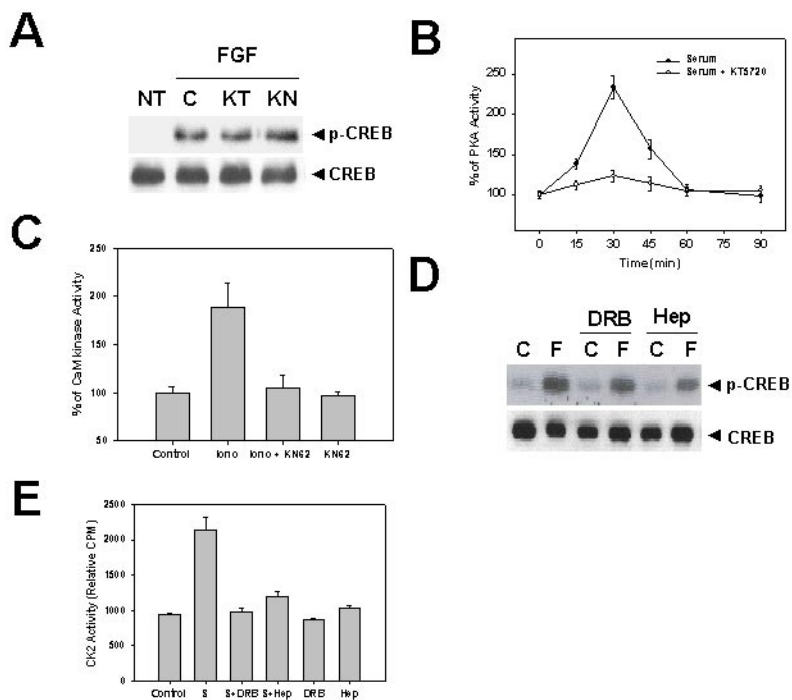


Fig. 8



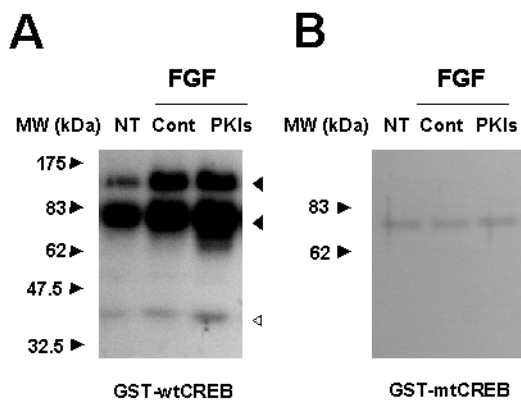


Fig. 9