
MECHANISMS OF SIGNAL TRANSDUCTION:

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# Protein Kinase Dyrk1 Activates cAMP Response Element-binding Protein during Neuronal Differentiation in Hippocampal Progenitor Cells*

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**Eun Jin Yang, Young Soo Ahn, and Kwang Chul Chung‡**

*From the Department of Pharmacology, Brain Research Institute and Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul 120-752, Korea*

**Dyrk is a dual specific protein kinase thought to be involved in normal embryo neurogenesis and brain development. Defects/imperfections in this kinase have been suggested to play an important role in the mental retardation of patients with Down's syndrome. The transcriptional factor cAMP response element-binding protein (CREB) has been implicated in the formation of many types of synaptic plasticity, such as learning and memory. In the present study we show that Dyrk1 activity is markedly induced during the differentiation of immortalized hippocampal progenitor (H19-7) cells. The addition of a neurogenic factor, basic fibroblast growth factor, to the H19-7 cells results in an increased specific binding of Dyrk1 to active CREB. In addition, Dyrk1 directly phosphorylates CREB, leading to the stimulation of subsequent CRE-mediated gene transcription during the neuronal differentiation in H19-7 cells. Blockade of Dyrk1 activation significantly inhibits the neurite outgrowth as well as CREB phosphorylation induced by basic fibroblast growth factor. These findings suggest that Dyrk1 activation and subsequent CREB phosphorylation is important in the neuronal differentiation of central nervous system hippocampal cells.**

The regulation of gene expression by specific signal transduction pathways is tightly connected to the cell phenotype. The response elicited by a given transduction pathway varies according to the cell type. One major signal transduction system utilizes cAMP as a secondary messenger and has as its ultimate target a DNA control element, the cAMP response element (CRE)[1] (1). The CRE-binding protein (CREB) is a transcription factor that activates CRE-mediated transcription. CREB activity is regulated by multiple kinases after various kinds of stimulation (2). Specific roles for CREB in neuronal development and differentiation have been revealed through *in vivo* and *in vitro* manipulations of CREB function. For example, the expression of a dominant-interfering CREB within certain pituitary neurons causes them to develop abnormally and die perinatally (3).

"Minibrain" (Mnb) is a mutant of *Drosophila* whose presence is exhibited by a specific and marked size reduction of the optic lobes and central hemispheres in the adult brain (4). The Mnb gene encodes a Ser/Thr protein kinase that possesses a Tyr-*X*-Tyr sequence in the activation loop (5). At least 7 closely related homologous mammalian kinases have since been isolated, and a novel superfamily of protein kinases called Dyrk(s) has been established (6). Dyrks possess Ser/Thr phosphorylation activity as well as autophosphorylation activity on Tyr residues, suggesting that Dyrk seems to be a dual specificity kinase (5, 6). The kinase activity of Dyrk is dependent on the Tyr-*X*-Tyr motif in the activation loop, suggesting the existence of a phosphorylation-dependent activation mechanism of Dyrk by certain upstream kinases (6). The most homologous protein among the Dyrks in the *Saccharomyces cerevisiae* genome is YAK1, which has been characterized as a negative regulator of growth (7). Interestingly, human Dyrk1A is mapped to the Down's syndrome (DS) critical region on chromosome 21 (4-megabase region containing 60~100 genes between the markers D21S17 and ETS2) (31) and, thus, could be a candidate gene responsible for the mental retardation of DS patients (8). Thus, from *Drosophila* to humans, it is suggested that Dyrk/Mnb is a key regulator of neuronal cell growth (36). Although the exact cellular function of the Dyrk kinases is still unknown, an understanding of the physiological role(s) of this protein kinase family will prove to be very relevant.

Although little is known about the mechanism by which the overexpression of Dyrks interferes with normal development in DS patients, Dyrk genes are strongly implicated in normal neuronal development by a mechanism that may involve a signal transduction pathway (36). This study examined the functional role played by Dyrk1 during the neuronal differentiation in hippocampal progenitor cells. We found that Dyrk1 is activated and phosphorylates the transcription factor CREB during neuronal differentiation. In addition, Dyrk1 activation induces CRE-mediated gene transcription. These findings suggest that Dyrk1 may play an important role during neurogenic factor-induced differentiation of central nervous system (CNS) neuronal cells.

## EXPERIMENTAL SECTION

*Materials*—The following materials were purchased. Peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (IgGs) were from Zymed Laboratories Inc. (San Francisco, CA); Dulbecco's modified Eagle's medium, fetal bovine serum, and cell culture reagents were from Life Technologies, Inc.; protein A-Sepharose was from Amersham Phar-

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, Yonsei University College of Medicine, Shinchon-dong 134, Seodaemun-gu, Seoul 120-752, Korea. Tel.: 82-2-361-5229; Fax: 82-2-313-1894; E-mail: kchung@yumc.yonsei.ac.kr.

[1] The abbreviations used are: CRE, cAMP response element; CREB, cAMP response element-binding protein; CNS, central nervous system; DS, Down's syndrome; ECL, enhanced chemiluminescence; GST, glutathione *S*-transferase; HA, hemagglutinin; Mnb, minibrain; TK, thymidine kinase; bFGF, basic fibroblast growth factor; TBST, Tris-buffered saline with Tween; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis.

macia Biotech; enhanced chemiluminescence (ECL) reagents and [$\gamma$-$^{32}$P]ATP were from PerkinElmer Life Sciences; anti-Dyrk IgG was from Becton Dickinson; anti-CREB IgG was from NEN Cell Signaling (Beverly, MA); anti-phosphorylated CREB antibodies were from Upstate Biotechnology (Lake Placid, NY); synthetic dropout medium (SD/−T, SD/−L, SD/−HLT) and yeast extract peptone dextrose containing adenine were from Bio101 (Vista, CA); 3-amino-1,2,4-triazole was from Sigma; 5-bromo-4-chloro-3-indoryl-D-galactoside and luciferase assay kit were from Promega (Madison, WI); human fetal brain cDNA library was from CLONTECH (Palo Alto, Calif.); human basic FGF was from Bachem (Bubendorf, Switzerland). As gifts, pCRE-TK-Luc and pTK-Luc constructs were received from K. Saeki (Research Institute, International Medical Center of Japan). The plasmids encoding hemagglutinin (HA)-tagged pSVL-HA-Dyrk1A and mutant type K188R were provided by W. Becker (Institute of Pharmacology and Toxicology, RWTH, Aachen, Germany).

*Yeast Two-hybrid Assay*—The bait vector for yeast two-hybrid assay was constructed by subcloning the mutant CREB cDNA, in which RRPSY (amino acid 130–134) was replaced by RRSLY, into pHybTrp/Zeo vector. Human fetal cDNA library subcloned into prey vector (pACT2) was purchased from CLONTECH. The yeast strain L40, containing the reporter genes *lacZ* and *HIS3* downstream of the LexA promoter, was sequentially transformed with bait vector followed by cDNA library vectors and then plated on a synthetic medium containing 5 mM 3-amino-1,2,4-triazole and lacking histidine, leucine, and tryptophan residue. After incubating the plates for 10–14 days at 30 °C, the transformants were tested with a synthetic medium lacking histidine, leucine, and tryptophan residue and containing 50 $\mu$g/ml 5-bromo-4-chloro-3-indoryl-D-galactoside. After incubation for 2–3 days at 30 °C, the yeast colonies showing blue color were selected as positive clones. The positive clone plasmids were extracted from yeast in lysis buffer containing 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, and 1.0 mM EDTA and then transformed into *Escherichia coli* DH5$\alpha$ using electroporation. Sequences of the inserts in positive library plasmids were analyzed by automatic DNA sequencer (ALF express, Amersham Pharmacia Biotech).

*Cell Culture and Preparation of Cell Lysates*—H19-7 cells were generated from rat embryonic hippocampal neurons (9). They were conditionally immortalized by stable transfection with temperature-sensitive SV40 large T antigen. They were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 units/ml penicillin-streptomycin under G418 selection throughout the experiments. To induce neuronal differentiation, the cells were placed in N2 medium and shifted to 39 °C before treatment with differentiating agents as previously described (10). To test the effect of Dyrk1 activation on the neuronal differentiation in H19-7 cells, the cells were transfected with a plasmid encoding either wild type or kinase-inactive Dyrk1 at 33 °C for 24 h. Then the cells were switched to N2 medium, cultured at 39 °C for 48 h, treated with bFGF, and analyzed for morphological changes with a optical microscope (Zeiss, Thornwood, NY). Differentiated cells were defined as cells with a rounded and refractory cell body containing at least one neurite whose length was greater than the cell body diameter. To prepare cell lysates, the cells were rinsed twice with ice-cold phosphate-buffered saline and solubilized in lysis buffer (20 mM Tris, pH 7.9, containing 1.0% Triton X-100, 1 mM $Na_3VO_4$, 137 mM NaCl, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 5 mM $Na_2EDTA$, 10% glycerol, 1 mM $\beta$-glycerophosphate, 0.1 g/ml *p*-nitrophenylphosphate, and 0.2 mM phenylmethylsulfonyl fluoride). The cells were scraped, and the supernatants were collected after centrifugation for 10 min at 14,000 $\times$ *g* at 4 °C. Protein concentrations were determined using the Bio-Rad detergent-compatible protein assay kit.

*DNA Transfection and Luciferase Reporter Assay*—The H19-7 cells were plated at a density of $2 \times 10^6$ cells per well in 100-mm-diameter dishes. When the cell confluency was 70~90%, the cells were transfected with suitable plasmid constructs using LipofectAMINE Plus reagent (Life Technologies) according to the manufacturer's instruction. Where indicated, luciferase reporter construct, pCRE-TK-Luc, was transiently co-transfected with kinase-inactive Dyrk1 mutant (K188R), and the luciferase activity was measured using a luciferase assay kit (Promega) and a luminometer (EG & G Berhold, Germany). In every transfection experiment the CRE-lacking thymidine kinase (TK) promoter construct (pTK-Luc) was used as a negative control.

*Immunoprecipitation*—One microgram of either monoclonal anti-Dyrk1 or polyclonal anti-CREB antibodies was incubated at 4 °C overnight with 300 $\mu$g of cell extracts prepared using lysis buffer. Forty microliter of a 1:1 suspension of protein A-Sepharose beads was added to the cell lysates and incubated for 2 h at 4 °C with gentle rotation. The beads were pelleted and washed extensively with cell lysis buffer. Bound proteins were dissociated by boiling the samples in PAGE sample buffer, and whole samples were separated on SDS-PAGE gel.

*Western Blot Analysis*—The whole cell lysates were separated through a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore, Japan). The membranes were blocked in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) containing 3% nonfat dry milk for 3 h and then incubated overnight at 4 °C in 3% nonfat dry milk containing either anti-phospho CREB, anti-CREB, or anti-Dyrk1 (Becton Dickinson). The membrane was then washed several times in TBST and incubated with anti-mouse IgG-coupled horseradish peroxidase antibodies. After 1 h, the blot was washed several times with TBST and developed with ECL reagents.

*In Vitro Dyrk Assay*—H19-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and switched to N2 medium before differentiation for 2 days at 39 °C. The cells were treated with 10 ng/ml of bFGF, harvested, and lysed in lysis buffer. Then 300 $\mu$g of protein was incubated with monoclonal Dyrk1 antibody overnight at 4 °C. The immuno-complexes were added to 40 $\mu$l of protein A-Sepharose beads. After incubation, the samples were washed three times in lysis buffer, and the kinase reactions were carried out at 30 °C for 1 h in 20 $\mu$l of kinase buffer containing 20 mM HEPES, pH 7.2, 5 mM $MnCl_2$, 200 $\mu$M sodium orthovanadate, 5 $\mu$g of acid-treated enolase, 10 $\mu$M ATP, 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP, and 5 $\mu$g of either bacterially expressed glutathione *S*-transferase (GST)-CREB or mutant GST-CREB (S133A) as a substrate. The reactions were stopped by adding SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by autoradiograpy.

## RESULTS

*Yeast Two Hybrid Assay to Identify Novel CREB Kinase(s) Activated during Neuronal Differentiation*—Immortalized hippocampal H19-7 cells differentiate in response to bFGF at 39 °C, the temperature at which the simian virus 40 large T antigen is not active (11). In an earlier work we have shown that CREB phosphorylation and subsequent CRE-mediated gene transcription play an important role during bFGF-induced neuronal differentiation in H19-7 cells and that the activation of novel protein kinase-signaling pathways is required for bFGF-responsiveness (12). During the differentiation of H19-7 cells, Ser-133 residue in the CREB protein was phosphorylated rapidly and sustained for 1–2 h after growth factor stimulation. In addition, the activation of two previously unreported CREB kinases in response to bFGF was observed by using *in vitro* in-gel kinase assays (12). In the present study, in order to isolate the novel CREB kinase(s) we undertook yeast two-hybrid assays by using mutant CREB mutant in

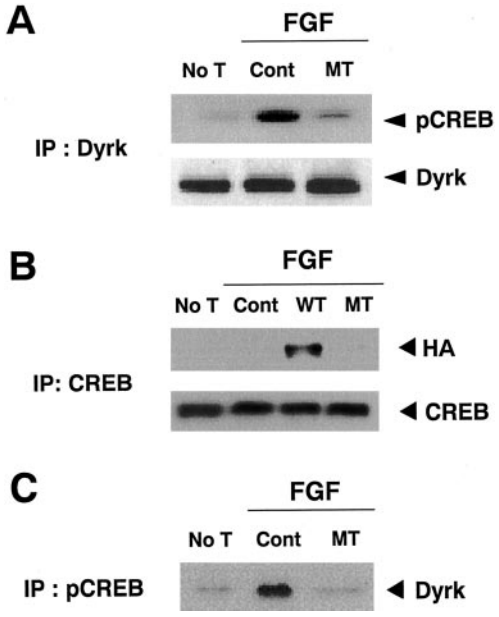


FIG. 1. **Dyrk1 interacts with active CREB in hippocampal H19-7 cells.** Where indicated, H19-7 cells were transfected with 5 $\mu$g of a plasmid encoding either HA-tagged wild type or kinase-deficient Dyrk1 for 24 h. The cells were then stimulated with 10 ng/ml bFGF for 1 h under differentiation conditions. Total cell lysates were immunoprecipitated with either monoclonal anti-Dyrk 1 IgG followed by blotting with polyclonal anti-Dyrk1 or anti-phospho-CREB antibodies (*A*) or anti- CREB followed by the blotting with anti-HA or anti-Dyrk1 antibodies (*B*). In *panel C*, the cell lysates were immunoprecipitated with anti-phospho-CREB and blotted with anti-Dyrk1 antibodies. All expressed results are representative of three independent experiments. *IP*, immunoprecipitation; *WT*, wild Dyrk1 type; *MT*, kinase-inactive Dyrk1 mutant; *No T*, no treatment; *Cont*, control.

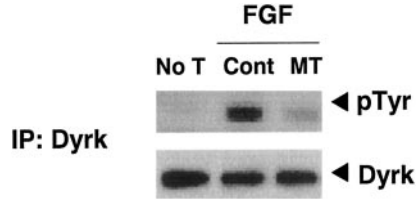


FIG. 2. **Basic FGF-induced tyrosine phosphorylation of Dyrk1 in H19-7 cells.** Where indicated, the cells were treated with vehicle or transiently transfected with 5 $\mu$g of plasmid encoding kinase-inactive Dyrk1 (K118R). The cells were treated with 10 ng/ml bFGF under differentiating conditions for 1 h. Total cell lysates were immunoprecipitated with monoclonal anti-Dyrk1 IgG, and bound proteins were resolved onto SDS-PAGE and detected with an anti-phospho-Tyr (*pTyr*) antibody. All expressed results are representative of three independent experiments. *IP*, immunoprecipitation; *MT*, Dyrk1 K118R mutant; *Cont*, control; *No T*, no treatment.

which the critically regulatory Pro-132–Ser-133 residues of CREB had been mutated to Ser-132–Leu-133 as bait. As a result of the screening of human fetal brain cDNA library, we identified a number of unknown as well as previously reported CREB-interacting genes, for example, histone deacetylase (34), retinoblatoma-binding protein 4 (35), and zinc finger protein ZEB. A BLAST search revealed that one of the isolated clones encodes a partial clone of mammalian homolog of yeast minibrain kinase, Dyrk1. Because Dyrk1 was known to be important in the cell cycle control in neuronal cells and implicated in the pathogenesis of DS mental retardation, we chose Dyrk1 as a target for further analysis.

*Specific Binding between CREB and Dyrk1 during the Neuronal Differentiation in H19-7 Cells*—Next we examined whether Dyrk1 specifically binds to CREB in mammalian neuronal cells and how the neurogenic growth factor affects the expression of Dyrk1 during neuronal differentiation. The H19-7 cell extracts obtained after the stimulation with neurogenic bFGF were immunoprecipitated against anti-Dyrk1 antibodies and then blotted with anti-phospho-CREB antibodies. As shown in Fig. 1*A*, the expression of Dyrk1 protein with 105 kDa was not induced by bFGF, whereas significant levels were constitutively expressed in H19-7 cells. Although there was no complex formation without any stimulation, the addition of bFGF led to an increase of Dyrk1 binding to phosphorylated CREB. In the following experiment, the cells were either not transfected (Fig. 1*C*) or transiently transfected with a plasmid encoding HA-tagged wild type Dyrk1 (Fig. 1*B*). Then the cells were stimulated with bFGF under differentiating conditions, the cell lysates were immunoprecipitated against either anti-CREB or anti-phospho-CREB antibodies, and the protein mixtures were analyzed using anti-HA tag (Fig. 1*B*) or anti-Dyrk1 IgG (Fig. 1*C*). Consistent with the previous finding, bFGF stimulation promoted the formation of binding complex between Dyrk1 and phospho-CREB in H19-7 cells. A construct encoding dominant-negative Dyrk1 was used to block the activation of Dyrk1. When the kinase-inactive K188R Dyrk1 (pSVL-HA-Dyrk1A/K188R), in which critical Lys-188 residue in the catalytic domain of Dyrk1 was converted to Arg-188 (13), was transiently transfected into the cells, the specific binding of Dyrk1 to active CREB markedly decreased in response to bFGF (Fig. 1, *A–C*). As a control for protein loading, we measured the amount of immunoprecipitated CREB by Western blot analysis. In all samples, CREBs were present at the same levels (Fig. 1*B*). These results indicate that Dyrk1 specifically binds to active CREB in response to neurogenic growth factor.

*Dyrk1 Is Selectively Activated by bFGF, but Not by EGF, in H19-7 Cells*—Because Dyrk1 is activated by tyrosine phosphorylation in the activation loop of the catalytic domain (14), we examined whether Dyrk1 is also activated by bFGF. After cells were stimulated with bFGF, the cell lysates were immunoprecipitated against anti-Dyrk1 antibodies, and the precipitated Dyrk1-bound mixtures were analyzed using anti-phosphotyrosine antibodies. As shown in Fig. 2, the tyrosine phosphorylation of Dyrk1 increased in response to bFGF. Compared with the control cells, the overexpression of kinase-dead Dyrk1 mutants by transient transfection extensively repressed the Dyrk1 phosphorylation, suggesting that the addition of bFGF induces Dyrk1 activation. Differences in CREB phosphorylation are known to be critical in the determination and regulation of both mitogenic factor-mediated proliferation and neurotrophin-mediated differentiation in neuronal PC12 cells (15). Like PC12 cells, H19-7 cells also respond differentially to EGF and bFGF (12). At the permissive temperature (33 °C) EGF treatment induces proliferation, whereas at the nonpermissive temperature (39 °C), the addition of either FGF or phorbol 12,13-dibutyrate, but not EGF, induces differentiation (9, 16). Furthermore, in contrast to prolonged CREB phosphorylation by neurogenic bFGF, the addition of EGF induces transient CREB phosphorylation. These findings from previous studies imply that stable CREB activation by bFGF is important in deciding the fate of hippocampal progenitor cell to terminally differentiate to neuronal cell (12). To determine whether Dyrk1 activation is selectively induced during neuronal differentiation in H19-7 cells, the cells were stimulated with either EGF under mitogenic conditions or bFGF under differentiation conditions. Then immunoprecipitation was performed with the cell lysates using anti-Dyrk1 antibodies followed by Western blot analysis against an antibody specific for phosphotyrosine residue to detect Dyrk1 activation. Compared with a stable but slight activation of Dyrk1 within 2 h after EGF stimulation (Fig. 3*A*), a transient but significant phosphorylation of Dyrk1 was observed upon the stimulation with bFGF reaching the maximum

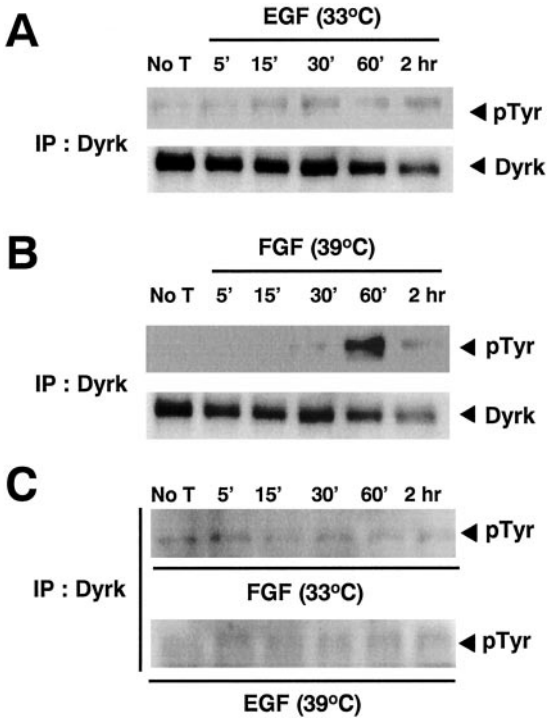


FIG. 3. **Selective activation of Dyrk1 in response to neurogenic bFGF, but not mitogenic EGF, in H19-7 cells.** Where specified, H19-7 cells were stimulated with either 50 ng/ml EGF or 10 ng/ml bFGF for the indicated times. The Dyrk1 was immunoprecipitated from cell lysates, and the Dyrk1-bound protein mixtures were blotted with monoclonal anti-phosphotyrosine (*pTyr*) antibodies. The quantity of immunoprecipitated Dyrk1 kinase was determined by Western blot analysis using anti-Dyrk1 antibodies (*lower panel*). All expressed results are representative of two independent experiments. *A*, EGF at 33 °C; *B*, bFGF at 39 °C; *C*, EGF at 39 °C (*top panel*) and bFGF at 33 °C (*bottom panel*). *IP*, immunoprecipitation; *No T*, no treatment.

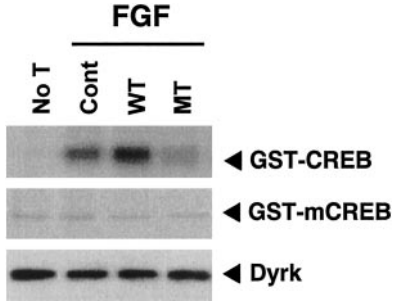


FIG. 4. **Dyrk1 directly phosphorylates CREB in response to bFGF in H19-7 cells.** Where indicated, H19-7 cells were transfected in a transient manner with 5 $\mu$g of expression plasmid encoding either wild type or kinase-deficient Dyrk1. The cells were then stimulated with 10 ng/ml bFGF for 1 h, and total cell lysates were prepared. *In vitro* CREB kinase assays were performed using bacterially expressed GST fusion protein with either wild type CREB (*GST-CREB*) or S133A CREB mutant (*GST-mCREB*) as an exogenous substrate. Kinase reaction products were resolved by 10% SDS-PAGE, and the levels of phosphorylated CREB were visualized by autoradiography. All expressed results are representative of three independent experiments. *WT*, wild type of Dyrk1; *MT*, mutant type of Dyrk1; *No T*, no treatment; *Cont*, control.

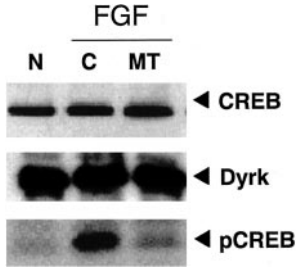


FIG. 5. **Effect of kinase-deficient Dyrk1 on bFGF-induced CREB phosphorylation.** Where indicated, H19-7 cells were treated with vehicle or transiently transfected with 5 $\mu$g of plasmid encoding kinase-deficient Dyrk1. Then, the cells were either untreated (*N*) or stimulated with 10 ng/ml of bFGF for 1 h under differentiating condition. Total cell lysates were resolved by SDS-PAGE and analyzed by Western blot analysis using the antibodies against CREB, Dyrk1, or phosphorylated CREB. *N*, no treatment; *C*, control; *MT*, kinase-inactive Dyrk1.

peak at 1 h (Fig. 3*B*). In addition, the addition of either EGF at 39 °C or bFGF at 33 °C failed to induce a significant tyrosine phosphorylation of Dyrk (Fig. 3*C*). These data indicate the differential activation of Dyrk1 in response to mitogenic and neurogenic growth factor. In particular, Dyrk1 activation may contribute to neuronal differentiation by neurogenic bFGF in the H19-7 cells.

*Dyrk1 Directly Phosphorylates CREB in Response to bFGF*—Next we investigated whether active Dyrk1 could phosphorylate CREB. The H19-7 cell lysates obtained after bFGF treatment were immunoprecipitated using anti-Dyrk1 antibodies, and immunocomplex kinase assays were performed using bacterially expressed GST fusion protein with either wild type CREB or S133A mutant CREB as a substrate. Phosphorylated substrates were visualized by autoradiography. As shown in Fig. 4, CREB phosphorylation was remarkably induced by bFGF. Furthermore, transient transfection of kinase-deficient Dyrk1 significantly diminished the bFGF-induced CREB phoshorylation, whereas overexpression, in a similar way, of wild type Dyrk1 proteins caused a remarkable induction of CREB phosphorylation compared with the control cells. When GST-CREB S133A, encoding CREB mutant, in which Ser-133 residue was substituted to Ala-133, was used as a substrate, there was no significant phosphorylation of CREB (Fig. 4). In addition, the effect of kinase-inactive Dyrk1 on the CREB phosphorylation was examined in H19-7 cells (Fig. 5). Consistent with the previous finding that phospho-CREB selectively binds to active Dyrk1, the addition of bFGF induced a marked CREB phosphorylation. Furthermore, the overexpression of dominant-negative Dyrk1 in a transient manner remarkably attenuated the CREB phosphorylation upon the stimulation with bFGF compared with the control cells (Fig. 5). These data suggest that Dyrk1 directly phosphorylates the Ser-133 residue of transcription factor CREB during neuronal differentiation in H19-7 cells.

*Effect of Dyrk1 Activation on the CRE-dependent Gene Transcription during Neuronal Differentiation*—To assess whether Dyrk1 exerts its stimulatory effect on CRE-mediated gene transcription as well as on CREB activation, the gene expression of CRE-containing TK promoter-reporter construct was assayed in response to bFGF (Fig. 6). 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 4 h (12). To test the role of Dyrk1 activation on CRE-mediated gene transcription, the cells were transfected transiently with pCRE-TK-Luc reporter plasmid plus an expression vector encoding kinase-inactive Dyrk1 mutants (pSVL-HA-Dyrk1A/K188R). Whereas the addition of bFGF led to an increase of CRE-mediated reporter luciferase activity, the expression of kinase-deficient Dyrk1 proteins with the mutation of critical Lys-188 residue significantly inhibited the activation of luciferase activity triggered by bFGF (Fig. 6). Taken together, these findings implied that neurogenic bFGF causes the activation of CRE-mediated gene transcription, possibly through the activation of Dyrk1 and subsequent CREB phosphorylation in embryonic CNS hippocampal cells.

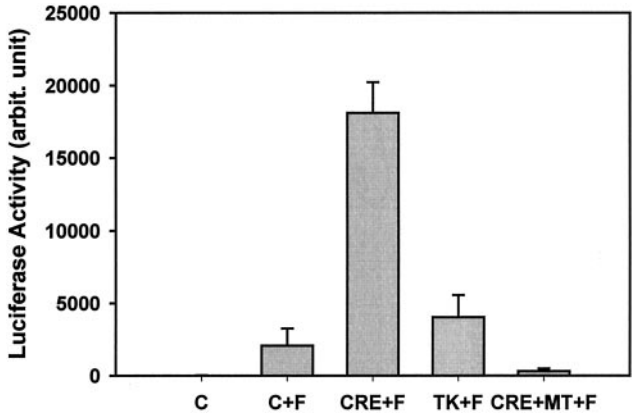


FIG. 6. **Effect on bFGF-induced activation of Dyrk1 on CRE-mediated gene transcription in H19-7 cells.** Where indicated, 1 $\mu$g of DNA of pCRE-TK-Luciferase (*CRE*) reporter plasmid was transiently transfected into H19-7 cells alone or along with 1 $\mu$g of kinase-inactive Dyrk1 K188R vector (*MT*). The cells were then untreated (*C*) or stimulated with 10 ng/ml of bFGF (*F*) for 4 h, and the luciferase activity of reporter plasmid was measured as described under "Experimental Procedures." In every transfection experiment, the CRE-lacking TK promoter construct, pTK-Luc (*TK*), was used as a negative control. Data are plotted as the percent of maximum luciferase activity and represent the mean plus range of samples from three independent experiments in triplicate.

*Effect of Dyrk1 Activation on Neuronal Differentiation in H19-7 Cells*—The functional role of Dyrk1 activation during neuronal differentiation in H19-7 cells was further examined. After treatment with bFGF, most of the H19-7 cells displayed neurite extension at 39 °C at which the large T-antigen is inactive (Fig. 7*A*). The differentiated cells were shown to be resistant to mitogenic stimulation by serum and to express neuronal markers such as neurofilament and brain type II sodium channel (9, 16). The capability of the H19-7 cells to differentiate in response to bFGF is similar to the response of primary hippocampal cells during late embryogenesis, since bFGFs act as a differentiating factor in certain CNS regions, such as the hippocampus, to express bFGF receptor (33). After a plasmid encoding either wild type or dominant-negative Dyrk1 was transfected into the cells, the formation of neurite outgrowth was subsequently analyzed. As shown in Fig. 7*A*, the vehicle-transfected control cells displayed a similar percentage of differentiated cells (~68%) in two separate transfection experiments. However, cells in the mutant Dyrk1-transfected population exhibited only 26% differentiated cells (Fig. 7*B*). Interestingly, transient transfection of wild type Dyrk1 had no apparent effect on the neuronal differentiation in H19-7 cells (Fig. 7*A*). These results suggest that Dyrk activation is likely to play an important role in the differentiation of neuronal H19-7 cells.

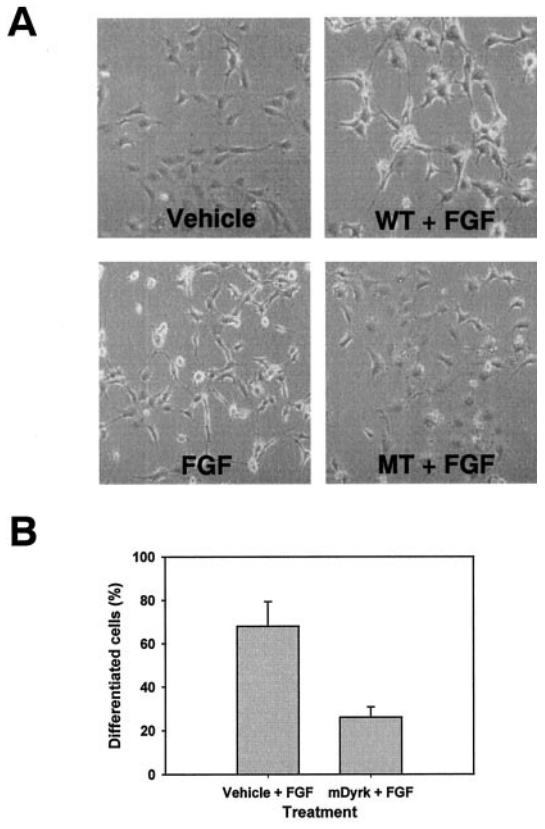


FIG. 7. **Effect of Dyrk1 activation on bFGF-induced neuronal differentiation.** Where indicated, H19-7 cells were mock-transfected (*Vehicle*) or transiently transfected with 5 $\mu$g of either pSVL-HA-Dyrk1A vector to express wild type Dyrk1 kinase (*WT*) or pSVL-HA-Dyrk1A to express K188R Dyrk1 mutant (*MT* or *mDyrk*). The cells were then either untreated or stimulated with 10 ng/ml bFGF (*FGF*) under differentiation condition for 48 h, and the change of cell morphology was observed by optical microscope (*A*). The differentiated cell percentages were the ratios relative to the total cell numbers (*B*). The results represent the mean plus the range of data from two independent experiments in triplicates.

## DISCUSSION

The participation of Dyrk1 or other Dyrk-related kinases in any particular signal transduction pathway has not been elucidated so far. The selective recognition of the correct substrate by protein kinase is an important biochemical mechanism that underlies the specificity of cellular responses to various stimuli (13). It is therefore important to determine the nature of Dyrk substrate to understand its function in the regulation of cellular responses. Previously, Dyrk was shown to be required for the proliferation of distinct neuronal cell types during postembryonic neurogenesis in *Drosophila* (4). Mutant flies with a reduced Dyrk1 expression have a reduced number of neurons in distinct areas of the adult brain and exhibit specific behavioral defects (4). In the present study we examined the cellular Dyrk1 substrate and its functional role during neuronal differentiation in hippocampal H19-7 cells. We have shown that Dyrk1 is activated by neurogenic bFGF but not by mitogenic EGF and that active Dyrk1 stimulates CREB phosphorylation and subsequent CRE-mediated gene transcription. Our data strongly suggest that Dyrk1 may play an important role during neurogenic factor-induced differentiation in CNS neuronal cells. Such a role was further supported by the finding that the expression of kinase-deficient Dyrk1 in a transient manner remarkably attenuates the formation of differentiated cells.

We have shown that the kinase-inactive Dyrk mutant could block bFGF-induced phosphorylation of CREB (Fig. 5). Since this mutant does not bind to CREB (Fig. 1), the effect in Fig. 5 might not be due to the competition between endogenous and kinase-deficient Dyrk1 for CREB. Rather, it shows that if the kinase-inactive mutant is working specifically, then it must act by binding to the site that normally activates Dyrk1 and competing with endogenous Dyrk1 there. Since there could be other effectors that might also be activated at this site, such as non-Dyrk1 proteins, then the kinase-minus Dyrk mutant might block more than Dyrk1-mediated effects.

The Dyrk1 protein contains several striking structural features, such as a bipartite nuclear localization signal, a PEST region, repetitively present 17-serine/threonine residues, and an activation loop (YQY motif) between subdomain VII and VIII (14). Dyrk1 is localized to the cell nucleus (17), hence gaining the potential to control the expression of other genes. A green fluorescent protein-Dyrk1A fusion protein was found in

the nucleus of transfected COS-7 or HEK293 cells (6, 17). Furthermore, this kinase has been shown to be a dual specificity protein kinase regulated by tyrosine phosphorylation in the activation loop (14). This suggests that Dyrk1 might be a component of a signaling pathway regulating nuclear events, but exactly how Dyrk1 is involved in this mechanism has not been elucidated so far. The present study, showing as it does that Dyrk1 is activated by bFGF and could activate CREB and subsequent CRE-mediated gene transcription, strongly supports the idea that Dyrk1 controls the expression of a certain gene(s). It is possible that gene dosage for such a protein could alter the expression of downstream genes and contribute to the welter of phenotypic effects seen in DS (17).

We also found that bFGF does not affect the expression of Dyrk1 and that significant levels of Dyrk1 continue to be constitutively expressed in H19-7 cells. This is in good accordance with previous reports that Dyrk1 is expressed strongly in the brain from embryonic rat (18) and ubiquitously but most abundantly in CNS regions of 17-day mouse embryo, such as in the cerebral cortex, cerebellum, and the hippocampus (19, 20).

Neuronal activity plays a critical role in many forms of synaptic plasticity such as learning and memory (21). Studies on the formation of long term memory indicate that the induction of immediate early genes is often associated with memory storage (22, 23). The immediate early gene products are thought to activate late effector genes, which alter the structural and functional properties of nerve cells. Although the molecular mechanism by which neuronal activity is coupled to the alteration in gene expression is poorly understood, CREB phosphorylation appears to be important in mediating the expression of several immediate early genes (24, 25). Moreover, CREB has been implicated in the formation of long term memory. CREB supports memory in various organisms that perform different behavioral tasks, from simple reflexes in mollusks to complex emotional behaviors in mammals. For example, blockade of CREB activation by injection of CRE-containing oligonucleotides has been found to impede long term facilitation, a correlate of memory in the marine mollusk, *Aplysia californica* (26). Knock-out mice with a deletion in the CREB gene and *Drosophila melanogaster* expressing a repressor form of CREB show deficits in long term memory without any effect on short term memory (27, 28, 32).

The CNS limbic system, including the hippocampus, plays a critical role in the processes of emotional behavior, memory, integration of homeostatic responses, sexual behavior, and motivation. The transgenic mice overexpressing Dyrk1 in the forebrain, including the hippocampus, show significant defects in learning and memory as well as long term potentiation (29, 30). In addition, functional magnetic resonance imaging indicates a lower level of metabolic activity in the hippocampus of these mice (29). Underlying these changes are anatomical deficits in the expression of hippocampal synaptic and axonal markers. Furthermore, with progressing age, transgenic mice show a significant reduction in hippocampal volume and a concomitant enlargement in ventricular size, suggesting that the overexpression of Dyrk may affect postnatal hippocampal development.

Down's syndrome is a developmental disorder. Individuals with DS have a higher incidence of complicated medical problems (31). For example, children with DS are at increased risk for congenital heart defects and have increased susceptibility to infection, respiratory problems, obstructed digestive tracts, and childhood leukemia. Adults with DS are at increased risk for Alzheimer's disease. Most people with DS have some level of mental retardation. The secondary messenger cAMP regulates a striking number of physiological processes, including intermediary metabolism, cellular proliferation, and neuronal signaling, by altering the basic pattern of gene expression via a conserved CRE motif. In addition, CREB phosphorylation appears to be important in mediating the expression of various kinds of genes and in the formation of long term memory, as described previously. Based on the findings presented here that Dyrk1 activates CREB during neuronal differentiation in CNS hippocampal cells, further analysis of the upstream signaling pathways activated by various neurogenic growth factors may give deeper insights into the mechanism of neuronal differentiation mediated by Dyrk1 activation.

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