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Bruton's Tyrosine Kinase Phosphorylates cAMP-responsive Element-binding Protein at Serine 133 during Neuronal Differentiation in Immortalized Hippocampal Progenitor Cells*

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Bruton's tyrosine kinase (BTK) is a member of the Tec family of kinases, which is a subgroup of the nonreceptor cytoplasmic protein tyrosine kinases. BTK has been shown to be important in the proliferation, differentiation, and signal transduction of B cells. Mutations in BTK result in B cell immune deficiency disorders, such as X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice. Although BTK plays multiple roles in the life of a B cell, its functional role in neuronal cells has not been elucidated. In the present study, we demonstrate that BTK activates transcription factor, cAMP response element (CRE)-binding protein (CREB), and subsequent CRE-mediated gene transcription during basic fibroblast growth factor (bFGF)-induced neuronal differentiation in immortalized hippocampal progenitor cells (H19-7). The kinase activity of BTK is also induced by bFGF, and BTK directly phosphorylates CREB at Ser-133 residue, indicating that BTK has a dual protein kinase activity. In addition, blockading BTK activation significantly inhibits CREB phosphorylation as well as the neurite outgrowth induced by bFGF in H19-7 cells. These results suggest that the activation of BTK and the subsequent phosphorylation of CREB at Ser-133 are important in the neuronal differentiation of hippocampal progenitor cells.

Growth factors act by binding to cell surface receptors to elicit the regulation of cell growth and differentiation (1). This, in turn, triggers a variety of intracellular signaling pathways that ultimately control cell physiology. The activation of signaling cascades changes gene expression patterns through the functional modulation of various transcription factors. These processes allow cells to coordinate long term physiological adaptation.

cAMP response element-binding protein (CREB)[1] is a central transcription factor that mediates cAMP- and calcium-dependent gene expression through the cAMP response element (2). Moreover, CREB activity is regulated by multiple kinases after various kinds of stimulation. Many Ser/Thr kinases can phosphorylate CREB, including protein kinase C (3), Ca^{2+}/calmodulin-dependent protein kinases (4, 5), Ras-dependent p105 kinase (6), p90rsk (7), and Rsk2 (8). CREB is believed to be necessary for long term potentiation in invertebrates and vertebrates (9). Furthermore, the activation of CREB plays an important role in neuronal differentiation (10, 11). Specific roles for CREB in neuronal development have been found by manipulating CREB function *in vivo* and *in vitro* (12).

Bruton's tyrosine kinase (BTK) family kinases are members of a group of nonreceptor tyrosine kinases, which include BTK, Tec, and Bmx. These kinases have a canonical pleckstrin homology (PH) domain at the amino terminus, followed by cysteine-rich and proline-rich regions, which together have been termed the Tec homology domain (13). The proline-rich portion of the Tec homology domain mediates interactions with the Src homology-3 (SH3) domains *in vitro* and may stabilize regulatory interactions with Src-type kinases *in vivo* (14, 15). The expression of BTK is restricted to a subset of B cells and myeloid cell (16) and is involved in signaling of B cell antigen receptor, mast cell FcεR, interleukin-5 receptor, and interleukin-6 receptor. Mutations in BTK are associated with X-linked agammaglobulinemia in humans and X-linked immunodeficiency and an impaired B cell proliferation in mice. A large number of proteins interact with BTK and are phosphorylated in a BTK-dependent manner. These include BAP-135, pp70, WASP, c-Cbl, Sam-68, vav, and EWS (16). Although these proteins are putative BTK regulators or effectors by virtue of their binding activity and/or BTK-dependent phosphorylation, there is no direct biological evidence that demonstrates their role in BTK signaling.

Although BTK is expressed ubiquitously in nearly all cells of the hematopoietic lineages, only B cells have been shown to be vulnerable with respect to functional integrity. In addition, the molecular mechanisms underlying BTK activation in other

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[1] The abbreviations used are: CREB, cAMP-responsive element-binding protein; bFGF, basic fibroblast growth factor; BTK, Bruton's tyrosine kinase; ca, constitutive active; CRE, cAMP-responsive element; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; GST, glutathione *S*-transferase; Luc, luciferase; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PH domain, pleckstrin homology domain; PI-3K, phosphatidylinositol 3-kinase; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyridine; SH domain, Src homology domain; siRNA, short RNA interference; TK, thymidine kinase.

cells, including neuronal cells, are not well understood. Interestingly, one of the genes in the breakpoint region of 7q11.23 in Williams-Beuren syndrome, a neurodevelopmental disorder with multisystemic manifestations, which is caused by a heterozygous deletion in 7q11.23, encodes BAP-135, a phosphorylation target of BTK (17). This finding suggests that the signal transduction mediated via BTK activation appears to be important for normal neural development. Recently it was shown that a small pool of BTK could translocate to the nucleus, although it continues to be present predominantly in the cytoplasm (18). BTK can also translocate to the plasma membrane upon growth factor stimulation (19). To identify the upstream signal transduction pathways in which CREB functions, we sought to identify proteins that interact specifically with and directly phosphorylate CREB *in vivo*. In the present study, we demonstrate that CREB is a substrate for BTK phosphorylation. Interestingly, active BTK directly phosphorylates CREB on Ser-133, suggesting that BTK could be a member of dual specificity protein kinases that are capable of phosphorylating both serine/threonine and tyrosine residues. Mutations of BTK which impair its activation or the suppression of endogenous BTK by siRNA duplexes lead to the abolishment of BTK-dependent phosphorylation of CREB and to a significant inhibition of neurite outgrowth induced by neurogenic growth factor. These findings suggest that CREB lies downstream of BTK in a signaling pathway started by neurogenic bFGF, and CREB activation via BTK is important for neuronal differentiation in central nervous system hippocampal progenitor cells.

EXPERIMENTAL PROCEDURES

Materials—Peroxidase-conjugated anti-rabbit and anti-mouse IgGs were purchased from Zymed Laboratories, Inc. (San Francisco). Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), and LipofectAMINE PLUS reagent from were from Invitrogen. Protein A-Sepharose was from Amersham Biosciences. Anti-CREB and anti-phospho-CREB antibodies were from PerkinElmer Life Sciences and Upstate Biotechnology (Lake Placid, NY), respectively. Anti-rabbit polyclonal anti-BTK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL reagents and [γ-^{32}P]ATP were from PerkinElmer Life Sciences. Selective Src kinase inhibitor PP1 was from A. G. Scientific, Inc. (San Diego). The synthetic yeast dropout medium (SD/-T, SD/-L, SD/-HLT) and yeast extract peptone dextrose containing adenine were from Bio 101, Inc. (Vista, CA). 3-Amino-1,2,4-triazole and 5-bromo-4-chloro-3-indolyl-D-galactoside were from Sigma and Promega, respectively. The human fetal brain cDNA library was from Clontech. Human bFGF was from Sigma, and the luciferase assay kit was purchased from Promega. pVP16-CREB and pVP16-CREB S133A were gifts from K. Saeki (Research Institute in International Medical Center, Tokyo, Japan). The hemagglutinin-tagged wild type or kinase-inactive BTK mutants (K430E, in which the Lys-430 in the kinase domain of BTK was mutated to Glu) were gifts from A. L. Roy (Tufts University School of Medicine, Boston). The double tyrosine-mutated BTK construct (pEGFP-BTK-2MT-Y223A/Y551F, in which Tyr-223 in the SH3 domain of BTK and Tyr-551 in the kinase domain were replaced by Ala and Phe, respectively) was generously provided by A. J. Mohamed (Karolinska Institute, Huddinge, Sweden). A eukaryotic expression vector encoding constitutive active BTK, pSR-MSVTK-caBTK, in which Glu-41 residue in the PH domain was replaced by Lys, was kindly provided by O. N. Witte (University of California, Los Angeles). A prokaryotic expression plasmid encoding bacterially recombinant GST-caBTK was constructed by inserting the NotI fragment of the caBTK sequence digested from pSR-MSVTK-caBTK into pGEX4T1 vector (Amersham Biosciences). The plasmids encoding v-Src (pMvSrc) and mutant Src (pcSrc295Arg) were gifts from D. Foster (Hunter College of the City University of New York).

Yeast Two-hybrid Assay—The bait vector for yeast two-hybrid assay was constructed by subcloning the mutant CREB cDNA, in which RRPSY (from amino acids 130–134) was replaced by RRSLY, into pHybTrp/Zeo. Human fetal cDNA library subcloned into prey vector (pACT2) was purchased from Clontech. All yeast two-hybrid screening protocols were performed as described previously (20). Sequencing of the inserts in positive library plasmids was performed using an automatic DNA sequencer (ALF express, Amersham Biosciences).

Cell Culture—Immortalized hippocampal neuronal cells (H19-7) were grown on poly-L-lysine-coated cell culture dishes in Dulbecco's modified Eagle's medium containing 10% FBS and 100 units/ml penicillin-streptomycin (21). The cells were maintained at 33 °C under 5% CO_2 and stimulated with 10 ng/ml neurogenic growth factor, bFGF, to induce neuronal differentiation, after being treated with serum-free N2 medium for 2 days, as described previously (22). To prepare the cell lysates, cells were rinsed twice with ice-cold phosphate-buffered saline and then 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 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 5 mM Na_2EDTA, 10% glycerol, 1 mM β-glycerophosphate, 0.1 g/ml *p*-nitrophenyl phosphate, and 0.2 mM phenylmethylsulfonyl fluoride). The cells were removed by scraping, and the supernatants were collected after centrifugation for 15 min at 14,000 $\times$ *g* at 4 °C. Primary cortical neurons were obtained from rat fetal brain on embryonic day 15 and triturated. Dissociated cells were plated in 24-well culture plates coated with 100 mg/ml poly-D-lysine and 4 mg/ml laminin and maintained in Eagle's minimal essential medium (Eagle's salts, glutamine-free) supplemented with 21 mM glucose, 5% FBS (Biofluid), 5% horse serum, and 2 mM glutamine, at 37 °C under a humidified atmosphere of 95% air plus 5% CO_2. Plating density was adjusted to 10^5 cells/culture well. After 2 days, the culture medium was replaced with growth medium identical to plating medium, but lacking fetal serum, and then fed twice a week. The U937 cell line was cultured in RPMI 1640 supplemented with 10% FBS and kept in a humidified incubator at 37 °C in 5% CO_2 plus 95% air. SH-SY5Y cell line was grown on Dulbecco's modified Eagle's medium containing 10% FBS and 100 units/ml penicillin-streptomycin. The cells were maintained in a humidified incubator at 37 °C under 5% CO_2 condition.

Immunohistochemistry—Adult mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and postfixed in the same buffer for 24 h at 4 °C. Thereafter, they were cryoprotected in 30% sucrose, frozen on dry ice, and sectioned on a cryostat. Serial coronal or sagittal sections (40 μm thick) were collected in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M phosphate buffer, pH 7.4). Immunohistochemistry was performed by using polyclonal anti-BTK antibodies and developed using avidin-biotin technique (Elite ABC kit, Vector Laboratories).

DNA Transfection and Luciferase Reporter Assay—H19-7 cells were plated at a density of 2 $\times$ 10^6 cells/well in 100-mm dishes, were transfected when 70~90% confluent with suitable plasmid constructs using LipofectAMINE PLUS reagent according to the manufacturer's instructions. The luciferase reporter construct, pCRE-TK-Luc, was transiently cotransfected with the kinase-inactive mutant BTK kinases (Y223A/Y551F, R28C, or K430E), and luciferase activity was measured using a luciferase assay kit (Promega) and a luminometer (EG & G Berhold, Germany). The CRE-lacking TK promoter construct (pTK-Luc) was used as a negative control throughout.

Microinjection of H19-7 Cells—Around 4.5 $\times$ 10^4 cells were seeded to poly-L-lysine-coated coverslips placed on 6-well plate and grown at 33 °C. After the cells grew to the desired densities, anti-rabbit polyclonal anti-BTK antibody or anti-mouse control IgG (0.5 mg/ml) was microinjected with the injection buffer (50 mM HEPES, pH 7.4, 40 mM NaCl) containing 0.5% dextran rhodamine into the cell nuclei. Microinjection was done with an Eppendorf Micromanipulator 5171, Microinjector FemtoJet, and Olympus DP50 microscope. The cells were recovered in 10% FBS at 33 °C overnight before being shifted to 39 °C in N2 medium and were treated with 10 ng/ml bFGF. The cells containing dextran rhodamine were examined by immunofluorescence microscopy.

Immunoprecipitation—1 μg of polyclonal anti-BTK antibody was incubated with 600 μg of cell extract prepared in lysis buffer overnight at 4 °C. 40 μl of a 1:1 suspension of protein A-Sepharose beads was added 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 in SDS-PAGE sample buffer, and whole protein samples were separated on SDS-polyacrylamide gel.

Western Blot Analysis—Whole-cell lysates were separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Japan). The membrane was blocked in TBST buffer containing 20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween 20, and 3% nonfat dry milk for 3 h, and then incubated overnight at 4 °C in 3% nonfat dry milk containing anti-phospho-CREB, anti-CREB, or anti-BTK antibodies (Santa Cruz Biotechnology). The membrane was washed several times in TBST and then incubated with secondary IgG-coupled horseradish peroxidase antibody (Zymed Laboratories Inc.). After 60 min, blots were washed several times with TBST and visualized by enhanced chemiluminescence.

Analysis of bFGF-induced Neuronal Differentiation in H19-7 Cells—H19-7 cells were grown on poly-L-lycine-coated 6-well dishes to reach 70~90% confluence. The cells were transfected with 1 μg of mammalian constructs encoding wild type or dominant negative BTK mutants, such as kinase-inactive K430E or Y223A/Y551F mutant of BTK using LipofectAMINE reagent. The next day the cells were switched to N2 medium and cultured at 39 °C for 2 days. They were then treated with 10 ng/ml bFGF for 48 h, and any morphological changes were noted. Differentiated cells were defined as cells with a round and refractory cell body and at least one neurite of length exceeding the diameter of the cell body.

In Vitro BTK Kinase Assay—H19-7 cells grown in serum-free N2 medium for 2 days at 39 °C were treated with bFGF. The cells were harvested and lysed in lysis buffer, and 600 μg of the proteins so obtained were incubated with polyclonal BTK antibody overnight at 4 °C. The immunocomplexes were then added to 40 μl of a 1:1 suspension of protein A-Sepharose. After washing the samples three times in lysis buffer, kinase reactions were carried out at 30 °C for 60 min in 20 μl of kinase buffer containing 20 mM HEPES, pH 7.2, 5 mM $MnCl_2$, 200 μM sodium orthovanadate, 5 μg of acid-treated enolase, 10 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 5 μg of wild type GST-CREB or mutant GST-CREB S133A as a substrate. The reactions were stopped by adding SDS-sample buffer and analyzed by SDS-PAGE and autoradiography.

Assay of Bacterially Recombinant Fusion GST-BTK Protein with Constitutive Active Kinase Activity—The Sepharose-4B beads prebound to bacterially expressed GST-BTK with a constitutive active kinase activity were prepared by using Bulk GST purification module (Amersham Biosciences). 30 μl of beads were suspended in 200 μl of binding buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 30 mM $MgCl_2$, 40 mM NaCl, 0.5% Nonidet P-40) and mixed with 300 μg of either wild type GST-CREB or mutant GST-CREB S133A proteins for 2 h at 4 °C. After the bead pellet was washed twice with kinase buffer containing 20 mM HEPES, pH 7.2, 5 mM $MnCl_2$, 200 μM sodium orthovanadate, and 10 μM ATP, the pellet was resuspended in 20 μl of kinase buffer, and kinase reactions were carried out by adding 5 μCi of [γ-^{32}P]ATP at 30 °C for 60 min. The reactions were terminated by adding of SDS-sample buffer, and the phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography.

Proteomic Analysis of BTK Immunocomplexes—The total cell lysates were immunoprecipitated with a polyclonal anti-BTK IgG. After the beads were washed three times with lysis buffer, bound proteins were dissociated by boiling of the beads in SDS-PAGE sample buffer, and the eluted whole proteins were separated on 8% SDS-polyacrylamide gel. Next the gel was stained with Coomassie Blue R-250 dye followed by destaining overnight, and the individual protein band was digested. Each gel piece was then immersed in 100 μl of acetonitrile, dried under vacuum centrifugation for 10 min, rehydrated in trypsin buffer containing 1 μg/μl trypsin plus 50 mM ammonium bicarbonate, immersed in 100 μl of 50 mM ammonium bicarbonate, pH 8.0, incubated for 18 h at 37 °C, and analyzed by MALDI-TOF MS.

In Vitro In-gel Kinase Assay—An 8.0% SDS-polyacrylamide gel was prepared in the presence of 50 μg/ml bacterial recombinant wild type GST-CREB or mutant GST-CREB-S113A proteins as a phosphorylation substrate. The total cell extracts were prepared after the stimulation of H19-7 cells with bFGF and applied to the gel. All gel renaturation and phosphorylation protocols were performed as described previously (22).

Construction of siRNA Duplexes and Transfection—The siRNA duplexes for BTK were designed as described elsewhere (50). The cDNA-targeted region and the sequence of the siRNA duplexes for BTK are as follows: targeted region (cDNA), 895-GGGAAAGAAGGAGGTTTCA-913; sense siRNA, 5′-GGGAAAGAAGGAGGUUU CAUU-3′; antisense siRNA, 3′-UUCCCUUUCUUCCUCCAAAGU-5′. The siRNA duplexes of BTK were transfected by using LipofectAMINE PLUS reagent according to the manufacturer's instructions.

RESULTS

Identification of Novel CREB Kinase(s) Activated during Neuronal Differentiation in Immortalized Hippocampal Progenitor Cells—The conditionally immortalized hippocampal cell line (H19-7) was generated by transducing temperature-sensitive SV40 large T antigen into rat embryonic day 17 hippocampal cells (21). Central nervous system progenitor H19-7 cells have the ability to differentiate terminally into neuronal cells, at a nonpermissive temperature (39 °C), in the presence of several agents, such as bFGF. Differentiating H19-7 cells express several neuronal markers, including neurite outgrowth and neurofilament proteins. In an earlier study we showed that CREB phosphorylation and subsequent CRE-mediated gene transcription play an important role during bFGF-induced neuronal differentiation in H19-7 cells (23). Interestingly, CREB phosphorylation is not likely to be mediated by any of the previously known signaling pathways such as the MAPK, protein kinase A, protein kinase C, $p70^{S6K}$, or Ca^{2+}/calmodulin-dependent protein kinase pathways. These findings suggest that the activation of a novel protein kinase-signaling pathway is required to induce bFGF-responsiveness (23). Furthermore, the activation of two previously unreported CREB kinases of ~76 and 120 kDa by bFGF was observed by *in vitro* in-gel kinase assay with recombinant GST-CREB fusion proteins as substrates. CREB phosphorylation on Ser-133 is considered to be a critical step in CREB transcriptional activation. Moreover, the modification of either the catalytic or the regulatory domain of the transcription factor is frequently used to enhance and to stabilize complex formation with its kinase in the yeast two-hybrid assay (24–26). The same assay was performed to identify the upstream signal transduction pathways leading to CREB phosphorylation and to isolate the novel CREB kinase(s), using CREB as bait, in which the critically regulatory Pro-132 and Ser-133 residues were changed to Ser-132 and Leu-133. As a result of screening of human fetal brain cDNA library, many CREB-interacting proteins were identified, including histone deacetylase and Dyrk1A (20). In addition, it was found that BTK specifically interacts with CREB in yeast cells (data not shown). Because the molecular size of BTK (77 kDa) is comparable with that of one of the novel CREB kinases (23) and BTK plays an integral role in the differentiation and signal transduction directed by antigen-receptor in B cells, its functional role during neuronal differentiation was further examined.

Specific Binding between Active CREB and BTK during Neuronal Differentiation in H19-7 Cells—First, we examined whether BTK is expressed in various types of mammalian primary and transformed neuronal cells. As shown in Fig. 1*A*, immunohistochemical analysis has shown that significant levels of BTK are expressed in a variety of mice central nervous system regions, such as neocortex, cerebral cortex, cerebellum, and hippocampus. Furthermore, considerable levels of BTK protein are normally present in transformed neuronal cells. These includes human neuroblastoma SH-SY5Y cell line, immortalized rat embryonic hippocampal H19-7 cells, and rat pheochromocytoma PC12 cell line, compared with that in several immune B cells as a control (Fig. 1*B*). We also observed that BTK is enriched in the cell extracts from rat whole brain and cerebellum (Fig. 1*B*). These observations suggest that, outside of hematopoietic cells, BTK could play a role during cell differentiation and signal transduction in neuronal cells.

To confirm the previous finding that bFGF exerts its stimulatory effect on the activation of CREB (23), Western blot analysis was performed using an antibody specific for the Ser-133-phosphorylated form of CREB. During the differentiation of H19-7 cells by bFGF, the Ser-133 residue in the CREB protein was phosphorylated, but endogenous CREB levels were not markedly changed (Fig. 1*C*). Then we examined whether BTK specifically binds to CREB in hippocampal H19-7 cells. The H19-7 cell extracts obtained after neurogenic bFGF stimulation were immunoprecipitated using anti-BTK antibodies and then blotted using anti-phospho-CREB antibodies. As shown in Fig. 1*C*, the expression of BTK is not markedly changed by bFGF. However, the addition of bFGF leads to a marked increase in the specific binding between BTK and phospho-CREB. Furthermore, when the cell lysates were immunoprecipitated with anti-phospho-CREB antibodies and an-

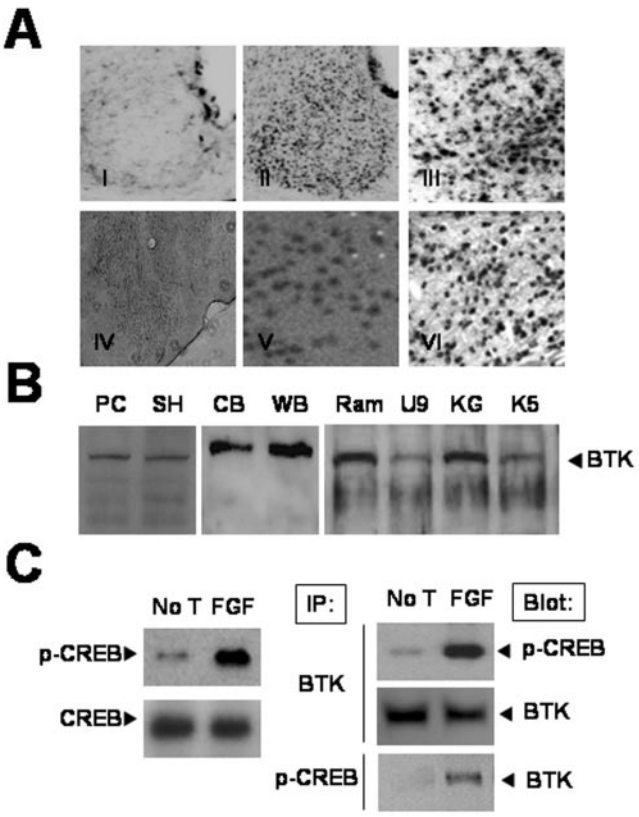

FIG. 1. **Bruton's tyrosine kinase, but not its kinase-inactive mutant, interacts with active CREB during neuronal differentiation in H19-7 cells.** *A*, localization of BTK in mouse brain. After perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, adult mice brains were fixed in the same buffer for 24 h at 4 °C. The brains were cryoprotected in 30% sucrose, frozen on dry ice, and sectioned on a cryostat. After serial sections were collected, immunohistochemical analysis was performed by using either preimmune serum (*I*) or polyclonal anti-BTK antibodies (*II–VI*). Micrographs are arranged with gross (*I*, *II*, *IV*) and enlarged structure (*III*, *V*, *VI*) of specified central nervous system regions. *I*, *II*, and *III*, cerebral cortex; *IV* and *V*, ventral neocortical area; *VI*, cerebellum. *B*, BTK expression in various neuronal cells. Where indicated, the cell extracts were isolated from either rodent brain, neuronal and hematopoietic cells, and the expression of endogenous BTK was examined by Western blot analysis. *PC*, rat pheochromocytoma PC12 cells; *SH*, human dopaminergic neuroblastoma SH-SY5Y cells; *CB*, rat cerebellum; *WB*, rat whole brain; *Ram*, human B lymphocyte Ramos cell; *U9*, human lymphocyte U937 cell line; *KG*, human leukemia KG1 cells; *K5*, human malignant myeloid K532 cells. *C*, specific binding between CREB and BTK in neuronal H19-7 cells. After H19-7 cells were stimulated with 10 ng/ml bFGF for 30 min, the phosphorylated and endogenous CREB proteins were identified by Western blot analysis (*left panel*). Where indicated, H19-7 cells were either untreated (*No T*) or stimulated with 10 ng/ml bFGF for 30 min. Total cell lysates were immunoprecipitated (*IP*) with polyclonal anti-BTK or anti-pCREB antibodies and blotted with an anti-phospho-CREB or anti-BTK IgG, as indicated. The quantity of immunoprecipitated BTK was determined by Western blot analysis using anti-polyclonal anti-BTK antibodies, respectively (*right panel*).

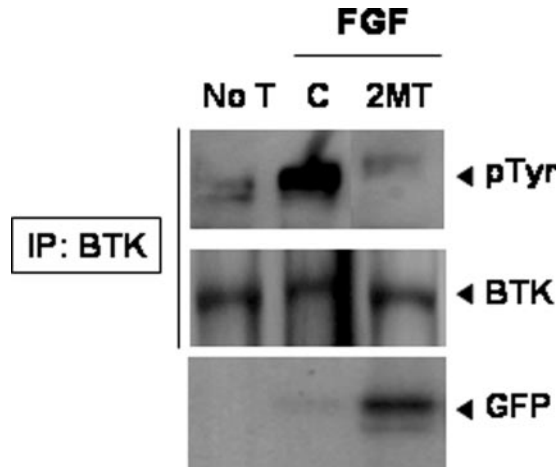

FIG. 2. **FGF-induced tyrosine phosphorylation of BTK in H19-7 cells.** Where indicated, the cells were either mock-transfected (*No T* or *C*) or transiently transfected with 5 μg of plasmids encoding kinase-inactive BTK (Y551F/Y223A). The cells were untreated or stimulated with 10 ng/ml bFGF for 30 min under differentiating condition. Total cell lysates were immunoprecipitated (*IP*) using BTK antibodies and detected with an anti-phospho-Tyr or anti-BTK IgG. The levels of exogenous added fusion GFP-BTK-Y551F/Y223A proteins were examined by polyclonal anti-GFP IgG. *No T*, no treatment; *C*, control; *2MT*, Y551F/Y223A.

alyzed by Western blot using anti-BTK IgG, it was found that the addition of bFGF resulted in specific binding between BTK and phospho-CREB (Fig. 1*C*). These results suggest that BTK interacts with active CREB in a specific way, during bFGF-induced neuronal differentiation in H19-7 cells.

BTK Is Activated Selectively by Neurogenic bFGF, but Not by Mitogenic EGF, in H19-7 Cells—Because BTK is activated by tyrosine phosphorylation in the activation loop of the catalytic domain, we assessed whether BTK is activated by neurogenic bFGF in H19-7 cells. The cells were stimulated with bFGF, and cell lysates were then immunoprecipitated with anti-BTK antibodies. The precipitated mixtures were separated by SDS-PAGE and analyzed using anti-phosphotyrosine antibody. As shown in Figs. 2 and 3*A*, tyrosine phosphorylation of endogenous BTK was observed during neuronal differentiation in H19-7 cells. The transient overexpression of dominant negative BTK mutants was used to block the activation of BTK (18, 27). The activity of BTK is partially regulated by transphosphorylation of its kinase domain (by Src family kinases) at residue Tyr-551 and subsequent autophosphorylation at Tyr-223. When the dominant negative BTK mutant BTK-Y551F/Y223A, in which Tyr-223 in the SH3 domain and Tyr-551 in the kinase domain were replaced by Ala and Phe, was overexpressed in a transient manner, the tyrosine phosphorylation of BTK was repressed extensively, compared with mock-transfected control cells (Fig. 2). These results suggest that neurogenic factor bFGF induces the activation of BTK in H19-7 cells.

Differences in CREB phosphorylation are known to be critical in the determination and regulation of EGF-mediated proliferation and bFGF-induced differentiation in neuronal H19-7 cell (23). In contrast to the prolonged CREB phosphorylation by neurogenic bFGF, mitogenic EGF treatment induces rapid but transient CREB phosphorylation in H19-7 cells (23). This finding suggests that stable CREB activation by bFGF is an important determinator of the fate of hippocampal progenitor cells, in terms of their terminal differentiation to neuronal cells. To verify that the activation of BTK occurs in a neurogenic growth factor-specific way, the effect of EGF stimulation on endogenous BTK phosphorylation was examined at mitogenic culture temperatures. As shown in Fig. 3*B*, EGF fails to phosphorylate the tyrosine residue of BTK at 33 °C, when the simian virus large T antigen is active. These results suggest that the activation of BTK is induced selectively by a neurogenic factor, but not by a mitogenic factor, and BTK plays a role during neuronal differentiation in H19-7 cells.

BTK Directly Phosphorylates CREB at Ser-133 in Response to bFGF—Next, we examined whether active BTK could phosphorylate CREB in H19-7 cells. The cell lysates obtained after bFGF treatment were immunoprecipitated using anti-BTK antibodies. Immunocomplex kinase assays were performed using bacterial recombinant GST-CREB as a substrate, and the phosphorylated substrate was visualized autoradiographically. As

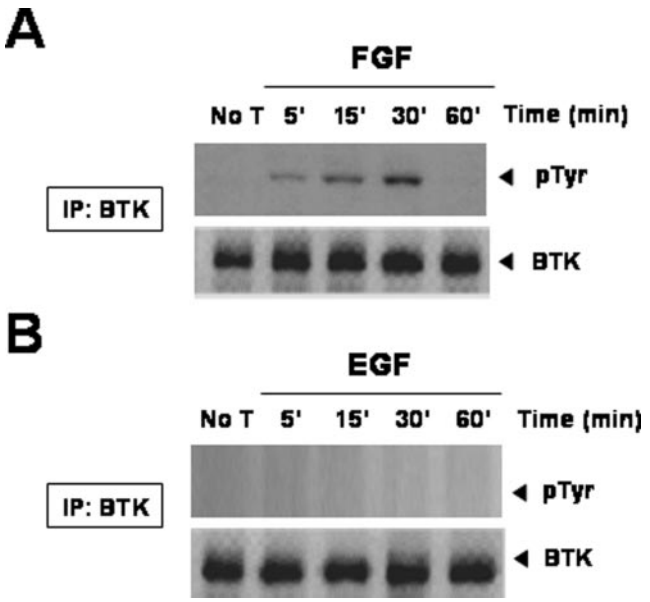

FIG. 3. **BTK is activated by neurogenic bFGF, but not by mitogenic EGF, in H19-7 cells.** Where specified, H19-7 cells were stimulated with either 50 ng/ml EGF under proliferation conditions or 10 ng/ml bFGF under differentiation conditions for the indicated times. Endogenous BTK was immunoprecipitated from cell lysates, and the BTK-bound protein mixtures were blotted with monoclonal anti-phosphotyrosine (*pTyr*) antibodies. As a control, the quantity of BTK immunoprecipitated was determined by Western blot analysis using anti-BTK antibodies (*lower panel*). *A*, bFGF at 39 °C; *B*, EGF at 33 °C; *IP*, immunoprecipitation; *No T*, no treatment.

shown in Fig. 4*A*, the phosphorylation of CREB significantly increased in the presence of bFGF. As a control for equal protein loading, we measured the amount of endogenous BTK enzymes in the immunoprecipitates by using anti-BTK IgG. Interestingly, when the phosphorylation-resistant GST-CREB mutant, in which the Ser-133 residue of CREB had been replaced by alanine, was used as a substrate, no significant CREB phosphorylation was observed compared with wild type CREB (Fig. 4*B*). To test the possibility that, rather than BTK, other contaminated Ser/Thr kinase(s) in the BTK-immunocomplexes phosphorylate CREB on Ser-133 residue in response to bFGF, the immunoprecipitated protein complexes prepared with anti-BTK antibodies were analyzed by SDS-PAGE followed MALDI-TOF MS. Although seven major proteins (aside from CREB) were detected in the BTK-protein complexes, including myosin, glucose-regulated protein precursor-78 immunoglobulin heavy chain binding protein, vimentin, platelet-derived growth factor receptor-β, actin-γ, minor striated-muscle tropomyosin-α, tropomyosin-β, and myosin regulatory light chain 2-A, we could not observe the presence of potential Ser/Thr kinase(s) in the BTK-immunocomplexes.

To verify the serine-specific kinase activity of BTK, an *in vitro* in-gel kinase assay was performed using polyacrylamide gel prepared in the presence of bacterially recombinant fusion GST-CREB or mutant GST-CREB S133A proteins. Equal protein-containing anti-BTK-IgG immunoprecipitates from H19-7 cells that had been stimulated with 10 ng/ml bFGF for 30 min were resolved by SDS-PAGE, renatured, and assayed for CREB phosphorylation in the gel. The results show that a 77-kDa band corresponding to the molecular size of BTK phosphorylate CREB in the gel (Fig. 4*C*). No significant kinase activity was detected when the mutant GST-CREB S133A was used as a substrate (Fig. 4*C*).

To ensure that the serine-specific kinase activity of BTK which we identified is not an artifact of the renaturation process, we have verified that active BTK is capable of phosphorylating CREB at Ser-133 *in vitro*. To do this, a prokaryotic GST fusion expression plasmid encoding caBTK mutant was constructed by replacing Glu-41 residue in the PH domain of BTK with Lys (28). Then, *in vitro* kinase activity of the recombinant caBTK proteins was examined using either bacterially recombinant wild type GST-CREB or GST-CREB S133A mutant as a substrate. As shown in Fig. 4*D*, the constitutive active GST-BTK (GST-caBTK) significantly phosphorylated GST-CREB *in vitro*, whereas mutant GST-CREB S133A proteins were not phosphorylated by GST-caBTK. Taken together, our data suggested that active BTK directly phosphorylates CREB on Ser-133, and thus BTK has a dual specific protein kinase activity.

Furthermore, the transient overexpression of the kinase-deficient BTK mutants, including BTK-K430E, in which the Lys-430 in the kinase domain was mutated to Glu, or BTK-Y223A/Y551F, significantly diminished bFGF-stimulated CREB phosphorylation, *versus* the control cells or the cells transfected with an expression vector encoding wild type BTK (Fig. 5*A*). When H19-7 cells were transfected with the plasmid encoding GFP fused with kinase-deficient BTK double mutant (Y223A/Y551F), *in vitro* kinase assay using GST-CREB as a substrate showed that the elevated levels of phospho-CREB induced by the addition of bFGF are significantly decreased by the overexpression of kinase-inactive BTK mutant (Fig. 5*B*). Overall these results suggest that BTK directly phosphorylates CREB during the differentiation of H19-7 cells, and its effect is specifically related to the CREB Ser-133 residue.

Effect of BTK Activation on CRE-dependent Gene Transcription during Neuronal Differentiation—To assess whether BTK exerts a stimulatory effect on CRE-mediated gene transcription as well as on CREB activation, we assayed the gene expression of CRE-containing thymidine kinase (TK) promoter-reporter construct. Treatment of mock-transfected control H19-7 cells with bFGF for 4 h resulted in increased CRE-mediated gene transcription (Fig. 6). To test the role of BTK activation in CRE-mediated gene transcription, H19-7 cells were transfected transiently with pCRE-TK-Luc reporter plasmid plus plasmid encoding a dominant negative BTK mutant, namely, K430E or Y223A/Y551F. The expression of any of these kinase-inactive BTK proteins significantly suppressed the activation of luciferase activity by bFGF, compared with mock-transfected control cells (Fig. 6). These results indicate that the stimulation of H19-7 cells with bFGF causes the activation of CRE-mediated gene transcription, possibly through the activation of BTK and subsequent CREB phosphorylation in embryonic hippocampal H19-7 cells.

Effect of BTK Activation on bFGF-induced Neuronal Differentiation in H19-7 Cells—The functional role of BTK activation during FGF-induced differentiation in H19-7 cells was examined further. Treatment of H19-7 cells with bFGF induced differentiation at 39 °C, at which large T antigen is inactivated. Differentiated cells are known to be resistant to mitogenic stimulation by serum and to express neuronal markers, such as neurofilament and brain type II sodium channels (21). In the present study, parental or mutant BTK expression vectors were transfected, and subsequently the formation of neurite outgrowth was analyzed in H19-7 cells (Fig. 7*A*). As shown in Fig. 7*A*, the addition of bFGF plus parental vector led to cells with a round and refractory cell body and with at least one neurite longer than the diameter of the cell body. As shown in Fig. 7*B*, mock-transfected control cells generated high levels of differentiated cells (~71%) in two separate transfection experiments. However, the cells in the mutant BTK-transfected population had remarkably reduced levels of differentiated cells (~36%) (Fig. 7, *A* and *B*). Taken together, these results suggest that relatively stable BTK activation by bFGF is likely to play

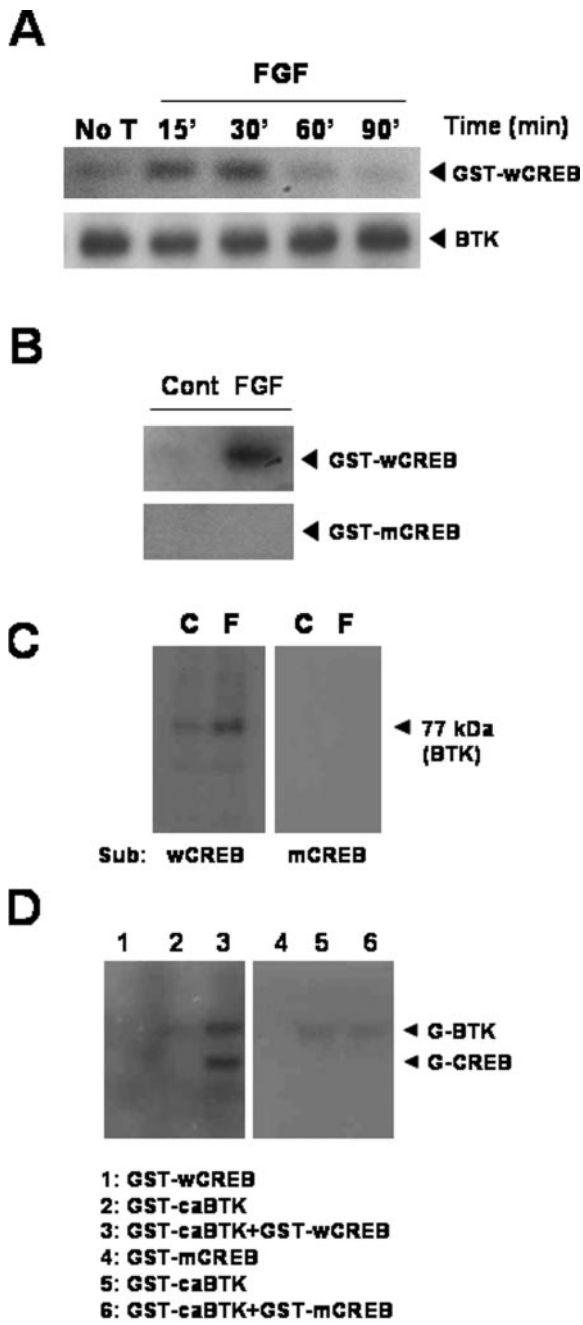

FIG. 4. **BTK directly phosphorylates CREB in response to bFGF in neuronal H19-7 cells.** *A* and *B*, H19-7 cells were stimulated with 10 ng/ml bFGF for the indicated times (*A*) or 30 min (*B*), and the cell lysates were isolated. Total cell lysates were immunoprecipitated with anti-BTK antibodies. The BTK levels in the immunocomplexes were identified using polyclonal anti-BTK antibodies. *In vitro* kinase assays were performed using bacterially expressed wild type GST-CREB (*wCREB*) or mutant CREB (*mCREB*), in which the Ser-133 residue was replaced by Ala, as an exogenous substrate. Kinase reaction products were resolved by 10% SDS-PAGE, and the levels of phosphorylated CREB were visualized autoradiographically. *IP*, immunoprecipitation; *No T*, no treatment; *Cont*, control. *C*, the H19-7 cells were untreated or stimulated for 30 min with 10 ng of bFGF/ml as indicated, and total cell lysates were immunoprecipitated with anti-BTK antibodies. The cell lysates containing 10–20 μg of proteins were resolved by SDS-PAGE on an 8% gel containing 50 μg of bacterially expressed wild type GST-CREB or mutant GST-CREB S133A (*mCREB*)/ml as a substrate. The in-gel kinase renaturation assay was performed as described under "Experimental Procedures." A 77-kDa CREB kinase activated by bFGF is indicated by an *arrow* (*C*, control; *F*, bFGF). *D*, Sepharose 4B beads prebound to bacterially expressed fusion proteins of wild type GST-CREB or the GST-CREB S133A mutant (*GST-mCREB*) were prepared as described under "Experimental Procedures" and used for substrates as indicated. 30 μl of Sepharose 4B beads was resuspended with Tris-buffered saline (1:1 ratio), and *in vitro* kinase assay was performed by adding bacterially recombinant GST-BTK with constitutive active kinase activity (*GST-caBTK*) for 60 min at 30 °C. The final eluates from the beads were resolved by SDS-PAGE (8% gel) and visualized by autoradiography. The phosphorylation products corresponding to the molecular sizes of fusion GST-caBTK (*G-BTK*, 105 kDa) and GST-CREB (*G-CREB*, 67 kDa) proteins are indicated by an *arrow*, respectively.

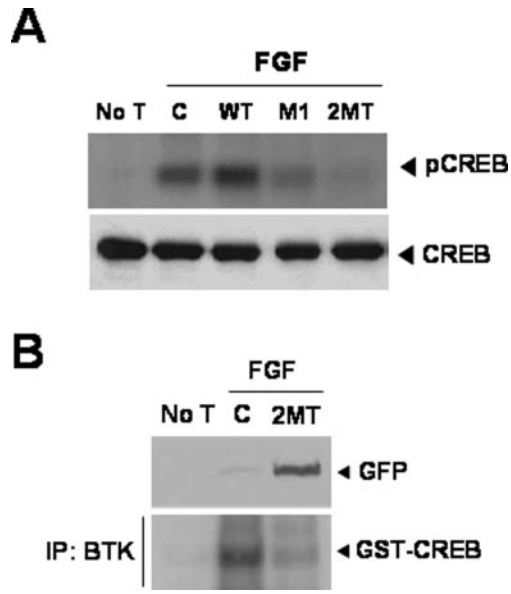

FIG. 5. **Effect of kinase-inactive BTK on FGF-induced CREB phosphorylation in H19-7 cells.** *A*, where indicated, H19-7 cells were mock-transfected (*No T* and *C*) or transfected in a transient manner with 5 μg of cDNA construct encoding wild type or kinase-deficient BTK, namely, K430E or Y551F/Y223A mutant. The cells were then stimulated with 10 ng/ml bFGF for 30 min and the cell lysates isolated. Total cell lysates were resolved by 10% SDS-PAGE and analyzed using either polyclonal anti-CREB or anti-pCREB IgG. *B*, H19-7 cells were mock-transfected or transfected with the plasmid encoding GFP fused to double tyrosine-mutated BTK (Y223A/Y551F). The cells were then either untreated or stimulated with 10 ng/ml bFGF for 30 min. Transfected protein levels of the double tyrosine-mutated BTK were identified by Western blotting using anti-GFP antibodies. Total cell extracts were immunoprecipitated (*IP*) with anti-BTK, and *in vitro* kinase assay was performed using GST-CREB as a substrate. *No T*, no treatment; *C*, control; *WT*, wild type; *M1*, K430E; *2MT*, Y551F/Y223A.

an important role in the differentiation of hippocampal progenitor H19-7 cells.

To confirm the hypothesis that the activation of BTK is required for neuronal differentiation in a single cell level, measurement was done on single cells where the individual cells expressed the protein of interest. The H19-7 cells were transiently transfected with a vector to express green fluorescent protein (GFP) fused with kinase-inactive BTK mutant, pEGFP-BTK-2MT, and subsequently the effect on bFGF-mediated differentiation was examined. Analysis of the GFP-fluorescent cells showed that the inhibition of BTK makes a remarkable inhibition of cellular differentiation. (Fig. 8, *A* and *C*). Meanwhile, the control cells transfected with an empty expression vector encoding GFP alone showed a characteristic neurite outgrowth, suggesting that the blockade of neuronal differentiation is caused by the expression of dominant negative BTK mutant but not by GFP. Similar results were obtained when H19-7 cells were microinjected with anti-BTK antibodies and differentiated by bFGF (Fig. 8, *B* and *C*).

Effect of BTK Silencing by siRNA Duplexes on CREB Phosphorylation and Neuronal Differentiation in H19-7 Cells—Besides using a dominant negative approach to implicate BTK, we

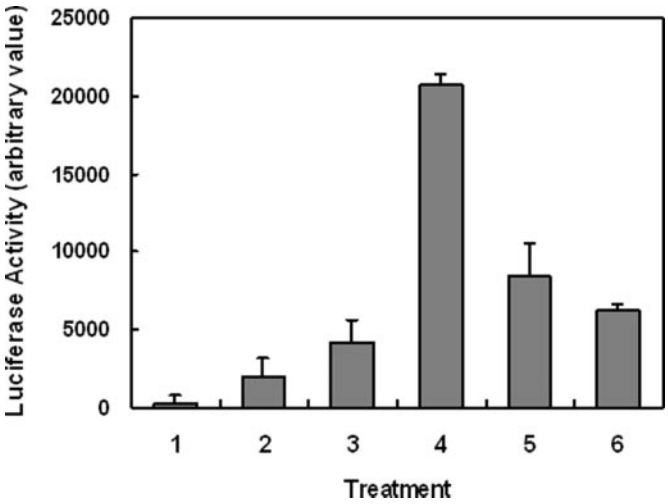

FIG. 6. **Effect of BTK activation on CRE-mediated gene transcription in H19-7 cells.** Where indicated, the cells were mock-transfected or 1 μg of CRE-reporter plasmid, pCRE-TK-Luciferase (CRE-Luc) was transiently transfected into H19-7 cells alone (Control) or with 1 μg of plasmid encoding hemagglutinin-tagged kinase-dead BTK mutant, namely K430E (M1), or double tyrosine-mutated Y223A/Y551F (2MT). The cells were then either untreated or stimulated with 10 ng/ml bFGF (FGF) for 4 h, and the luciferase activities of the reporter plasmid were measured. The CRE-lacking TK promoter construct, pTK-Luc (TK-Luc), was used as a negative control in every transfection experiment. Data are plotted as percentages of maximum luciferase activity and represent the means plus the range of sample results obtained from independent experiments performed in triplicate. *1*, Control; *2*, FGF; *3*, TK-Luc + FGF; *4*, CRE-Luc + FGF; *5*, CRE-Luc + M1 + FGF; *6*, CRE-Luc + 2MT + FGF.

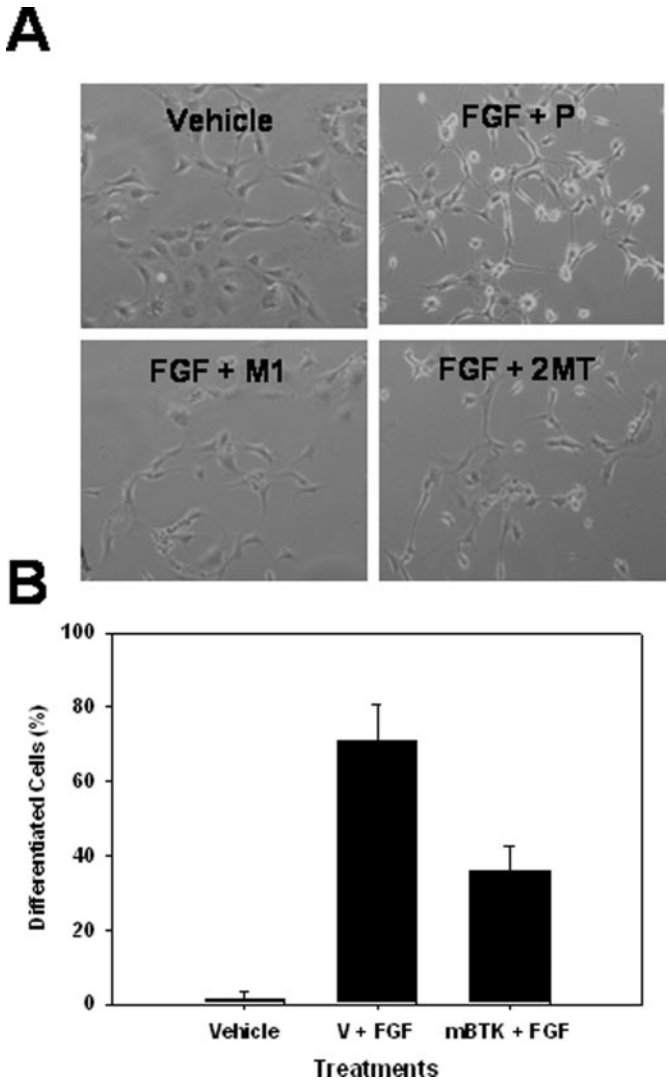

FIG. 7. **Activation of BTK is important for neuronal differentiation in hippocampal H19-7 cells.** Where indicated, H19-7 cells were mock-transfected (*Vehicle*) or transiently transfected with 5 μg of vectors expressing parental vector (*P*) or kinase-inactive BTK mutant, namely, K430E (*M1*) and Y223A/Y551F (*2MT*). The cells were then either untreated or stimulated with 10 ng/ml bFGF (FGF) under differentiation conditions for 48 h, and changes in cell morphology were observed under the optical microscope (*A*). Differentiated cell percentages are expressed as ratios relative to the total cell numbers (*B*). The results represent the means and the ranges in the data from two independent experiments performed in triplicate.

attempted to prove that BTK is the kinase responsible for phosphorylating and activating CREB during neuronal differentiation using double-stranded short RNA interference (siRNA) technology. First, we examined whether endogenous BTK protein can transiently be depleted using BTK siRNA oligonucleotides. We synthesized siRNA duplexes specific for BTK mRNA, the site being conserved on both the human and the mouse gene (50). After the transfection of BTK siRNA duplexes, Western blotting analysis demonstrated that BTK silencing was remarkable up to 90% in H19-7 cells (Fig. 9*A*), suggesting the specificity of siRNA silencing of endogenous BTK expression. Second, to study the biological relevance of endogenous BTK suppression by siRNA duplexes, we investigated its effect on CREB phosphorylation and neurite outgrowth in H19-7 cells under neuronal differentiation. In bFGF-stimulated cells, *in vitro* kinase assay showed that elevated levels of phosphorylated CREB were significantly diminished by the presence of siRNA for BTK (Fig. 9*A*). Furthermore, the neurite outgrowth was also significantly decreased (by $\sim$ 65%) when BTK expression was down-regulated by BTK siRNA duplexes (Fig. 9*B*). These results validated the specificity and accuracy of our current findings, when siRNA down-regulation was compared with the overexpression of kinase-deficient BTK mutant proteins. Taken together, these results indicate that BTK kinase activity is required for efficient CREB phosphorylation and therefore possibly influences neuronal differentiation.

The Activation of BTK in Response to bFGF Is Linked to the Signaling Pathways Activated by Extracellular Signal-regulated Protein Kinase, Phosphatidylinositol 3-Kinase, and Src Kinase—To characterize the possible "cross-talk" between BTK and the intracellular signaling pathways mediated by other protein kinases, such as MAPK (or extracellular signal-regulated protein kinase, ERK) or phosphatidylinositol 3-kinase (PI-3K) in neuronal H19-7 cells, we examined the blockade effect of each signaling pathway on the bFGF-induced activation of BTK and BTK-mediated CREB phosphorylation by using its synthetic chemical inhibitor or kinase-inactive mutant. BTK activation has been reported to depend on PI-3K in immune B cells (19, 29). In addition, sustained activation of ERK2 requires BTK during B cell antigen receptor-induced signaling events in chicken DT40 B cell line (30). As shown in Fig. 10*A*, when H19-7 cells were pretreated with the specific inhibitor of MEK, PD98059, the activation of BTK and its kinase activity was suppressed by bFGF to the level in control cells. Previously it has been shown that bFGF exerts its stimulatory effect on the activation of MEK-ERK (31). Furthermore, the same concentration of PD98059 was shown to inhibit the activation of ERK by bFGF completely (32). In a similar way, pretreatment of H19-7 cells with LY294002, the specific inhibitor of PI-3K, completely inhibited the activation of BTK in response to bFGF (Fig. 10*A*). As a control, it was shown that the activation of PI-3K is induced upon bFGF stimulation and that the addition of LY294002 blocks the activation of PI-3K by bFGF in H19-7

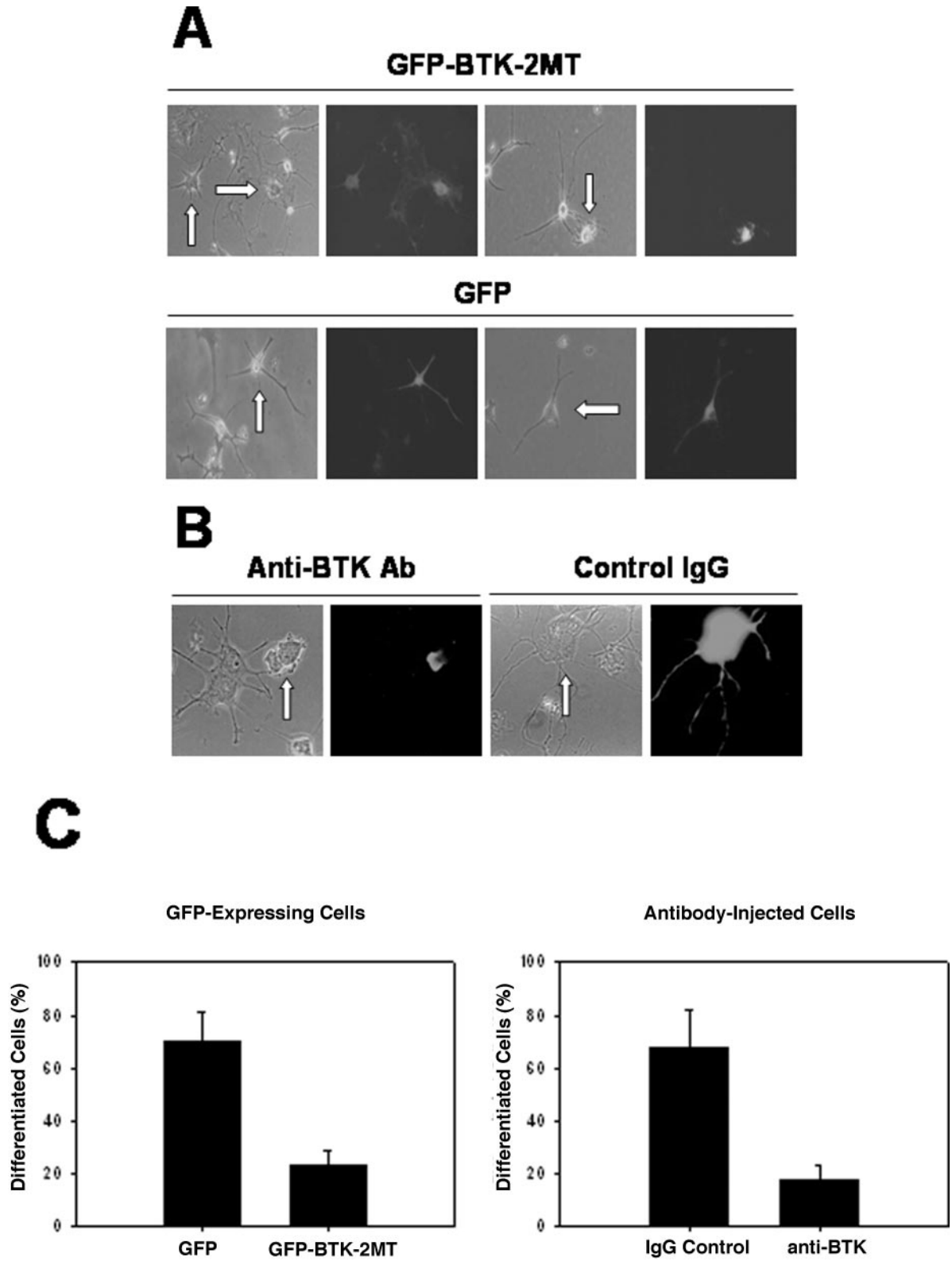

FIG. 8. **Microinjection of kinase-deficient BTK or anti-BTK antibodies blocks the neurite outgrowth in H19-7 cells.** *A*, where indicated, H19-7 cells were transiently transfected with 2 μg of pEGFP or pEGFP-BTK 2MT-Y223A/Y551F vector encoding GFP fused with kinase-deficient BTK mutant, in which two Tyr residues in SH3 domain (Tyr-223) and kinase domain (Tyr-551) were mutated to Ala and Phe, respectively. The cells were then either untreated or stimulated with 10 ng/ml bFGF under differentiation conditions for 48 h, and changes in cell morphology of GFP-expressed were observed under the optical and fluorescence microscope (Olympus-DP50). *B*, H19-7 cells in Dulbecco's modified Eagle's medium and 10% FBS were given microinjections of the affinity-purified cst-1 (4 mg/ml) and coinjection of rat gig 91 mg/ml) as a marker for the injected cells. At 1 h after microinjection, the cells were switched to N2 medium, treated with bFGF, and shifted to 39 °C for 24–48 h. Cells from the same field were visualized by immunofluorescence (*left panel*) and phase-contrast microscopy (*right panel*). Injected cells stained with fluorescein isothiocyanate-conjugated anti-rat antibodies (*Ab*) are indicated by *arrows*. *C*, the percentages of differentiated cells in GFP-BTK-2MT and BTK antibody microinjection were obtained from dividing the number of differentiated, GFP-expressing- and antibody-staining cells by the total number of injected cells. A total of 101 GFP-BTK-2MT-injected cells and 109 anti-BTK IgG-microinjected cells were analyzed. As a control, the total numbers of cells expressing GFP and control IgG counted were 124 and 111, respectively.

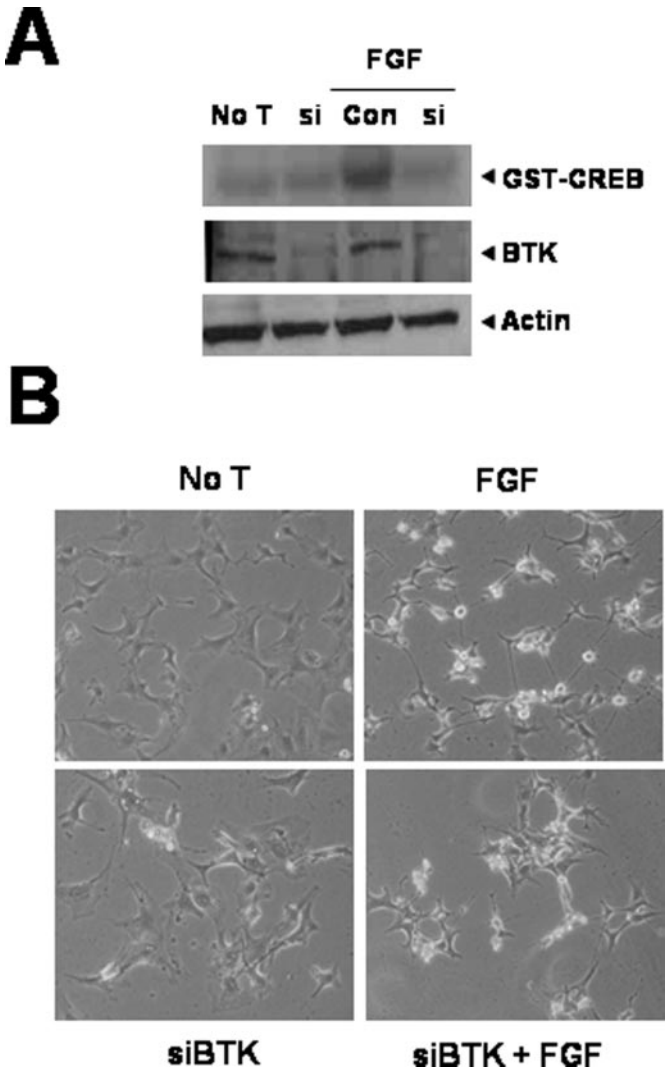

FIG. 9. **Effect of the suppression of endogenous BTK by siRNA duplexes on bFGF-induced CREB phosphorylation and neuronal differentiation.** *A*, where indicated, H19-7 cells were mock-transfected (*No T* or *Con*) or transfected with siRNA duplexes specific for BTK mRNA (*si*). The cells were then either untreated (*No T*) or stimulated with 10 ng/ml bFGF for 30 min. The endogenous BTK expression in cell lysates and its suppression by siRNA duplexes were detected by Western blotting using anti-BTK antibodies. The kinase activity of BTK in the immunocomplexes obtained with anti-BTK antibodies was analyzed by *in vitro* kinase assay using GST-CREB as a substrate. *B*, the changes in cell morphology under differentiation conditions for 48 h were observed under the optical microscope, as indicated.

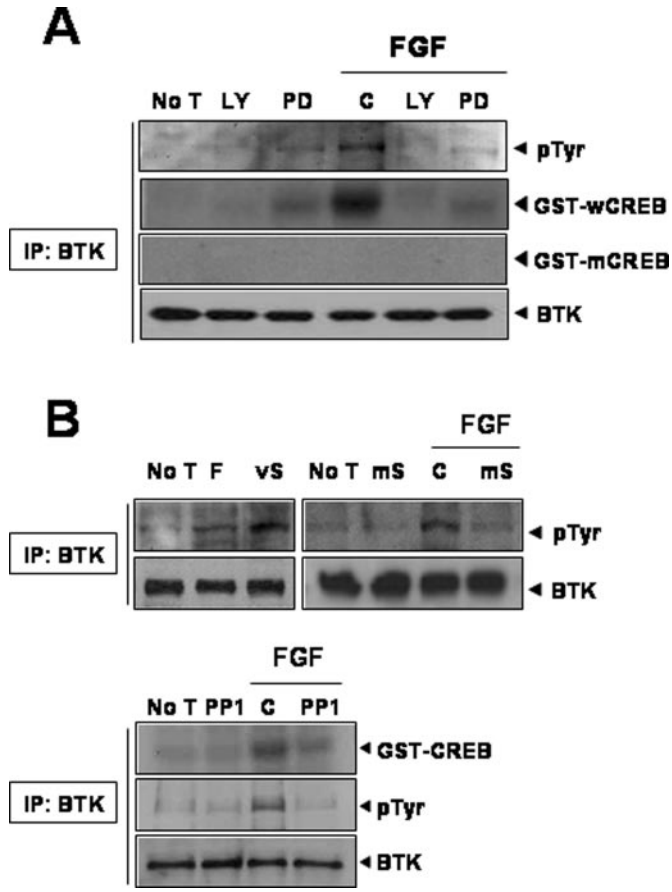

FIG. 10. **Effect of ERK, PI-3K, or Src kinase on FGF-induced BTK activation in H19-7 cells.** *A*, H19-7 cells were untreated or preincubated with either 30 μM MEK inhibitor PD98059 (*PD*) or 10 μM PI-3K inhibitor LY294002 (*LY*) for 30 min prior to stimulation. The cells were then stimulated with 10 ng/ml bFGF for 30 min and immunoprecipitated with anti-BTK antibodies. The immune complexes were resolved on 8% SDS-PAGE, blotted onto a nitrocellulose filter, and detected with either anti-phosphotyrosine antibodies or anti-BTK IgG. An *in vitro* CREB kinase assay was performed using bacterially expressed wild type recombinant CREB proteins fused with GST as an exogenous substrate. *B*, where indicated, the H19-7 cells were mock-transfected (*No T*, *F*, and *C*) or transiently transfected with 5 μg of plasmids encoding viral Src kinase (*vS*) or kinase-inactive Src kinase (*mS*). The cells were then either untreated or stimulated with bFGF (or *F*) for 30 min. The BTK was immunoprecipitated from the cell lysates and blotted with monoclonal anti-phosphotyrosine antibodies. Where specified, H19-7 cells were untreated or preincubated with 2.5 μM PP1 for 30 min before stimulating with 10 ng/ml bFGF for 30 min. Total cell lysates were immunoprecipitated with anti-BTK antibodies and then detected with anti-BTK or anti-phospho-Tyr (*pTyr*) IgG. *In vitro* kinase assay was performed by using bacterially recombinant GST-CREB as a substrate of BTK. *C*, control; *IP*, immunoprecipitation; *No T*, no treatment.

cell (33). Taken together, the above indicates that the activation of both ERK and PI-3K is linked to and required for the induction of BTK during neuronal differentiation in H19-7 cells.

The Src kinase family is involved in a broad range of cellular responses ranging from cell division and cytoskeletal rearrangement in fibroblasts, to the differentiation of neuronal cells (34–36). Many studies have demonstrated a role for pp60Src tyrosine kinase (Src) in BTK-induced B cell mitogenesis via the phosphorylation of the Tyr-133 residue of BTK (13). It was shown previously that Src kinase is also activated in response to bFGF and that its activation is very important for neuronal differentiation in H19-7 cell (32). To determine whether Src kinase functions as a mediator of BTK signaling in H19-7 cells, we assayed the effect of the inhibition of Src kinase activity on BTK activation in response to bFGF. Fig. 10*B* shows that the transfection of kinase-deficient Src mutant resulted in the inhibition of FGF-induced BTK activity to the basal level in H19-7 cells compared with mock-transfected control cells. Similar results were obtained when the Src kinase activity was inhibited using PP1, the selective Src kinase inhibitor (Fig. 10*B*). Furthermore, when v-Src, which is a constitutively active tyrosine kinase, was transfected into H19-7 cells, the BTK activity increased significantly in a constitutive manner and was comparable with that induced by bFGF (Fig. 10*B*). These findings indicate that FGF may use Src kinase for its downstream signaling leading to BTK activation.

DISCUSSION

Although the cellular functions of BTK family kinases have been elucidated in immune cells, such as B cells, mast cells, and platelets, their physiological roles are not known in neuronal cells. In the present study, we show that endogenous BTK

is remarkably expressed in various mammalian neuronal cells. In addition, BTK is activated by neurogenic bFGF, but not by mitogenic EGF, and active BTK stimulates CREB phosphorylation and subsequent CRE-mediated gene transcription in immortalized hippocampal H19-7 cells. Knowing that CREB plays a crucial role in neuronal differentiation (17) and the formation of learning and memory in various organisms (9), our results strongly suggest that the activation of BTK plays an important role during neuronal differentiation in embryonic central nervous system progenitor cells. Such a role is supported further by the finding that the expression of kinase-inactive BTK in a transient manner attenuates the formation of differentiated cells.

In the present we also demonstrate that active BTK directly phosphorylates CREB on Ser-133 by using *in vitro* kinase and *in vitro* in-gel kinase assay. These data indicated that BTK is classified as a dual specific protein kinase rather than protein tyrosine kinase. Dual specificity protein kinases are usually divided into three groups: kinases that show true dual specificity by phosphorylating Tyr and Ser/Thr residues of exogenous substrates; kinases that exhibit dual specificity only through autophosphorylation; and kinases that possess the structural motif characteristic of dual specific kinases (1–3, 37, 38), and our data suggested that BTK belongs to the first group. Further identification and characterization of its unique substrates should resolve other physiological role(s) and signaling network mediated by this kinase in neuronal cells.

BTK can translocate to the nucleus and utilizes functional chromosomal region maintenance-dependent nuclear export signals to shuttle between the nucleus and the cytoplasm (18). This finding has implications regarding potential targets inside the nucleus, which may be critical for gene regulation during the development and differentiation of neuronal cells as well as B cells. In accordance with this speculation, BTK is known to phosphorylate many transcription factors, such as STAT5A, BAP-135, Bright, and nuclear factor-κB (27, 35–41). In addition, the present work shows that CREB is a phosphorylation target of BTK in neuronal cells.

BTK family kinases contain a PH domain, which is found in a wide range of signaling molecules including protein kinases, phospholipases, GTPases, adaptor proteins, and cytoskeletal proteins (42). The mutations in the PH domain of BTK have been shown to associate with X-linked agammaglobulinemia, suggesting an important role for the PH domain in the regulation of BTK kinase activity (43, 44). Although the exact function of the PH domain has yet to be elucidated, accumulating evidence suggests that PH domains are able to bind to phospholipids and many proteins including heterotrimeric G proteins, protein kinase C isoforms, STAT3, F-actin, Fas, and FAK (16). The kinase domain of BTK appears to be critical for CREB phosphorylation and subsequent CRE-mediated gene transcription. Given the multimodular structure of the BTK family kinases and the multitude of signaling molecules that can interact with them, it is to be expected that these kinases impact multiple signaling pathways and generate pleiotropic effects (16). The BTK family kinases have the potential to activate all of the major signaling pathways.

The current findings, namely, that the activation of BTK is linked to the signaling pathways mediated by ERK, PI-3K, and Src kinase, strongly suggest that BTK appears not to be a previously identified novel CREB kinase, whose activation is not linked to either ERK or PI-3K (23). Several studies have found that BTK-activated signaling cascades are coupled with MAPK and PI-3K. For example, the activation of BTK has been correlated with the association of BTK and the cellular membrane (16). Moreover, PI-3Kγ activates BTK together with Src family kinase (45). In addition, B cell antigen receptor-mediated Akt stimulation is regulated by BTK, which regulates Akt both proximal and distal to PI-3K activation, and BTK functions downstream of PI-3K (46).

In terms of the regulation of BTK by MAPKs, multiple signaling pathways, including the BTK, protein kinase C, and SEK1 and MKK7 pathway, are suggested to be involved in the activation of JNK in mast cells (47). In the chicken DT40 B cell line c-Jun N-terminal kinase-1 (JNK-1) is activated via a BTK-dependent pathway in response to B cell antigen receptor cross-linking (30). In some cases, BTK appears to act upstream and regulate the activation of MAPKs, such as ERK or JNKs in immune B cells. For example, BTK directly regulates c-Jun NH_2-terminal kinase activity upon FcRI cross-linking, growth factor stimulation, or growth factor deprivation (48). On the contrary, the activation of BTK appears to be regulated by ERK in neuronal H19-7 cells, suggesting that BTK is present downstream of ERK. This discrepancy may be possible by the differences in specific cellular environments.

We showed previously that the expression of dominant negative Src kinase or a microinjection of an anti-Src antibody blocks the differentiation of H19-7 cells by bFGF, indicating that bFGF signals through a Src kinase-mediated pathway (32). Although constitutively activated v-Src was insufficient to cause the differentiation of H19-7 cells, the coexpression of v-Src with constitutively activated MEK induced neurite outgrowth. These results suggest that Src kinase activation is necessary for differentiation by bFGF and that the differentiation of H19-7 neuronal cells by bFGF requires signaling pathways activated by Ras and Src (32). In addition, our work indicates that the differentiation signals of Src kinase are transmitted through its downstream target BTK and the subsequent activation of CREB in response to bFGF in H19-7 cells.

The overall consequences of the various signals transmitted by the BTK family kinases are varied and include growth, differentiation, and apoptosis (49). The involvement of BTK in growth, differentiation, and development has long been recognized in X-linked immunodeficiency and X-linked agammaglobulinemia cells, as BTK deficiency leads to the failure of B cells to proliferate upon antigen stimulation and subsequent maturation. The ability of BTK to complement a weakly oncogenic Src in the transformation of fibroblasts and the observation that Etk/Bmx is the key effector of Src in the transformation of hepatocytes and fibroblasts suggest their participation in anchorage-independent growth (39, 45). On the basis of the present finding that BTK activates CREB during neuronal differentiation in central nervous system hippocampal cells, further investigation of the upstream signaling pathways and of the possible cross-talk between the many intracellular signaling pathways may give deeper insight into the mechanism of neuronal differentiation mediated by BTK activation.

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