LIM Kinase 1 Activates cAMP-responsive Element-binding Protein during the Neuronal Differentiation of Immortalized Hippocampal Progenitor Cells*

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LIM kinase 1 (LIMK1), a novel member of a subclass of the protein-serine/threonine kinases, is known to play a role in the development and maintenance of neuronal circuits that mediate cognitive function. Genetic studies have implicated a mutation of LIMK1 as a causative factor in the impairment of visuospatial cognition in a neurodevelopmental disorder, Williams syndrome. A transcriptional factor, cAMP-responsive element-binding protein (CREB), is thought to be involved in the formation of many types of synaptic plasticity involving learning and memory. In the present study we show that the LIMK1 activity is markedly induced during the differentiation of immortalized hippocampal progenitor (H19-7) cells. We found that the addition of neurogenic growth factor to H19-7 cells induces specific binding between LIMK1 and active CREB, that LIMK1 directly phosphorylates CREB, and that this leads to the stimulation of subsequent cAMP-responsive element-mediated gene transcription during H19-7 cell neuronal differentiation. In addition, we also found that LIMK1 activation occurs through Rac/Cdc42- and p21-activated kinase-mediated signaling pathways. Moreover, when the plasmid encoding kinase-inactive LIMK1 was transfected to block the activation of endogenous LIMK1, the neuronal differentiation of H19-7 cells was significantly suppressed. These findings suggest that LIMK1 activation and subsequent CREB phosphorylation are important in the neuronal differentiation of central nervous system hippocampal progenitor cells.

The abbreviations used are: CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; bFGF, basic fibroblast growth factor; CNS, central nervous system; GFF, green fluorescent protein; HA, hemagglutinin; LIMK1, LIM kinase 1; PAK1, p21-activated kinase; PID, PAK1-inhibitory domain; TK, thymidine kinase; GST, glutathione S-transferase; RBD, Rho-binding domain.

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RESULTS

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factor (bFGF), in H19-7 cells. These findings suggest that active LIMK1 plays an important role during the neurogenic factor-induced neuronal differentiation of central nervous system (CNS) hippocampal progenitor cells.

EXPERIMENTAL PROCEDURES

Materials— Peroxidase-conjugated anti-rabbit and anti-mouse IgGs were purchased from Zymed Laboratories Inc. (San Francisco, CA). Dulbecco’s modified Eagle’s medium, fetal bovine serum, and Lipo effectAMINE Plus reagent were from Invitrogen. Protein A-Sepharose was from Amersham Biosciences. Anti-CREB and anti-phospho-CREB antibodies were from Cell Signaling Technology (Beverly, MA) and Upstate Biotechnology (Lake Placid, NY), respectively. Polyclonal antiserum against LIMK1 was obtained from Upstate Biotechnology, and enhanced chemiluminescence reagents and γ-[32P]ATP were from PerkinElmer Life Sciences. The synthetic yeast drop out medium (SD/-T, SD/-L, and SD/-HIL) and yeast extract peptone dextrose containing adenine were purchased from BIO101 (Vista, CA). 3-Amino-1,2,4-triazole and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were from Sigma and Promega (Madison, WI), respectively. Human fetal brain cDNA library was purchased from Clontech, human bFGF was from Sigma, and the luciferase assay kit was from Promega. The CRE reporter constructs, pCRE-TK-Luc and pTK-Luc, were gifts from K. Saeki (Research Institute, International Medical Center of Japan), and the expression vectors encoding dominant-negative and constitutive-active Rho family members were graciously provided by J. H. Kim (Korea University, Seoul, Korea). Plasmids encoding hemagglutinin (HA)-tagged or green fluorescent protein (GFP)-fusion wild type LIMK1 or kinase-inactive LIMK1-D460A mutant, in which the catalytic site of domain (PAK1) inhibitory domain (PID), pEGFP-C2/PID, was generously provided by E. G. Kim (Chungbuk National University, Chung-Ju, Korea).

Yeast Two-hybrid Assay—The bait vector for the yeast two-hybrid assay was constructed by subcloning the mutant CREB cDNA, in which RBSL1 extending from amino acids 130–134 was replaced by RRSLY, into pHybTrpZeo. The human fetal cDNA library subcloned into prey vector (pACT2) was purchased from Clontech. All yeast two-hybrid screening protocols were performed as described previously (21).

Cell Culture and the Preparation of Cell Lysates— Immortalized hippocampal neuronal cells (H19-7) were grown on poly-L-lysine-coated 6-well dishes, and before harvesting were treated with 10 ng/ml bFGF for 48 h and analyzed for morphological changes. Differentiated cells were defined as those with a rounded and refractile cell body containing at least one neurite with a length greater than that of the cell body.

In Vivo Kinase Assay—H19-7 cells grown in serum-free N2 medium for 2–3 days were treated with 10 ng/ml bFGF. Cells were then harvested and lysed in lysis buffer, and lysate protein (600 μg) was incubated with polyclonal anti-LIMK1 antibodies overnight at 4 °C. Twenty microliters of a 1:1 suspension of Protein A-Sepharose were then added to the immunocomplexes, and after washing the samples with lysis buffer, kinase reactions were carried out at 30 °C for 60 min in a total volume of 20 μl, which contained kinase buffer (20 mM HEPES, pH 7.2, 5 mM MnCl2, 200 μM sodium orthovanadate, 10 μM of γ-[32P]ATP, and 5 μg of GST-CREB or GST-CREB-S133A as a substrate. Reactions were stopped by adding SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography.

In vitro Kinase Assay—The SDS-polyacrylamide gel was prepared containing 50 μg/ml GST-CREB or GST-CREB-S133A as an in-gel phosphorylation substrate. The total cell extracts were applied to the gel and resolved. All gel renaturation and phosphorylation protocols were performed as described previously (22).

Rac, Cdc42, and Rho Activation Assays—Rac, Cdc42, and RhoA activities were measured as described previously (23). The amounts of the GDP-bound form of Rac, Cdc42, and RhoA were assessed by using either GST fused to the PAK1-binding domain, for active Cdc42 and Rac, or GST protein fused with the Rho-binding domain (RBD) of rhotekin, for active Rho, respectively. H19-7 cell lysates prepared with ice-cold cell lysis buffer were incubated with 50 μg of glutathione-Sepharose 4B bound either to Cdc42/Rac-binding domain of rat PAK1–rhotekin–GST-BDB (BD) or to RBD (BD) of GST. The beads were then washed with wash buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 30 mM MgCl2, 40 mM NaCl, 0.5% Nonidet P-40), and the bound proteins were resolved by 12% SDS-PAGE and immunoblotted using anti-mouse monoclonal Rac, Rho, or Cdc42 antibodies.

RESULTS

Identification of Novel CREB Kinase(s) Activated during the Neuronal Differentiation of Immortalized Hippocampal Progenitor Cells—A conditionally immortalized hippocampal progenitor cell line (H19-7) was generated by transducing temperature-sensitive SV40 large T antigen into rat embryonic day 17 hippocampal cells (24). These H19-7 cells had the ability to terminally differentiate into neuronal cells at a nonpermissive temperature (39 °C) in a defined medium when induced by several agents, which included bFGF (25). On differentiation H19-7 cells expressed several neuronal markers, including neurite outgrowth and neurofilament proteins (24, 26). In earlier work we showed that CREB phosphorylation and subsequent CRE-mediated gene transcription play important roles during bFGF-induced neuronal differentiation in H19-7 cells (27). Interestingly the CREB phosphorylation was not mediated by any of the previously known signaling pathways, i.e. mitogen-activated protein kinases, protein kinase A, protein kinase C, p70S6K, or Ca2+/calmodulin-dependent protein kinase. These findings suggest that the activation of a novel protein kinase signaling pathway is required for this bFGF responsiveness (27). To identify upstream signal transduction pathways leading to CREB phosphorylation and to isolate the novel CREB kinase(s), a yeast two-hybrid assay was performed using mutant CREB in which the critically regulatory Pro132-Ser133 residues of CREB were changed to Ser132-Leu133 as bait. The modification of either the catalytic or regulatory domains of transcription factors, including CREB, is frequently used to enhance the interaction and to stabilize the complex formation with its kinase in yeast two-hybrid assays (21, 28–30). By

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screening the human fetal brain cDNA library, we detected several known and several novel CREB-interacting proteins. These included Dyrk1A (21), Bruton’s tyrosine kinase (60), and LIMK1. As LIMK1 is known to play an integral role in the differentiation and signal transduction directed by nerve growth factor or epidermal growth factor in PC12 cells, we further examined its functional role during neuronal differentiation in CNS-derived hippocampal progenitor cells. Specific Binding between Active CREB and LIMK1 during the Neuronal Differentiation of H19-7 Cells—First, we examined whether LIMK1 is expressed in H19-7 cells or selectively induced under neuronal differentiation conditions. Cells were treated with bFGF for various times, and Western blot analysis was performed using anti-LIMK1 antibodies. As shown in Fig. 1A, LIMK1 expression was not markedly changed by stimulating H19-7 cells with bFGF, although LIMK1 protein was expressed highly without stimulation (Fig. 1A). Second, to confirm a previous finding that bFGF stimulated the activation of CREB (27), 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 CREB protein was found to be significantly phosphorylated, whereas endogenous CREB levels were not markedly changed (Fig. 1B). Next we examined whether LIMK1 specifically binds to CREB in mammalian H19-7 cells. Cell extracts obtained after neurogenic bFGF stimulation were immunoprecipitated against either anti-LIMK1 or anti-CREB antibodies and then blotted with anti-LIMK1 antibodies. The addition of bFGF led to a marked increase in the specific binding between endogenous LIMK1 and CREB (Fig. 1C). When cells were immunoprecipitated with anti-phospho-CREB antibodies and blotted with anti-LIMK1 antibodies, LIMK1 was found to selectively bind to active CREB within 30 min of bFGF stimulation (Fig. 1C). The transient overexpression of kinase-deficient LIMK1 mutants (LIMK1-D460A), in which the catalytic residue Asp^{460} had been replaced with Ala (31), caused a large reduction in phosphorylated CREB levels and blocked the interaction between LIMK1 and CREB induced by bFGF stimulation (Fig. 1D). Furthermore, when H19-7 cell lysates were mock-transfected or transfected with plasmids encoding either HA-tagged wild type or dominant-negative LIMK1 mutant were immunoprecipitated by anti-active CREB antibodies and analyzed by Western blot analysis using anti-HA IgG, it was found that the addition of bFGF results in specific binding between HA-tagged LIMK1 and phospho-CREB but not between kinase-inactive LIMK1 and CREB (Fig. 1E). These results suggest that LIMK1 interacts with active CREB in a specific way during the bFGF-induced neuronal differentiation of H19-7 cells.

Active LIMK1 Directly Phosphorylates CREB in Response to Neurogenic bFGF in H19-7 Cells—As LIMK1 is activated via the phosphorylation of the Thr^{208} residue located in its kinase domain (32), we investigated whether LIMK1 is also activated by bFGF in H19-7 cells. After stimulating H19-7 cells with bFGF, cell lysates were immunoprecipitated against anti-LIMK1 antibodies, and the precipitated mixtures were then separated by SDS-PAGE and analyzed using anti-phospho-Tyr or anti-phospho-Ser/Thr antibody. As shown in Fig. 2, the levels of Ser/Thr-phosphorylated LIMK1 were markedly increased by bFGF stimulation in H19-7 cells, whereas no tyrosine phosphorylation of endogenous LIMK1 was observed, suggesting that treating H19-7 cells with the neurogenic growth factor bFGF induces the LIMK1 activation.

We next investigated whether active LIMK1 directly phosphorylates CREB in H19-7 cells. The cell lysates obtained after bFGF treatment were immunoprecipitated using anti-LIMK1 antibodies. Immunocomplex kinase assays were performed using bacterial recombinant GST-CREB fusion proteins as a sub-
Fig. 3. LIMK1 directly phosphorylates CREB in response to bFGF in H19-7 cells. A, H19-7 cells were stimulated with 10 ng/ml bFGF (Fgf) for the indicated times, and total cell lysates were immunoprecipitated with anti-LIMK1 antiseraum. Where specified, an in vitro CREB kinase assay was performed using LIMK1-bound immunoprecipitates and bacterially expressed GST fusion protein containing 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 phosphorylated CREB levels were determined by autoradiography. B, H19-7 cells were either left untreated (Con) or treated with 10 ng/ml bFGF (Fgf) for 30 min or the indicated times, and total cell lysates were immunoprecipitated with anti-LIMK1 IgG. The in-gel kinase renaturation assay was performed as described under “Experimental Procedures.” The 72-kDa CREB kinase activated by bFGF is indicated by an arrow (upper panel). Immunocomplexes obtained using anti-LIMK1 IgG were resolved by SDS-PAGE and detected using anti-LIMK1 antibodies as indicated (lower panel). The data shown are representative of three experiments. IP, immunoprecipitation.

strate, and phosphorylated substrates were visualized by autoradiography. As shown in Fig. 3A, CREB phosphorylation was significantly induced by bFGF in a time-dependent manner and reached a maximum level at 30 min. When mutant GST-CREB-S133A, in which the Ser133 residue of CREB was replaced by alanine, was used as a substrate, no significant CREB phosphorylation was observed versus wild type CREB (Fig. 3B). These results suggest that LIMK1 directly phosphorylates the transcription factor CREB during the differentiation of H19-7 cells and that its effect specifically involves the Ser133 residue of CREB.

To test the possibility that, rather than LIMK1, other contaminated Ser/Thr kinase(s) in the LIMK1 immunocomplexes phosphorylate CREB at Ser133 in response to bFGF, we performed an in vitro in-gel kinase assay using a polyacrylamide gel prepared in the presence of bacterially recombinant GST fused with either wild type CREB or with CREB-S133A mutant protein. Equal amounts of protein-containing anti-LIMK1 IgG immunoprecipitates from H19-7 cells, which had been stimulated with 10 ng/ml bFGF for 30 min, were resolved by SDS-PAGE, renatured, and then assayed for CREB phosphorylation in the gel. The results obtained showed that a 72-kDa band, corresponding to LIMK1, markedly phosphorylated CREB in the gel (Fig. 4C). Moreover no significant kinase activity was detected when the mutant GST-CREB-S133A was used as a substrate (Fig. 4C). An analysis of the immunoprecipitated protein complexes prepared using anti-LIMK1 antibodies followed by Western blot analysis against anti-LIMK1 antibodies revealed that the 72-kDa CREB kinase band in the gel was identical to that expected for LIMK1. Overall these results show that active LIMK1 can specifically phosphorylate CREB at its Ser133 residue when H19-7 cells are stimulated with bFGF.

Effect of LIMK1 Activation on CRE-dependent Gene Transcription during Neuronal Differentiation—To assess whether LIMK1 exerts its stimulatory effect on CRE-mediated gene transcription as well as on CREB activation, we assayed the gene expression of the CRE-containing TK promoter construct, pTK-Luc (TK-Luc), which was used as a negative control (lane 6). B, where indicated, the cells were transiently transfected with pCRE-TK-Luciferase (CRE) plasmid alone or with a construct (1 µg) encoding wild type LIMK1 (LK1). The cells were then either left untreated (Control) or stimulated with 10 ng/ml bFGF (Fgf) for 4 h, and the luciferase activity of the control was measured. In every transfection experiment, the CRE-lacking TK promoter construct, pTK-Luc (TK-Luc), was used as a negative control (lane 6). Where indicated, the cells were transiently transfected with pCRE-TK-Luciferase (CRE) plasmid alone or with a construct (1 µg) encoding wild type LIMK1 (LK1). The cells were then either left untreated or stimulated with 10 ng/ml bFGF (Fgf) for 4 h, and the luciferase activity of the reporter plasmid was measured. Data are plotted as percentages of maximum luciferase activity and represent mean ± SD of three independent experiments performed in triplicate.

Effect of LIMK1 Activation on CRE-mediated Gene Transcription—H19-7 cells were transiently transfected with a dominant-negative mutant LIMK1 expression vector plus either pCRE-TK-Luc reporter plasmid or pTK-Luc lacking the CRE motif as a control. The expression of kinase-inactive LIMK1 mutant proteins was found to significantly inhibit the bFGF-induced activation of luciferase activity to the control levels. However, the overexpression of wild type LIMK1 in the absence of bFGF stimulation did not induce the gene transcription of CRE-containing luciferase reporter plasmid (Fig. 4B). These results show that the stimulation of H19-7 cells by bFGF activates CRE-mediated gene transcription possibly through the activa-
tion of LIMK1 and subsequent CREB phosphorylation in embryonic hippocampal H19-7 cells.

bFGF-induced LIMK1 Activation Occurs through a Rac/Cdc42-PAK1 Signaling Pathway and Not through Rho and Its Associated Kinase(s)—Previous studies have shown that Rho-associated, coiled-coil-forming protein kinase and PAK1 activate LIMK1 by phosphorylating Thr508 within the kinase domain (33–35). To determine whether the small GTPase Rho family, consisting of Rho, Rac, and Cdc42, also regulate LIMK1-mediated CREB phosphorylation in H19-7 cells, their individual blocking effects on LIMK1 activation and subsequent CREB phosphorylation were examined in response to bFGF. H19-7 cells were transiently transfected with a plasmid encoding a GTPase-inactive Rho family protein (Rho, Rac, or Cdc42), respectively. The cells were subsequently stimulated with 10 ng/ml bFGF for 30 min, and cell extracts were subjected to immunoprecipitation using anti-LIMK1 antibody, and this was followed by an in vitro kinase assay using GST-CREB as an exogenous substrate. As shown in Fig. 5A, transient transfection with plasmids encoding dominant-negative Rac and Cdc42 mutants markedly inhibited LIMK1-mediated CREB activation; however, dominant-negative Rho mutants had no marked effect on CREB phosphorylation versus mock-transfected control cells (Fig. 5). To further explore the effects of Rac and Cdc42 activity on LIMK1-induced CREB phosphorylation, H19-7 cells were similarly transfected with plasmids encoding constitutive-active mutants of the Rho family members (Rho-V12, Rac-V12, or Cdc42-V12) and immunoprecipitated with anti-LIMK1 antibody, and then an in vitro kinase assay was performed. Consistent with previous findings, anti-LIMK1 immunoprecipitates from cells overexpressing constitutive-active Rac or Cdc42 mutants phosphorylated GST-CREB without bFGF stimulation, whereas constitutively active Rho did not affect the levels of phosphorylated CREB proteins (Fig. 5A).

To assess whether Cdc42, Rac, or Rho is activated by bFGF, we measured the amounts of GTP-bound Rac, Cdc42, and Rho by using either GST fused to PAK1-binding domain to assay active Cdc42 and active Rac or GST fused with the binding domain of rhotekin to assay active Rho. As shown in Fig. 5, B, C, and D, the addition of bFGF did not stimulate the Rho activity but significantly increased the amounts of active Rac and Cdc42. Furthermore, the bFGF-induced activations of Rac and Cdc42 were significantly inhibited by the overexpression of each dominant-negative mutant protein (Fig. 5, B and C). As a control for Rho activation, the transient transfection of cells with plasmids encoding constitutive active Rho mutant resulted in an increase in intracellular activated Rho levels (Fig. 5D). In summary, these results suggest that the activation of LIMK1 and the consequent CREB phosphorylation occurs via a Rac/Cdc42-mediated pathway and not through a Rho-mediated pathway during the neuronal differentiation of H19-7 cells.

It is known that PAK1, a downstream effector of Rac/Cdc42, regulates LIMK1 activity (33). For example, PAK1 phosphorylates LIM kinase at Thr508 residue within the activation loop of LIM kinase and increases the LIM kinase-mediated phosphorylation of the actin-regulatory protein cofillin. Moreover in vitro it was found that activated Rac or Cdc42 increases the association between PAK1 and LIM kinase and that a PAK1-specific inhibitor blocks LIM kinase-induced cytoskeletal changes (33). Thus, we examined whether PAK1 affects the bFGF-induced activation of LIMK1. After mock transfection or transiently transfecting H19-7 cells with a plasmid encoding the PID, we performed immunoprecipitation with anti-LIMK1 IgG followed by Western blot analysis using anti-phospho-Ser/Thr antibodies. The results obtained indicated that LIMK1 activation is inhibited in the presence of PID (Fig. 6). In a similar way, an in vitro kinase assay showed that the LIMK1-mediated CREB phosphorylation by bFGF was remarkably inhibited upon the expression of PID (Fig. 6). These findings indicate that the activation of LIMK1 by bFGF stimulation is linked to the activations of Rac/Cdc42 and subsequently PAK1.

Effect of LIMK1 Activation on bFGF-induced Neuronal Differentiation in H19-7 Cells—The functional role of LIMK1 activation was examined during bFGF-induced neuronal differentiation in H19-7 cells. After being treated with bFGF, most H19-7 cells displayed neurite extension at 39°C at which the large T antigen is inactive (Fig. 7A). Differentiated cells are characterized by a round and refractive cell body containing at least one neurite longer than the diameter of the cell body, whereas undifferentiated cells are flat with no extended neurites (25). The differentiated cells were shown to be resistant to mitogenic stimulation by serum and to express neuronal markers like neurofilament and brain type II sodium channel (26). The ability of H19-7 cells to differentiate in response to bFGF

FIG. 5. bFGF-induced LIMK1 activation occurs through a Rac/Cdc42 signaling pathway and not through a Rho pathway in H19-7 cells. A, where indicated, H19-7 cells were mock-transfected (No T and C) or transiently transfected with a plasmid (5 μg) encoding either constitutive-active (V12) or dominant-negative (N17) Rho family members, including Rac, Cdc42, and Rho. The cells were then either left untreated (No T and V12) or stimulated with 10 ng/ml bFGF (FGF) for 30 min, and the cell extracts were immunoprecipitated using anti-LIMK1 antibody. In vitro kinase assay was followed by using GST-CREB as an exogenous substrate. B, to assay Rac activity, H19-7 cells were treated with 10 ng/ml bFGF for the indicated times. Total cell lysates were then incubated with GST-PBD, and the amount of GTP-bound Rac was determined by immunoblotting using a monoclonal antibody against Rac (left panel). Where specified, cells were mock-transfected (No T and C) or transiently transfected with a plasmid (5 μg) encoding either constitutive-active (V12) or dominant-negative (N17) Rac. Cells were then left untreated (No T or V12) or stimulated with 10 ng/ml bFGF for 30 min. Total cell lysates were analyzed by Western blotting with anti-Rac IgG (right panel). C and D, to examine the activities of Cdc42 and Rho, either constitutive-active (V12) or dominant-negative (N17) Cdc42 and Rho expression constructs were transfected into H19-7 cells as indicated. After 24 h of transfection, the cells were left untreated (No T or V12) or stimulated with 10 ng/ml bFGF for 30 min. Cell lysates were incubated with GST-PBD or GST-RBD, and the amounts of GTP-bound Cdc42 or Rho were determined by immunoblotting using anti-Cdc42 or anti-Rho antibodies as indicated. The data shown are representative of three experiments.
Fig. 6. The Rac/Cdc42 downstream effector, PAK1, regulates LIMK1 in H19-7 cells. Where indicated, H19-7 cells were treated with vehicle (Con) or transiently transfected with 5 μg of a plasmid encoding PID for 24 h. The cell extracts were immunoprecipitated with polyclonal anti-LIMK1 IgG. Bound proteins were resolved by SDS-PAGE and detected using either an anti-phospho-Ser/Thr (pSer/Thr) or anti-LIMK1 antibodies as indicated. Where specified, in vitro kinase assays were performed using anti-LIMK1 IgG-bound immunocomplexes and GST-CREB as a substrate. The data represent the summary of three independent experiments. IP, immunoprecipitation.

Fig. 7. Effect of LIMK1 on bFGF-induced neuronal differentiation in H19-7 cells. A, where indicated, H19-7 cells were mock-transfected (Control, FGF, Vehicle, or V) or transiently transfected with a plasmid to express either HA-tagged wild type LIMK1 (wLIMK) or the kinase-inactive LIMK1-D460A mutant (mLIMK). The cells were then either left untreated (Control, Vehicle, or wtLIMK) or stimulated with 10 ng/ml bFGF (FGF) under differentiating conditions for 48 h, and changes in cell morphology were observed under an optical microscope. B, the percentages of differentiated cells are expressed as ratios of the total cell numbers. The results represent the means and the ranges of two independent experiments performed in triplicate.

Fig. 8. Kinase-deficient LIMK1 blocks the neurite outgrowth in H19-7 cells. Where indicated, H19-7 cells were transiently transfected with 2 μg of plasmid encoding either green fluorescent protein (pEGFP-C1) or GFP-fused wild type (wLIMK) or kinase-inactive LIMK1-D460A (in which the catalytic residue Asp660 had been replaced by Ala; mLIMK). Cells were then either left untreated (Control or wtLIMK) or stimulated with 10 ng/ml bFGF (FGF) under differentiating conditions for 48 h as indicated. Changes in cell morphologies (left panel) and GFP expression (right panel) were observed by optical and fluorescence microscopy (Olympus-DP50), respectively. The data shown are representative of three independent experiments.

wild type LIMK1 had no apparent effect on the neuronal differentiation (Fig. 7A). In addition, the overexpression of wild type LIMK1 without bFGF stimulation did not induce the neurite outgrowth, suggesting that the up-regulation of LIMK levels is not sufficient to induce neuronal differentiation in H19-7 cells (Fig. 7A). As shown in Fig. 7B, whereas mock-transfected control cells contained a high level of differentiated cells (~77%), mutant LIMK1-transfected cells showed remarkably lower levels of differentiated cells (~36%).

To confirm the effect of LIMK1 activation on neuronal differentiation in H19-7 cells, we investigated single cells expressing the protein of interest. H19-7 cells were transiently transfected with a plasmid encoding either GFP or GFP fused with wild type LIMK1 or kinase-inactive LIMK1 mutant and then examined the effect on bFGF-mediated differentiation. The GFP-fluorescent cells showed that the inhibition of endogenous LIMK1 activity by the transient transfection of dominant-negative LIMK1 mutant proteins remarkably inhibited cellular differentiation (Fig. 8). Meanwhile the control cells transfected with a GFP expression vector showed characteristic neurite outgrowth, thus suggesting that the blockade of neuronal differentiation is caused by the expression of the kinase-inactive LIMK1 mutant but not by GFP (Fig. 8). In addition, the overexpression of GFP fusion protein with wild type LIMK1 in the absence of bFGF stimulation did not induce the neurite outgrowth (Fig. 8), suggesting that the activation of LIMK1, but not the mere increase of endogenous LIMK1 levels, is necessary for the induction of neuronal differentiation in H19-7 cells. Taken together, these results suggest that relatively stable LIMK1 activation by bFGF is likely to play an important role in the differentiation of hippocampal progenitor H19-7 cells.
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**DISCUSSION**

Williams syndrome is a rare genetic disorder caused by the microdeletion of about 20 genes on chromosome 7q11.23. The disorder is characterized by mental retardation or learning difficulties and extreme weakness in visuospatial constructive cognition (36–38). In man, five of the genes in the 7q11.23 region, Fzd3, Bcl7b, Stx1a, LIMK1, and Cyn2, are known to be expressed in neurons and thus are of interest as candidate factors underlying the neurobehavioral features of Williams syndrome. In particular, LIMK1 has been implicated in the impaired visuospatial constructive cognition of Williams syndrome by both familial and gene expression studies (39). Consistent with the physiological deficits of Williams syndrome, LIMK1 knock-out mice exhibited abnormalities in behavioral responses, including impaired fear conditioning, decreased learning reversal, and altered fear responses (40, 41). Consistent with the putative role of LIMK1 during neural development, the present studies show that active LIMK1 plays an important role by phosphorylating CREB at the Ser332 residue during the neuronal differentiation of hippocampal neuronal H19-7 cells.

The CREB is known to be an important regulatory component of proliferation and differentiation in a variety of cellular systems (42). CREB-dependent gene expression also plays a role in the learning and memory of vertebrates and invertebrates. Whereas the Lim domains in LIMK1 mediate protein-protein interactions, CREB activity is also modulated by interacting with tissue-specific cofactors of the four and a half domain (FHL) family (43). The present study into the relationship between LIMK1 and CREB indicates that CREB is a substrate of LIMK1 and that the interaction between these two proteins contributes to the proper progression of neuronal differentiation in hippocampal H19-7 cells. While the mechanism by which a disruption of CREB function may affect cognitive functions, such as learning and memory, is poorly understood, our results imply that learning and memory defects in Williams syndrome may be associated with the cognitive function of CREB. It is possible that the down-regulation of active LIMK1 levels and the consequent insufficient activation of CREB caused by the hemizygous deletion of LIMK1 in the chromosome results in the alteration of the downstream gene expression pattern.

LIMK1 is composed of one PDZ domain and two LIM domains in the amino-terminal half and a kinase domain in the carboxyl-terminal region (44). The LIM and PDZ domains in LIMK are involved in the regulation of the kinase activity or in its subcellular localization through protein-protein interactions (45, 46). LIMK1 is localized mainly in the cytoplasm (47), and its PDZ domain probably has nuclear exporting activity (46), suggesting that LIMK1 shuttles between the nucleus and cytoplasm. Moreover LIMK1 regulates actin dynamics and neuronal differentiation by directly phosphorylating ADF/cofilin, the actin depolymerization factor that is widely expressed in neurons and its PDZ domain probably has nuclear exporting activity (46), suggesting that LIMK1 shuttles between the nucleus and cytoplasm. Moreover LIMK1 regulates actin dynamics and neuronal differentiation by directly phosphorylating ADF/cofilin, the actin depolymerization factor that is widely expressed in neurons and invertebrates.

In summary, here we show that active LIMK1 directly phosphorylates the transcription factor CREB during the neuronal differentiation of H19-7 cells and that LIMK1 activation induces subsequent CRE-mediated gene transcription. Furthermore the blockade of LIMK1 activation was found to significantly inhibit CREB phosphorylation and the neurite outgrowth formation induced by bFGF in H19-7 cells, which strongly suggests that active LIMK1 may play an important role during the neurogenic factor-induced neuronal differentiation of CNS-derived hippocampal progenitor cells. Further studies of the upstream signaling pathways and of possible “cross-talk” between the many intracellular signaling pathways, including those involving mitogen-activated protein kinases, protein kinase A, protein kinase C, phosphatidylinositol 3-kinase-p70, calcium/calmodulin-dependent protein kinase, and casein kinase 2, may give us a deeper insight into the mechanism of neuronal differentiation induced by LIMK1 activation.

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