The Csk Homologous Kinase Associates with TrkA Receptors and Is Involved in Neurite Outgrowth of PC12 Cells*

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Csk homologous kinase (CHK), a member of the Csk regulatory tyrosine kinase family, is expressed primarily in brain and hematopoietic cells. The role of CHK in the nervous system is as yet unknown. Using PC12 cells as a model system of neuronal cells, we show that CHK participates in signaling mediated by TrkA receptors. CHK was found to be associated with tyrosine-phosphorylated TrkA receptors in PC12 cells upon stimulation with NGF. Binding assays and far Western blotting analysis, using glutathione-S-transferase fusion proteins containing the Src homology 2 (SH2) and SH3 domains of CHK, demonstrate that the SH2 domain of CHK binds directly to the tyrosine-phosphorylated TrkA receptors. Site-directed mutagenesis of TrkA cDNA, as well as phosphopeptide inhibition of the in vitro interaction of the CHK-SH2 domain or native CHK with TrkA receptors, indicated that the residue Tyr-785 on TrkA is required for its binding to the CHK-SH2 domain upon NGF stimulation. In addition, overexpression of CHK resulted in enhanced activation of the mitogen-activated protein kinase pathway upon NGF stimulation, and microinjection of anti-CHK antibodies, but not anti-Csk antibodies, inhibited neurite outgrowth of PC12 cells in response to NGF. Thus, CHK is a novel signaling molecule that participates in TrkA signaling, associates directly with TrkA receptors upon NGF stimulation, and is involved in neurite outgrowth of PC12 cells in response to NGF.

Nerve growth factor (NGF)1 regulates the survival, development, and differentiation of the sympathetic and sensory neurons in the peripheral nervous system and the differentiation of certain cholinergic neurons in the central nervous system (1, 2). In addition, NGF promotes neuronal differentiation of the rat pheochromocytoma cell line, PC12, which has been used extensively to investigate ligand-receptor interactions and cellular differentiation in response to NGF (3, 4). Upon stimulation with NGF, these cells acquire the phenotype of sympathetic neurons as characterized by neurite outgrowth and persistent activation of the ERK family of mitogen-activated protein (MAP) kinase (5). Two cell surface receptors for NGF have been identified: the receptor tyrosine kinase, TrkA, and the low-affinity neurotrophin receptor, p75NTR (6–9). NGF exerts its growth- and survival-promoting effects on neurons through activation of TrkA and subsequent biochemical events that ultimately influence the expression of various genes, including those encoding ion channels, neurotransmitter-synthesizing enzymes, and cytoskeletal components (10). The binding of NGF to the p140 TrkA receptor induces dimerization of TrkA receptors and stimulates rapid tyrosine autophosphorylation of the receptor (11). The phosphotyrosines on activated TrkA serve as docking sites for signaling substrates such as phospholipase C-γ1 (PLC-γ1), phosphatidylinositol 3-kinase (PI-3 kinase), and the Shc adaptor protein (6, 12). These molecules trigger kinase cascades resulting in the phosphorylation and activation of transcription factors that direct gene expression. Shc triggers the activation of Ras and the subsequent sequential phosphorylation and activation of the kinases Raf, mitogen- and extracellular-regulated kinase, and ERK (13, 8). The Ras-ERK pathway plays a major role in the activation of transcriptional events by NGF and in NGF-induced neuronal differentiation. Mutation analysis of TrkA has defined critical tyrosines that specifically regulate the activities of PLC-γ1, PI-3 kinase and Shc (14–18). PLC-γ1 and Shc regulation appears to play a major role in NGF-mediated neurite outgrowth (17, 18). In addition, sustained PI-3 kinase activity is necessary for the neurite outgrowth of PC12 cells induced by NGF (19).

The Csk homologous kinase (CHK) (previously referred to as megakaryocyte-associated tyrosine kinase (MATK)) is a recently identified protein tyrosine kinase that shares high homology with Csk (COOH-terminal SRC kinase). CHK was independently identified as MATK (20, 21), Lsk (22), Hyl (23), Ctk (24), Ntk (25), and Batk (26). Like Csk, CHK contains Src homology 3 (SH3), SH2, and tyrosine kinase domains and lacks the Src family NH2-terminal myristylation and auto-phosphorylation sites (20, 21). Csk is ubiquitously expressed, whereas expression of CHK is restricted to hematopoietic cells and the nervous system. The expression of CHK in brain increases postnatally, whereas the expression of CHK decreases with age (27). Although the function of CHK is still unclear, recent studies indicated that, unlike Csk, CHK interacts with receptor tyrosine kinases, such as c-Kit in megakaryocytes (28, 29) or the ErbB-2/neu receptor in breast cancer cells (30, 31) via its SH2 domain. Because CHK is abundantly expressed in the nervous system, we sought to identify the signaling pathways that involve CHK and to characterize its function in neuronal cells. In this report, we identified and characterized the association of CHK in TrkA signaling and investigated CHK involvement in neuronal differentiation of PC12 cells.

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¶ The abbreviations used are: NGF, nerve growth factor; CHK, Csk homologous kinase; SH, Src homology domain; GST, glutathione S-transferase; PI-3 kinase, phosphatidylinositol 3-kinase; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation; WB, Western blot; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein.
**EXPERIMENTAL PROCEDURES**

**Antibodies—**Anti-GST monoclonal antibodies were produced as described previously (32). Affinity-purified polyclonal anti-TrkA antibodies against the COOH-terminal peptide of the TrkA receptor were purchased from Oncogene Science (Cambridge, MA). Anti-CHK, anti-Csk, anti-p58/S1-3 kinase, anti-ERK1, and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phosphorylated anti-rabbit Ig antibodies, horseradish peroxidase-conjugated anti-mouse Ig antibodies, horseradish peroxidase-conjugated anti-rabbit Ig antibodies, and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech.

**Cell Culture and Stimulation—**PC12-12 cells were grown as described (3). PC12-TrkA cells were stably transfected with TrkA cDNA (33). These cells expressed 100000 TrkA receptors/cell. Both PC12 and PC12-TrkA cells were grown on 10-cm dishes coated with poly-1-lysine. For stimulation, cells were incubated with 100 ng/ml NGF and then lysed and precipitated with various GST fusion proteins, bound proteins were transferred onto PVDF-Plus membranes (Micron Separations, Inc.), and subjected to far Western blotting. Ten micrograms of GST fusion protein bound to glutathione-Sepharose beads was used for each binding assay. After lysate protein content was normalized and precipitated with GST fusion proteins, bound proteins were microinjected with GFP vector plasmid (pEGFP-C2) (CLONTECH, Palo Alto, CA) as a marker in a final concentration of 100 ng/ml. IgGs were purified from control antiserum, Csk and CHK antisera using protein G columns (Mab Trap, Amersham Pharmacia Biotech). Pooled fractions containing electrophoretically pure IgGs were dialyzed against phosphate-buffered saline and concentrated using Centricon concentrators (Amicon Corp., Danvers, MA). Following microinjection, cells were incubated for 2 h at 37 °C. Cells were refed with medium containing 1% horse serum overnight. The medium was then changed to RPMI medium containing 10% horse serum and 5% fetal bovine serum, with or without NGF (100 ng/ml) as indicated. Cells were monitored and analyzed after 24, 36, and 48 h. Differentiated cells were defined as cells with refractile cell bodies extending at least two processes, one of which had to be longer than the diameter of the cell body. Subsequent phase-contrast micrographs were recorded on Technical Pan film (2415) (Eastman Kodak Co.).

**RESULTS**

**CHK Is Associated with Activated TrkA Receptors upon Stimulation with NGF—**Because CHK is a second member of the Csk family and both kinases are expressed in brain, we investigated whether CHK and/or Csk might be involved in one of the main signaling pathways in neuronal cells that is mediated by the NGF receptor. Antibody for NGF. To identify proteins that associate with NGF-stimulated PC12-TrkA cell lysates were added to the GST-CHK-SH2 fusion protein (10 μg/incubation), which was preincubated with each phosphopeptide. The final concentration of phosphopeptides was 100 μM for each incubation. Lysates were incubated for 1 h at 4 °C and then precipitated by the addition of glutathione-Sepharose 4B for 30 min at 4 °C. Precipitates were washed and separated by 7% SDS-PAGE.

**MAP Kinase Assay—**MAP kinase assay was performed as described (34). Briefly, starved PC12 and PC12-CHK cells (three independent clones that overexpress CHK) were stimulated with NGF (100 ng/ml). PC12-CHK cells were incubated with 50 μg/ml anti-TrkA, anti-CHK, or anti-Csk antibodies. The blots were developed using the ECL system.

**Microinjection—**Synchronized PC12 cells were microinjected with purified NGF-specific antibodies (at a concentration of 100 μg/ml) in microinjection buffer with GFP vector plasmid (pEGFP-C2) (CLONTECH, Palo Alto, CA) as a marker in a final concentration of 100 μg/ml. IgGs were purified from control antiserum, Csk and CHK antisera using protein G columns (Mab Trap, Amersham Pharmacia Biotech). Pooled fractions containing electrophoretically pure IgGs were dialyzed against phosphate-buffered saline and concentrated using Centricon concentrators (Amicon Corp., Danvers, MA). Following microinjection, cells were incubated for 2 h at 37 °C. Cells were refed with medium containing 1% horse serum overnight. The medium was then changed to RPMI medium containing 10% horse serum and 5% fetal bovine serum, with or without NGF (100 ng/ml) as indicated. Cells were monitored and analyzed after 24, 36, and 48 h. Differentiated cells were defined as cells with refractile cell bodies extending at least two processes, one of which had to be longer than the diameter of the cell body. Subsequent phase-contrast micrographs were recorded on Technical Pan film (2415) (Eastman Kodak Co.).

**CHT Association with TrkA Signaling and Neurite Outgrowth**

Amino acid sequences of TrkA (33) obtained by automated NovaSeq (Applied Biosystems) and that of Csk (35) obtained by sequence analysis are shown in Fig. 1. Amino acid sequences were used to design a synthetic oligonucleotide: 5'-CAT AATG GAG AAG CCA CAA TTT TCC TTC ATG GAT GCC TGT GTT CAC-3' (Y490F), 5'-TGC CCA CCA GAG GTC TTC GCC ATG CGG GGC-3' (Y751F), and 5'-CAG GCA CCT CTT GTC CTG GAT GTC CTG GGC-3' (Y785F). The mutations were confirmed by DNA sequencing. The wild-type and mutant type TrkA receptors were excised from PC12 vectors and subcloned into the pcDNA3 vector. Transfected COS-7 cells was then transfected using Lipo-2000 (Life Technologies, Inc.) according to the manufacturer’s protocol.

**Inhibition of the CHK-SH2 Domain/TrkA Interaction by Phosphopeptides—**Tyrosine-phosphorylated synthetic peptides were obtained from the Molecular Biology Core Facility at Dana Farber Cancer Institute. Peptides were analyzed for purity by high pressure liquid chromatography, mass spectroscopy, and amino acid analysis. Two phosphopeptides corresponding to autophosphorylation sites of TrkA, including the Shc association site and the PLC-γ1 association site, were synthesized. In addition, nonphosphorylated peptide corresponding to the PLC-γ1 association site was also synthesized. Unstimulated and NGF-stimulated PC12-TrkA cell lysates were added to the GST-CHK-SH2 fusion protein (10 μg/incubation), which was preincubated with each phosphopeptide. The final concentration of phosphopeptides was 100 μM for each incubation. Lysates were incubated for 1 h at 4 °C and then precipitated by the addition of glutathione-Sepharose 4B for 30 min at 4 °C. Precipitates were washed and separated by 7% SDS-PAGE.
is associated with activated TrkA receptors upon NGF stimulation.

Characterization of the Association of the CHK-SH2 Domain with Activated TrkA Receptors—In order to determine which domain of CHK interacts with the activated TrkA receptors, we used GST fusion proteins containing the SH2 domain of CHK (CHK-SH2). Serum-starved PC12 cells were stimulated with 100 ng/ml NGF for 10 min. The lysates were divided into two equal portions. One-half of the samples were immunoprecipitated with either anti-CHK antibodies, affinity-purified anti-TrkA antibodies, anti-Csk antibodies, or control antibodies. Immunoprecipitates were separated by 7% SDS-PAGE and immunoblotted with anti-TrkA antibodies (A). The same blots were stripped and reprobed with monoclonal phosphotyrosine antibodies (PY-20) (B). The second half of the lysates were immunoprecipitated as detailed above, and the samples were diluted 1:2 (v/v) with 2× SDS sample buffer without β-mercaptoethanol. The samples were separated by 10% SDS-PAGE and immunoblotted with anti-CHK antibodies (C). The same blots were stripped and reprobed with anti-Csk antibodies (D). Molecular mass markers are indicated on the right (kDa).

These results indicate that the CHK-SH2 domain specifically precipitated activated TrkA receptors upon NGF stimulation.

CHK Is Directly Associated with Activated TrkA Receptors—In order to determine whether the binding between activated TrkA receptors and the CHK-SH2 domain is direct or indirect, we performed far Western blotting analysis. The phosphorylation of TrkA upon NGF stimulation was rapid, and reached maximum levels after 20 min (Fig. 4A and B). In the absence of NGF, the SH2 domain of CHK did not bind to TrkA, as observed by far Western blotting (Fig. 4D). However, upon NGF stimulation, binding of the CHK-SH2 domain to TrkA was observed at 2 min. This association was maintained up to 60 min after stimulation with NGF (Fig. 4D). In contrast, the CHK-SH3 domain or GST fusion protein alone did not bind to activated TrkA (Fig. 4, C and E). These results indicate that the
CHK-SH2 domain directly interacts with activated TrkA receptors upon NGF stimulation. Far Western blotting were performed to identify the association of the CHK-SH2 domain with activated TrkA receptors. Starved PC12 cells were stimulated with 100 ng/ml NGF for the indicated periods and lysed. TrkA receptors were immunoprecipitated from cell lysates and then immunoblotted with anti-TrkA antibodies (A) or phosphotyrosine (PY-20) (B). The membrane was stripped, and far Western blotting were performed using GST fusion proteins containing the CHK-SH3 domain (C), the CHK-SH2 domain (D), or GST alone as a control (E).

CHK-SH2 domain directly interacts with activated TrkA receptors upon NGF stimulation.

**CHK Is Associated with Tyrosine 785 of the TrkA Receptor**—In order to determine the binding site of the CHK-SH2 domain to TrkA receptors, COS-7 cells were transfected with plasmids encoding wild-type TrkA or with mutated TrkA having tyrosine-phenylalanine mutations at the Shc association site Tyr-490 (Y490F), the p85/PI-3 kinase interaction site Tyr-751 (Y751F), the PLC-γ1 interaction site Tyr-785 (Y785F), or mutations at both Tyr-490 and Tyr-751 (Y490F/Y751F), Tyr-490 and Tyr-785 (Y490F/Y785F), or Tyr-751 and Tyr-785 (Y751F/Y785F). The transfected COS-7 cells were starved and then stimulated with 100 ng/ml NGF for 10 min. Unstimulated and NGF-stimulated cell lysates were divided into two aliquots, and either precipitated with the GST fusion protein containing the CHK-SH2 domain or immunoprecipitated with anti-TrkA antibodies.

Tyrosine phosphorylation of wild-type TrkA upon stimulation with NGF was observed in transfected cells. Furthermore, although the CHK-SH2 domain did not precipitate the unstimulated wild-type TrkA receptor, it did precipitate the tyrosine-phosphorylated wild-type TrkA receptor upon NGF stimulation (Fig. 5A). These results demonstrate that the CHK-SH2 domain interacts with activated TrkA receptors upon NGF stimulation in TrkA-transfected COS-7 cells.

Experiments in COS-7 cells transfected with TrkA wild-type or mutant constructs revealed that the CHK-SH2 domain precipitated TrkA Y490F and Y751F in the same manner as the wild-type TrkA receptor (Fig. 5B). The same results were observed in the binding of the CHK-SH2 domain to TrkA Y490F/Y751F. However, the CHK-SH2 domain could not precipitate mutant TrkA that had a Y785F mutation, such as TrkA Y785F, TrkA Y490F/Y785F, or TrkA Y751F/Y785F. These results indicate that the CHK-SH2 domain binds to phosphorylated Tyr-785 on the TrkA receptor. In order to confirm the expression of wild-type and mutant TrkA, the same amount of cell lysates was first immunoprecipitated and then immunoblotted with anti-TrkA antibodies.

Further, we have studied the ability of synthetic phosphopeptides to compete for the binding of the CHK-SH2 domain to TrkA receptors. We synthesized two kinds of tyrosine-phosphorylated peptides derived from the Shc binding site and the PLC-γ1 association site of TrkA receptors. A peptide containing the Shc binding site of TrkA receptors (Y*FSDTCV, where the asterisk indicates a phosphotyrosine peptide) could

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**FIG. 4.** CHK is directly associated with the activated TrkA receptors upon NGF stimulation. Far Western blotting were performed to identify the association of the CHK-SH2 domain with activated TrkA receptors. Starved PC12 cells were stimulated with 100 ng/ml NGF for the indicated periods and lysed. TrkA receptors were immunoprecipitated from cell lysates and then immunoblotted with anti-TrkA antibodies (A) or phosphotyrosine (PY-20) (B). The membrane was stripped, and far Western blotting were performed using GST fusion proteins containing the CHK-SH3 domain (C), the CHK-SH2 domain (D), or GST alone as a control (E).

**FIG. 5.** Analysis of the binding site of the CHK-SH2 domain on the activated TrkA receptors. A–C, point mutation analysis: untreated and NGF-treated COS-7 cells transfected with wild-type (WT) TrkA cDNA were lysed and either precipitated with the CHK-SH2 domain or immunoprecipitated with anti-TrkA antibodies. Bound proteins were immunoblotted with anti-TrkA antibodies (A, upper panel) or phosphotyrosine (PY-20) (A, lower panel). COS-7 cells were transfected with cDNA for wild-type TrkA; for mutated TrkA with either a single mutation at Tyr-490 (Y490F), Tyr-751 (Y751F), or Tyr-785 (Y785F) or double mutations (Y490F/Y751F, Y490F/Y785F, and Y751F/Y785F); or for the vector control. NGF-treated cell lysates were lysed and either precipitated with the CHK-SH2 domain (B) or immunoprecipitated with anti-TrkA antibodies (C) and then immunoblotted with anti-TrkA antibodies. D, phosphotyrosine peptide inhibition assay: Unstimulated and NGF-stimulated COS-7 cell lysates were added to the CHK-SH2-GST fusion protein (10 μg) preincubated with the indicated phosphopeptides (100 μM), respectively, as detailed under “Experimental Procedures.” Lysates were precipitated by the addition of glutathione-Sepharose 4B. Precipitates were immunoblotted with anti-TrkA antibodies.
not compete the binding of the SH2 domain of CHK to the activated TrkA receptors (Fig. 5D). The non-tyrosine-phosphorylated control peptide (SYLDVLG) also failed to compete the binding of the SH2 domain of CHK to the activated TrkA receptors. On the other hand, a peptide containing the PLC-1 association site of TrkA receptors (SY*LDVLG) was able to compete with the binding of the SH2 domain to the activated TrkA receptors (Fig. 5D). To further test the binding of CHK to the Tyr-785 site, we linked the tyrosine-phosphorylated SY*LDVLG peptide or the nonphosphorylated SYLDVLG peptide to Affi-Gel 15 beads, and the association of either the CHK-SH2 GST fusion protein or native CHK to the beads was analyzed. GST-CHK-SH2 was associated in a phosphotyrosine dependent manner to the phosphorylated SY*LDVLG peptide (data not shown). Similar specificity was observed when we tested the association of native CHK to the peptide beads. The phosphorylated SY*LDVLG was able to associate with native CHK from extracts of PC12 or PC12-CHK cells, whereas the nonphosphorylated peptide SYLDVLG did not associate with native CHK (data not shown). This specificity was in agreement with the peptide inhibition experiments. Taken together, these results, along with the site-directed mutagenesis of TrkA and the far Western blotting analysis, indicate that CHK binds to Tyr-785 of TrkA upon NGF stimulation.

**Overexpression of CHK Results in Enhanced Activation of the MAP Kinase Pathway upon NGF Stimulation**—Differentiation of PC12 cells upon NGF treatment requires activation of the MAP kinase pathway (17, 18, 35). Because CHK is associated with TrkA receptors upon NGF stimulation, we assessed the effects of overexpression of CHK on activation of the p44 and p42 MAP kinases following stimulation with NGF. We stably transfected PC12 cells with CHK-pcDNA3neo, generating several PC12-CHK clones that overexpress CHK. PC12 cells and PC12-CHK clones (three independent clones of PC12-CHK) were cultured in the absence or the presence of NGF (100 ng/ml) for the indicated times, and MAP kinase activity using myelin basic protein as a substrate was assayed. Although no MAP kinase activity was detected in control PC12 or PC12-CHK cells, an increase in this activity was observed in PC12 cells upon NGF stimulation, with a peak activity at 10 min, followed by a subsequent decline in activity (Fig. 6A). In PC12 cells overexpressing CHK, MAP kinase activity was enhanced as compared with untreated PC12 cells, and this activity was maintained for longer periods of up to 60 min (Fig. 6A). In addition, p44/42 MAP kinase activity was measured using antibodies specific to the activated p44/42 MAP kinases. The phosphokinase antibodies detect p44 and p42 MAP kinases only when they are activated by phosphorylation at Tyr-204 (36). PC12 cells and three independent PC12-CHK clones were cultured in the absence or presence of NGF (100 ng/ml) for the indicated times, and MAP kinase activity was assayed. In PC12 cells overexpressing CHK, tyrosine phosphorylation of MAP kinases was enhanced compared with untreated PC12 cells, and this phosphorylation was maintained for longer periods of up to 60 min (Fig. 6B). These results indicate a role of CHK in enhancing MAP kinase activity in PC12 cells upon NGF stimulation and thus its involvement in PC12 cell differentiation.

**Microinjection of CHK-specific Antibodies Inhibited Neuronal Differentiation of PC12 Cells**—To determine the requirement for the binding of the CHK-SH2 domain to TrkA receptors during neurite outgrowth, we examined the effects of decreasing the intracellular levels of CHK by microinjecting purified anti-CHK antibodies, anti-Csk antibodies, control preimmune rabbit antiserum, or control monoclonal antibody into living PC12 cells. Differentiation of these cells upon NGF stimulation was inhibited, as indicated by microinjection of purified anti-

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**DISCUSSION**

CHK protein tyrosine kinase is found predominantly in neuronal and hematopoietic cells. In the present study, we demonstrated direct binding of CHK to the TrkA receptors, upon NGF stimulation. Furthermore, we identified the binding site of the CHK-SH2 domain to the activated TrkA receptors as residue Tyr-785. In addition, overexpression of CHK resulted in an enhanced activation of the MAP kinase pathway upon NGF stimulation of PC12 cells, whereas microinjection of CHK-specific antibodies inhibited the neurite outgrowth of PC12 cells upon NGF stimulation. These results indicate for the first time that CHK, but not Csk, is involved in TrkA signaling and in neurite outgrowth of PC12 cells.

The association of the SH2 domain of CHK with activated...
TrkA was demonstrated using GST fusion proteins containing various domains of the CHK molecule. The association of CHK with TrkA occurred within the same time period in which tyrosine phosphorylation of TrkA was observed upon NGF stimulation. The phosphorylation of TrkA upon NGF stimulation was rapid and declined slowly, as previously reported (11). Our results indicated that the CHK-SH2 domain specifically interacts with the tyrosine-phosphorylated TrkA receptors upon NGF stimulation. The CHK-SH2 domain also precipitated other proteins, namely p112, p89, and p77. Although we were not yet able to identify these proteins, the possibility of their involvement in the association of CHK with TrkA signaling remains to be investigated in future studies.

Far Western blotting revealed that the binding between activated TrkA and the CHK-SH2 domain is direct (Fig. 4). The SH2 domain was found to bind to activated TrkA receptors, which were tyrosine-phosphorylated upon NGF stimulation, whereas the SH3 domain or GST fusion protein failed to bind to TrkA. Therefore, the CHK-SH2 domain can bind to the activated and phosphorylated TrkA.

Upon interaction with NGF, TrkA, a receptor tyrosine kinase, becomes autophosphorylated on cytoplasmic tyrosine residues (11, 36), such as Tyr-490, Tyr-670, Tyr-674, Tyr-675, or Tyr-785 (14, 18, 38). Of the several autophosphorylated residues of TrkA, three tyrosine residues, Tyr-490, Tyr-751, and Tyr-785, have been demonstrated to associate with Shc, p85/PI-3 kinase, and PLC-γ1, respectively (15, 16, 18, 33). All three signaling molecules, Shc, PLC-γ1, and PI-3 kinase, are demonstrated to play a major role in NGF-mediated neurite outgrowth (14, 17–19). In the present study, mutation analysis revealed that residue Tyr-785 on TrkA is required for it to associate with the CHK-SH2 domain (Fig. 5). This association was also confirmed by peptide inhibition assay (Fig. 5D). The Tyr-785 site is known to bind to PLC-γ1. These results suggest that CHK and PLC-γ1 share the same tyrosine residue for binding with TrkA receptors. Interestingly, platelet-derived growth factor receptor, a receptor tyrosine kinase like TrkA, also autophosphorylates several tyrosine residues, and the tyrosine residue sites Tyr-579 and Tyr-581 are known to bind to the SH2 domains of Shc and Src family kinases (32–39). Therefore, the signal transduction pathway through Tyr-785 on TrkA needs to be further investigated for its potential interactions with additional signaling molecules.

CHK is undetectable in early embryos and begins to be expressed around E15 (27, 40, 41). It then increases progressively up to the time of birth and remains high in the adult central nervous system. CHK expression correlates with late stage development and neuronal differentiation. In contrast, Csk is expressed throughout embryonic development and remains high in the central nervous system until birth. Csk is then dramatically down-regulated in the adult brain, except in the olfactory bulb (27, 41). Such diametrically opposite tempo-
eral expression patterns of Csk and CHK suggest distinct roles for both these proteins, presumably mediated via different substrates. Csk is known to inhibit Src family kinases, and this inhibition results in a down-regulation of the MAP kinase pathway (42). However, our results indicate that overexpression of CHK resulted in enhanced activation of the MAP kinase pathway in PC12 cells upon NGF stimulation. In addition, we have observed that the neuronal differentiation of PC12 cells induced by NGF was inhibited by microinjection of anti-CHK antibodies, but not anti-Csk antibodies (Fig. 7), suggesting that CHK and Csk regulate different targets in the signaling pathways of neuronal cells. Thus, our findings strongly suggest that CHK plays a role in regulating the differentiation of PC12 cells. Future studies will further investigate the molecular mechanisms of the involvement of CHK in neuronal differentiation.

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