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Glycogen Synthase Kinase-3β Induces Neuronal Cell Death via Direct Phosphorylation of Mixed Lineage Kinase 3[*,S]

Rajakishore Mishra[‡,§,1], **Manoj K. Barthwal**[‡,§,1], **Gautam Sondarva**[‡,§], **Basabi Rana**[‡,§], **Lucas Wong**[¶], **Malay Chatterjee**[||], **James R. Woodgett**[**], and **Ajay Rana**[‡,§,2]

[‡]Department of Internal Medicine, Cardiovascular and Cancer Research Institute, The Texas A & M University System-HSC, College of Medicine

[§]Central Texas Veterans Health Care System, Temple, Texas 76504

[¶]Department of Internal Medicine, The Texas A & M University System-HSC, College of Medicine, Scott and White Clinic, Temple, Texas 76504

[**]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada

[||]Division of Biochemistry, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India

Abstract

Mixed lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase member that activates the c-Jun N-terminal kinase (JNK) pathway. Aberrant activation of MLK3 has been implicated in neurodegenerative diseases. Similarly, glycogen synthase kinase (GSK)-3β has also been shown to activate JNK and contribute to neuronal apoptosis. Here, we show a functional interaction between MLK3 and GSK-3β during nerve growth factor (NGF) withdrawal-induced cell death in PC-12 cells. The protein kinase activities of GSK-3β, MLK3, and JNK were increased upon NGF withdrawal, which paralleled increased cell death in NGF-deprived PC-12 cells. NGF withdrawal-induced cell death and MLK3 activation were blocked by a GSK-3β-selective inhibitor, kenpaullone. However, the MLK family inhibitor, CEP-11004, although preventing PC-12 cell death, failed to inhibit GSK-3β activation, indicating that induction of GSK-3β lies upstream of MLK3. In GSK-3β-deficient murine embryonic fibroblasts, ultraviolet light was unable to activate MLK3 kinase activity, a defect that was restored upon ectopic expression of GSK-3β. The activation of MLK3 by GSK-3β occurred via phosphorylation of MLK3 on two amino acid residues, Ser^{789} and Ser^{793}, that are located within the C-terminal regulatory domain of MLK3. Furthermore, the cell death induced by GSK-3β was mediated by MLK3 in a manner dependent on its phosphorylation of the specific residues within the C-terminal domain by GSK-3β. Taken together, our data provide a direct link between GSK-3β and MLK3 activation in a neuronal cell death pathway and identify MLK3 as a direct downstream target of

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[2]To whom correspondence should be addressed: Dept. of Pharmacology, Bldg. 102, Loyola University Medical Center, 2160 South First Ave., Maywood, IL 60153. Tel.: 708-216-5761; Fax: 708-216-6596; arana@lumc.edu.
[1]These authors contributed equally to this work.

GSK-3*β*. Inhibition of GSK-3 is thus a potential therapeutic strategy for neurodegenerative diseases caused by trophic factor deprivation.

The decision by cells to either undergo cell death or to allow survival is governed by a myriad of extracellular cues such as growth factor availability, heat shock, and cytotoxic insults. The extracellular changes are recognized by specific cellular receptors and transmitted to intracellular targets leading to prevalence of death or survival. Activation of family members of the c-Jun N-terminal kinases (JNKs)[3] by their upstream kinases has been implicated in cell death responses (1–4). JNK is stimulated by pro-inflammatory cytokines, oxidative stress, growth factor deprivation, heat shock, UV, *γ*-radiation, and other cellular stress (1, 2). The signals in the stress-activated JNK pathway are transmitted via three core protein kinase modules: MAP3K, MAP2K, and MAPK. In the JNK pathway, the activating stimuli activate the MAP3K members, such as members of mixed lineage kinases (MLKs) or MEKKs, which, in turn, phosphorylate MAP2K members such as SEK1/MKK4 and MKK7 (5, 6). The activated MAP2Ks then phosphorylate JNK at a tyrosine and threonine residue leading to activation and phosphorylation of transcription factors, such as, c-Jun, ELK1, and ATF2. The phosphorylated transcription factors bind to their cognate DNA targets and modulate gene transcription and cellular fate.

MLKs are members of a significant MAP3K family and have been implicated in neuronal cell death pathways (7–10). Among all the MLK family members, MLK3 has been investigated in most detail (6). Recently, it has been shown that ectopic expression of MLK3 or NGF withdrawal causes neuronal cell death, which is blocked by treatment with a small molecule inhibitor of MLKs, CEP-1347 (7, 9). Similarly, CEP-11004, an analog of CEP-1347, has also been shown to have neuroprotective roles in NGF withdrawal-induced cell death (11). Previously, we showed that cell death induced by MLK3 activation is attenuated by insulin via PI3K-AKT pathway activation (12). The cell survival protein AKT/PKB directly phosphorylates Ser^{674} in the C-terminal regulatory domain of MLK3. The phosphorylation of MLK3 by AKT attenuates MLK3 kinase and its cell death induction properties (12). *β*-Amyloid peptide has been implicated in promoting neuronal loss during Alzheimer's disease (13). Inhibition of MLKs by CEP-1347 can prevent neuronal cell death, evoked by *β*-amyloid peptide (14, 15). These results indicate a significant and a direct role of MLKs in controlling a major cell death promoting pathway. However, the detailed mechanism by which different effectors modulate MLK3 activity and regulate cell death is poorly defined.

Analogous to MLKs, GSK-3*β* activation also promotes neuronal cell death (16–19). Overexpression of GSK-3*β* in PC-12 cells (16) or its regulated expression in different parts of the brain in transgenic GSK-3*β* mice induces neuronal cell death (20). It has been shown that NGF withdrawal-induced cell death in PC-12 cells can be ameliorated by the GSK-3 inhibitor, lithium (18, 21). GSK-3*β* has also been shown to activate the JNK pathway

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[3]The abbreviations used are: JNK, c-Jun N-terminal kinase; HEK, human embryonic kidney; MAP3K, mitogen-activated protein kinase kinase kinase; MAP2K, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; DAPI-4′,6-dia-midino-2-phenylindole; MLK, mixed lineage kinase; GSK, glycogen synthase kinase; NGF, nerve growth factor; MEF, mouse embryonic fibroblast; MEKK, MAPK/extracellular signal-regulated kinase kinase kinase; GST, glutathione *S*-transferase; HA, hemagglutinin; WT, wild type; AKT, AKT/protein kinase B.

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through MEKK1 activation (22). Neurons subjected to severe apoptotic paradigms such as serum deprivation, combined with exposure to an *N*-methyl-D-aspartate receptor antagonist or treatment with PI3K inhibitors, exhibit elevated GSK-3*β* activation, followed by induction of apoptosis (23). Similar to MLKs, *β*-amyloid peptide also activates GSK-3*β* kinase activity and induces cell death (24, 25); this effect is attenuated by lithium treatment (26). These results indicate that GSK-3*β*, which is an important enzyme for glucose metabolism and transcriptional regulation, also plays an important role in cell death pathways. However, the detailed mechanism by which GSK-3*β* activation induces neuronal cell death and the functional relationship, if any, between GSK-3*β* and MLK3 in cell death pathway remain to be determined.

In this study, we demonstrate that MLK3 is a direct substrate of GSK-3*β*, and their functional interaction is regulated by apoptotic stimuli (*i.e.* NGF withdrawal) in PC-12 cells. This regulatory event that is induced by GSK-3*β* has measurable consequences on MLK3 downstream signaling, including activation of JNK and apoptosis. Our data also suggest that MLK3 is a cellular target of GSK-3*β* and raises the possibility that under pathophysiological conditions such as Alzheimer's disease, neuronal loss may be controlled by directly inhibiting MLK3 kinase activity using small molecule inhibitors.

EXPERIMENTAL PROCEDURES

Cell Culture

PC-12 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum, as described earlier (9). PC-12 cells were differentiated for 5–7 days in RPMI containing 0.2% fetal bovine serum and 50 ng/ml of 2.5S-NGF (Promega, Madison, WI). Human embryonic kidney 293 (HEK-293) cells, GSK-3*β*-deficient (GSK-3$\beta^{-/-}$), and normal mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum as described earlier (27).

Transfection and Treatments

HEK-293 and MEFs were transfected with the appropriate expression vectors, as indicated, using Lipofectamine 2000 (Invitrogen). Kenpaullone (Calbiochem, San Diego, CA) and CEP-11004 (kind gift from Cephalon, PA) pretreatments were given for the indicated time periods before starting the NGF withdrawal. Apoptosis was induced by washing the cells with NGF-free medium and was accelerated by the addition of anti-NGF antibodies (1:1000) (Sigma).

Immunoprecipitation and Immunoblot Analyses

HEK-293, PC-12, and MEFs were lysed in lysis buffer as described earlier (5). The cell extracts were clarified by centrifugation at 15,000 × *g* for 5 min, and the protein contents were measured using Bradford method. Immunoprecipitation and glutathione *S*-transferase (GST) pull downs were performed either by specific antibodies or glutathione-Sepharose (GSH) beads. The immunoprecipitates were washed thoroughly and processed either for immunoblotting or kinase assay. For immunoblotting, equal protein content of cell extracts

or the immunoprecipitated protein samples were taken. The proteins were separated on denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membrane and blotted with antibodies as indicated. The antibodies used were: JNK (Santa Cruz Biotechnology, CA), GST (Upstate Biotechnology, Inc.), HA tag (BAbCO), cleaved caspase-3 (Cell Signaling Technology), and GSK-3*β* (BD Biosciences). Antibody for immunoprecipitating endogenous JNK was provided by Dr. Joseph Avruch (Massachusetts General Hospital, Boston, MA). For immunoprecipitating endogenous MLK3, antibody against the C-terminal peptide of the protein was developed in the laboratory as described earlier (12, 28).

Kinase Assays

GSK-3*β* kinase assay was done as described elsewhere (29), using GST-*β*-catenin as the substrate. MLK3 kinase assay was performed using GST-SEK1 (K-R) as the substrate as described earlier (5). For *in vitro* kinase assay, prokaryotic recombinant proteins were expressed and purified as described earlier (5). The purified GSK-3*β* (active kinase) was purchased from Upstate Biotechnology, Inc.

Metabolic ^{32}P Labeling

Transfected HEK-293 cells were transferred to phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) containing [^{32}P]orthophosphate (100 *μ*Ci/ml) (Perkin Elmer Life Sciences) and were incubated for 3 h as described earlier (12).

Plasmids and Site-directed Mutagenesis

GSK-3*β* (WT) and GSK-3*β* (K-A) constructs were used as reported earlier (27, 30). All MLK3 constructs were generated in the laboratory by PCR and subcloning. Site-directed mutagenesis of human MLK3 was performed with a QuikChange™ kit (Stratagene, La Jolla, CA). The MLK3 T680A, S789A, and S793A mutants were generated with the oligonucleotide 5′-CAACACCCCCCgCGCCAACGCCCGCG-3′, 5′-GGGCCACGGCCTgCTCCCCTGCCATC-3′, 5′-CTCCCCTGCCAgCACCACAGCCTGC-3′ (mismatch with the wild type MLK3 template is indicated by a lowercase letter). The single point mutants were used as a template to generate double and subsequently triple mutants. Mutations were confirmed after sequencing the clones (Davis Sequencing, Davis, CA).

Generation of GST-β-Catenin

Full-length *β*-catenin was subcloned in pGEX4T2 bacterial expression vector between BamH1 and NotI sites. The HA-tagged wild type *β*-catenin clone was used as a template for PCR amplification. The primers used for full-length *β*-catenin (amino acids 1–781) were as follows: 5′-CGGGATCCGCCATGGCTACTCAAGCTGATTTGATG-3′ and 5′-ATAGTTTAGCGGCCGCCAGGTCAGTATCAAACCAGGC-3′. Following subcloning of the *β*-catenin in the pGEX4T2 vector, its GST-fused protein was purified and used as the substrate for *in vitro* GSK-3*β* kinase assays.

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Apoptotic Cell Death

The NGF withdrawal-induced cell death in differentiated PC-12 cells was measured by estimating the mono- and oligonucleosomes specifically released in cytoplasm, using a cell death detection kit following manufacturer's protocol (Roche Applied Science catalog number 1774425) and as described (21, 31). The cell extracts were, in addition, blotted for cleaved and total caspase-3. Cell death was also measured in differentiated PC-12 cells under overexpressed conditions. 2×10^6 differentiated PC-12 cells were transfected with pEGFP-C1 (Promega, WI) along with the vectors encoding the proteins, as indicated, using AMAXA biosystem (AMAXA, Germany) following manufacturer's protocol. Cell death was measured in the GFP-positive cells using DAPI stain. Briefly, the cells were fixed in 4% paraformaldehyde and subsequently permeablized with 0.1% Triton-X-100 followed by staining with ProLong Gold antifade reagent, containing DAPI (Molecular Probes, Eugene, OR). The DAPI-stained nuclei in GFP-positive cells were analyzed for apoptotic morphology by fluorescence microscopy. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells.

RESULTS

NGF Withdrawal Induces GSK-3β, MLK3, and JNK Protein Kinase Activities and Promotes Cell Death in PC-12 Cells

A widely used experimental model of neuronal apoptosis is trophic factor withdrawal-induced cell death in cultured neuronal cells. To examine the effects of trophic factor withdrawal on the protein kinase activities of GSK-3*β*, MLK3, and JNK and its consequential effect on neuronal cell death, NGF was withdrawn at different time intervals from differentiated PC-12 cell culture medium. NGF depletion was accelerated by adding anti-NGF antibody to the cell culture medium, and the cells were harvested at different time intervals ranging from 30 min to 24 h (Fig. 1). The kinase activities of endogenous JNK, GSK-3*β*, and MLK3 were determined using specific substrate for each kinase. There was a time-dependent activation of JNK activity (Fig. 1*A*), which peaked (~4.5-fold) at 4 h of NGF withdrawal. NGF withdrawal also activated GSK-3*β* (Fig. 1*B*) and MLK3 (Fig. 1*C*) kinase activities in a time-dependent manner. GSK-3*β* was activated about 4-fold at 4 h of NGF withdrawal, and MLK3 kinase activity also peaked (~5-fold) at 4 h of withdrawal. To determine whether the activation of JNK, GSK-3*β*, and MLK3 correlated with NGF withdrawal-induced cell death in PC-12 cells, we measured the kinetics of PC-12 cell death. NGF withdrawal-induced cell death in differentiated PC-12 cells was observed as early as 1 h following withdrawal, which was amplified with the time, and sustained up to 24 h, with a 12–15-fold increase in cell death compared with the control (Fig. 1*D*) cells. Together, these results indicate a possible correlation between induction of cell death and activation of GSK-3*β*, JNK, and MLK3 in PC-12. These results also suggest that once the process of cell death is initiated by the activation of these death-associated kinases, it is sustained via modulating downstream effectors of cell death pathways.

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NGF Withdrawal-induced Cell Death Is Attenuated by MLK3 and GSK-3 Inhibitors

To examine whether NGF withdrawal-induced GSK-3*β* and MLK3 activation play any role in PC-12 cell death, the cells were treated either with kenpaullone (32, 33), a GSK-3 inhibitor, or CEP-11004 (11), a MLK3 inhibitor, before NGF withdrawal. The inhibition of either GSK-3 or MLK3 almost completely inhibited NGF withdrawal-induced cell death (Fig. 2*A*) in PC-12 cells, suggesting that these protein kinases play a significant role in trophic factor deprivation-induced neuronal cell death. Furthermore, NGF withdrawal-induced JNK activation was also inhibited by both these inhibitors (Fig. 2*B*). Kenpaullone and CEP-11004 also inhibited NGF withdrawal-induced MLK3 activation significantly, an effect that was synergized in the presence of both of the inhibitors (Fig. 2*C*). Interestingly, although kenpaullone was able to inhibit MLK3 activation (Fig. 2*C*), the MLK3 inhibitor CEP-11004 failed to block NGF withdrawal-induced GSK-3*β* activation (Fig. 2*D*). These results indicate that GSK-3*β* might be functioning upstream of MLK3.

GSK-3β Is an Upstream Activator of MLK3

There are two isoforms of GSK-3, GSK-3*α* and GSK-3*β*. To determine whether GSK-3*β* played a direct role in MLK3 activation, we took advantage of MEFs derived from GSK-3*β*-deficient mice (27). First, the expression of GSK-3*β* and MLK3 were determined by immunoblotting. As expected, GSK-3*β* was detected in normal but not in GSK-3*β*-deficient MEFs (Fig. 3*A*). MLK3 expression in GSK-3*β*-deficient MEFs was comparable with the normal MEFs (Fig. 3*A*). Recently, it has been shown that UV irradiation activates GSK-3*β* kinase activity (22, 34). To understand whether activation of GSK-3*β* by UV influences MLK3 kinase activity, GSK-3*β*-deficient and normal MEFs were exposed to 80 J/m^2 of UV irradiation. The endogenous MLK3 in GSK-3*β*-null MEFs was not activated by UV. However, in normal MEFs, MLK3 was significantly activated (~3-fold) upon UV exposure (Fig. 3*A*). This MLK3 activation was not induced by an unknown UV-inducible kinase, because in GSK-3*β*-deficient MEFs, the basal and UV-induced MLK3 kinase activities were comparable (Fig. 3*A*). Interestingly, the basal MLK3 kinase activity in normal MEFs was higher than that of GSK-3*β*-deficient MEFs (Fig. 3*A*). To further corroborate the requirement for GSK-3*β* for MLK3 activation, the GSK-3*β*-deficient MEFs were complemented with either kinase-active (GSK-3*β*, WT) or kinase-inactive (GSK-3*β*, K-A) GSK-3*β* expression plasmids. The kinase-active, but not kinase-inactive GSK-3*β* complementation restored UV-induced MLK3 activation (Fig. 3*B*), suggesting that GSK-3*β* function is necessary for MLK3 activation.

GSK-3*β* Interacts with and Phosphorylates MLK3

Three stringent putative consensus GSK-3 phosphorylation sequences are present in the C-terminal half of human MLK3 (see Fig. 5*A*). Because NGF withdrawal activated both MLK3 and GSK-3 (Fig. 1), we postulated that the activation of MLK3 may be mediated proximally through GSK-3*β* phosphorylation of MLK3. To test this hypothesis, we tested for association between endogenous MLK3 and GSK-3*β* in differentiated PC-12 cells following NGF withdrawal. GSK-3*β* co-precipitated with MLK3 in response to NGF withdrawal (Fig. 4*A*), but this association was completely blocked by the GSK-3 inhibitor,

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kenpaullone, suggesting that GSK-3*β* interacts with MLK3 in a kinase-active-dependent manner.

Because the potential GSK-3*β* phosphorylation sites on MLK3 are present in the C-terminal half of the protein, we examined whether the interaction between MLK3 and GSK-3*β* is mediated through this region of MLK3. The N-terminal half (amino acids 1–511) or the C-terminal half (amino acids 511–847) of MLK3 were separately transfected along with a GSK-3*β* expression plasmid into HEK-293 cells and serum-starved for 12 h (Fig. 4*B*). The full-length MLK3 and C-terminal half of MLK3 were found to interact with wild type but not with kinase-inactive GSK-3*β*, whereas the N-terminal half of MLK3 failed to interact with GSK-3*β* (Fig. 4*B*). These results further support our endogenous interaction data (Fig. 4*A*), indicating that the interaction between C-terminal regulatory domain of MLK3 and GSK-3*β* is dependent upon the activation state of GSK-3*β*.

Next, we examined whether GSK-3*β* directly phosphorylates the C-terminal half of MLK3 protein. The recombinant, truncated C-terminal MLK3 protein was purified from bacteria, and *in vitro* kinase assays were performed in presence of purified active GSK-3*β* proteins. The kinase-active purified GSK-3*β* proteins phosphorylated the C-terminal half but not the N-terminal half of the recombinant MLK3 proteins, which was inhibited in presence of kenpaullone (Fig. 4*C*). These data indicate that GSK-3*β* has the capacity to directly phosphorylate MLK3 *in vitro*. To assess whether this reaction occurred *in vivo*, we employed metabolic ^{32}P labeling experiments in HEK-293 cells. These experiments showed that active GSK-3*β* was capable of phosphorylating full-length, kinase-inactive MLK3 under *in vivo* conditions, whereas phosphorylation of MLK3 by inactive GSK-3*β* was insignificant (Fig. 4*D*). These results strongly suggest that MLK3 is not only an *in vitro* substrate of GSK-3*β* but is also targeted at the cellular level by GSK-3*β*. To further strengthen our *in vitro* and *in vivo* phosphorylation data and demonstrate that GSK-3*β* is a physiologically relevant kinase of MLK3, we measured the phosphorylation of endogenous MLK3 in differentiated PC-12 cells under NGF withdrawal condition. The differentiated cells were pretreated either with kenpaullone to inhibit endogenous GSK-3*β* activity or with vehicle as a control. These cells were metabolically labeled with ^{32}P, and NGF was withdrawn for 4 h. The phosphorylation of endogenous MLK3 was measured after immunoprecipitation. The NGF withdrawal induced measurable increased phosphorylation of MLK3, which was blocked by kenpaullone (Fig. 4*E*). These results strongly signify GSK-3*β* as a physiological kinase of MLK3 under growth factor deprivation condition.

GSK-3*β* Phosphorylates Specific Sites on C-terminal Regulatory Domain of MLK3

The three stringent putative GSK-3*β* phosphorylation sites in the C-terminal half of MLK3 are Thr^{680}, Ser^{789}, and Ser^{793} (Fig. 5*A*). To examine whether these putative sites are indeed targeted by GSK-3*β*, we mutated these residues to nonphosphorylable alanine residue in full-length MLK3 and the C-terminally truncated mutant of MLK3. These point mutants were created as single residue mutations or in combination, as indicated in Fig. 5*B*. First, we examined their interaction with full-length GSK-3*β* to assess whether these mutations disrupted the interaction with GSK-3*β*. The wild type and MLK3 mutants interacted

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comparably with GSK-3*β*, but the relative interactions between GSK-3*β* and triple MLK3 mutant were less than other MLK3 mutants (Fig. 5*B*).

To examine whether Thr^{680}, Ser^{789}, and Ser^{793} are targeted by GSK-3*β*, the C-terminal half of MLK3 proteins containing individual or multiple point mutations were expressed in bacteria. The recombinant bacterial proteins were used in *in vitro* kinase assays with purified active GSK-3*β* protein. The C-terminal half of wild type and T680A mutant MLK3 proteins were strongly phosphorylated by GSK-3*β*. However, the other ala-nine mutant MLK3 proteins showed different degrees of decreased phosphorylation when compared with wild type or T680A C-terminal MLK3 proteins (Fig. 5*C*). To examine whether *in vitro* GSK-3*β* phosphorylation sites on MLK3 are also targeted under *in vivo* conditions, HEK-293 cells were co-transfected with GSK-3*β* along with full-length, kinase-inactive MLK3, containing GSK-3*β* phosphorylation site mutations, as described in Fig. 5*D*. *In vivo* metabolic ^{32}P labeling of these transfected cells showed that GSK-3*β* was able to phosphorylate kinase-inactive MLK3, whereas phosphorylation of single or multiple point mutants, except the T680A mutant of MLK3, was decreased significantly (Fig. 5*D*). Specially, the phosphorylation of MLK3 (K-A) S793A mutant was very low compared with other two single point mutants: T680A and S789A. These results suggest that Ser^{793} may be the major *in vivo* GSK-3*β* phosphorylation site on MLK3.

GSK-3*β* Regulates JNK Activation via MLK3 Phosphorylation

It has been reported that GSK-3*β* facilitates activation of JNK and that this effect contributes to apoptotic signaling (22, 35). We and others have reported that MLK3 is a potent activator of JNK (5, 36). Recently, we have demonstrated that the activation of JNK in response to apoptotic stimuli, like ceramide or tumor necrosis factor *α*, is specifically mediated via MLK3 (28). To examine whether phosphorylation of MLK3 by GSK-3*β* contributes to JNK activation, the GSK-3*β* phosphorylation site mutants of MLK3 or wild type MLK3 were transfected along with wild type GSK-3*β* into HEK-293 cells (Fig. 6*A*). The endogenous JNK protein was immunoprecipitated, and *in vitro* kinase assays were performed using GST-c-Jun protein as the substrate. Overexpression of wild type MLK3 or GSK-3*β* alone activated JNK potently, which was synergized in the presence of both MLK3 and GSK-3*β* together (Fig. 6*A*). The activation of JNK by GSK-3*β* was attenuated to different degrees by single or multiple phosphorylation-deficient point mutants of MLK3 (Fig. 6*A*). Remarkably, the GSK-3*β*-induced JNK activation was significantly blocked by the MLK3 mutant containing two GSK-3*β* phosphorylation sites (*i.e.* MLK3 S789A,S793A) mutated to alanine (Fig. 6*A*, compare *lane 4* with *lane 16*). Collectively, these results suggest that the phosphorylation/activation of MLK3 by GSK-3*β* contributes to the activation of JNK. The T680A MLK3 mutant also partially blocked JNK activation; however, our data (Fig. 5, *C* and *D*) suggest that this site may not be targeted by GSK-3*β*. The Thr^{680} site conforms to Pro-directed kinases, and thus it is possible that this site is regulated by some unknown Pro-directed kinase, other than GSK-3*β*. To examine how the phosphorylation of MLK3 by GSK-3*β* regulates the activation of MLK3, we also measured the protein kinase activities of MLK3 mutants from these experiments. The activation of all the MLK3 mutants by GSK-3*β* was less than wild type MLK3 (Fig. 6*B*).

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GSK-3β-induced Cell Death Is Mediated via MLK3 Phosphorylation/Activation

To determine whether phosphorylation/activation of MLK3 by GSK-3β contributes to neuronal cell death, differentiated PC-12 cells were transfected with either GSK-3β or MLK3 by using Amaxa Nucleofection System. Because we observed that MLK3 is a downstream effector of GSK-3β (Fig. 2*D*), transfected PC-12 cells were treated with either vehicle or CEP-11004 to block GSK-3β-induced cell death. Overexpression of MLK3 or GSK-3β alone significantly induced cell death, and this effect was blocked by CEP-11004 (Fig. 7*A*), suggesting that activation of MLK3 by GSK-3β contributes to cell death.

We next tested whether the phosphorylation of MLK3 by NGF withdrawal-induced GSK-3β was necessary for cell death. Because our data suggested that Ser^{793} on MLK3 might be the major *in vivo* GSK-3β phosphorylation site (Fig. 5*D*) and that the S789A,S793A phosphorylation mutant of MLK3 was insignificantly phosphorylated by GSK-3β (Fig. 5*D*), we transfected these mutants or wild type MLK3 into differentiated PC-12 cells. The MLK3 S793A mutant partially prevented NGF withdrawal-induced cell death, whereas the double phosphorylation mutant (*i.e.* MLK3 S789A,S793A) was less potent than wild type MLK3 in promoting NGF withdrawal-induced cell death in PC-12 cells (Fig. 7*B*). Taken together, these results strongly suggest that phosphorylation/activation of MLK3 by GSK-3β plays a significant role in neuronal cell death pathways.

DISCUSSION

GSK-3 activation has been linked to neuronal cell death during pathogenesis of Alzheimer's disease (18, 37, 38). Activation of MLK3 has also been shown to play a major role in neuronal cell loss in 1-methyl-4-phenyl tetrehydropyridine-treated Par-kinson's mice model (10, 39). A functional correlation, if any, between GSK-3 and MLK3 in neuronal or non-neuronal cell death pathways has never been identified. In this study, we demonstrate that trophic factor withdrawal activates GSK-3β, which in turn phosphorylates distinct residues on the C-terminal regulatory domain of MLK3. This GSK-3β-mediated phosphorylation of MLK3 appears to play an important role in trophic factor deprivation-induced cell death in differentiated PC-12 cells.

Neuronal cells die in response to various insults, including ischemia, trophic factor deprivation, heat shock, and many other stress stimuli. NGF deprivation-induced neuronal cell death in cell culture model systems mimics *in vivo* trophic factor loss-induced neurodegeneration (40). We investigated the role of death inducing protein kinases, specifically GSK-3β, MLK3, and JNK, in NGF withdrawal-induced cell death pathway in the PC-12 cell model system. It has been reported that the NGF deprivation causes neuronal cell death in PC-12 cells via GSK-3β activation. Based on these results, we investigated the time kinetics of GSK-3β, MLK3, and JNK activation in differentiated PC-12 cells, in response to NGF deprivation. The kinase activities of all three protein kinases followed similar time kinetics, with significant activation around 4 h of NGF deprivation (Fig. 1). These results suggested the possibility that these kinases are regulated through a common pathway in response to NGF withdrawal.

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CEP-1347 (KT7515), a derivative of the natural product K-252a found in broths of *Narcodiopsis* bacterium, exhibits neuroprotective properties (41). During initial characterization, it was observed that CEP-1347 potently inhibits the JNK group of kinases at low nanomolar concentration (42, 43). Subsequently, it was found that CEP-1347 specifically targets the MLK group of kinases that act upstream in the JNK pathway (7) and prevents neuronal loss in the 1-methyl-4-phenyl tetrehydropyridine-treated mice model of Parkinson's disease (44). It is also reported that CEP-11004 and its analogs also induce TrkA receptor expression and AKT activation in sympathetic neurons and down-regulates MLK downstream signaling, including JNK activation (45). Interestingly, in random human trials, it was observed that CEP-1347 was well tolerated and had no significant side effects (46). We used CEP-11004, which is an analog of CEP-1347, to investigate the role of MLK3 in the GSK-3*β*-induced cell death pathway. NGF withdrawal-induced cell death in PC-12 was almost completely blocked by CEP-11004 or by the GSK-3 specific inhibitor, kenpaullone (Fig. 2*A*). These results suggested that GSK-3 and MLK3 were somehow interlinked to induce neuronal cell death. Interestingly, when PC-12 cells were treated with the GSK-3 inhibitor, kenpaullone, the kinase activity of MLK3 and cell death (Fig. 2, *A* and *C*) were attenuated. However, pretreatment with the MLK3 inhibitor, CEP-11004, although preventing cell death, was unable to down-regulate GSK-3*β* kinase activity (Fig. 2*D*). These results indicated that MLK3 was likely downstream of GSK-3*β* in the trophic factor deprivation-induced cell death pathway. This important observation was corroborated by using GSK-3*β*-deficient MEFs, where UV, an activator of GSK-3*β*, could not activate MLK3 in absence of GSK-3*β* (Fig. 3*A*); however, re-expression of kinase-active GSK-3*β* restored UV-induced MLK3 activation (Fig. 3*B*).

Because our data suggested that MLK3 was downstream of GSK-3*β* and that both the kinases were activated in response to NGF deprivation, we postulated that GSK-3*β* probably activates MLK3 via phosphorylation. An interesting feature of GSK-3 is its substrate specificity. Many GSK-3 substrates require prior phosphorylation by different kinases, generating the motif (S/T)*XXX*(S/T)(P) (*X* is any amino acid), where the upstream S/T motif acts as the phosphorylation site for GSK-3 (47). A second category of GSK-3 substrates seem not to require prior phosphorylation for their recognition by GSK-3 (47). Examination of the MLK3 sequence revealed three highly stringent putative GSK-3 phosphorylation sites, clustered in the C-terminal regulatory domain of MLK3 (Fig. 5*A*). In light of these observations, we tested whether the endogenous MLK3 and GSK-3*β* associated in response to NGF deprivation, which was, indeed, found to be the case (Fig. 4*A*). Moreover, this interaction was blocked by a GSK-3 inhibitor, kenpaullone, indicating that GSK-3*β* interacts with MLK3 in a protein kinase functionally dependent manner (Fig. 4*A*). Because the C-terminal domain of MLK3 contained the putative GSK-3*β* phosphorylation sites, we next examined whether the bacterially expressed C-terminal fragment of MLK3 was a direct substrate for GSK-3*β*. The purified active GSK-3*β* phosphorylated the C-terminal half of MLK3 (Fig. 4*C*).

There are few enzymes that exert as broad a regulatory influence on cellular function as GSK-3 (48, 49). More than 40 proteins have been reported to be phosphorylated by GSK-3, including more than 12 transcription factors (17, 18). However, most of these proteins have not yet met all the criteria set out by Frame and Cohen (47), which are important to

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demonstrate that a protein is an *in vivo* substrate of GSK-3β. Our metabolic labeling data under overexpression conditions demonstrated that MLK3 was a cellular substrate of GSK-3β, because the kinase-inactive MLK3 was specifically phosphorylated by kinase-active GSK-3β, whereas kinase-inactive GSK-3β failed to phosphorylate MLK3 under *in vivo* conditions (Fig. 4*D*). These results were further corroborated with more physiologically relevant experiments, where endogenous MLK3 was observed to be phosphorylated by GSK-3 in differentiated PC-12 cells, upon NGF withdrawal (Fig. 4*E*). These results collectively fulfill at least one of the important criteria for MLK3 to be designated as a "physiological substrate" of GSK-3β.

To add further weight to the possibility that MLK3 is a physiological substrate of GSK-3β, the three stringent putative sites, Thr^{680}, Ser^{789}, and Ser^{793}, were mutated to nonphosphorylatable alanine, either individually or in combination, as indicated in Fig. 5. The *in vitro* phosphorylation with the point mutants of C-terminal half of MLK3 suggested that of three putative sites only two sites (*i.e.* Ser^{789} and Ser^{793}) were directly targeted by GSK-3β (Fig. 5*C*). However, when the *in vivo* metabolic labeling experiments with the kinase-inactive point mutants of MLK3 along with GSK-3β were carried out, they showed that of the three putative GSK-3β sites, Ser^{793} was the primary target under *in vivo* conditions, and Thr^{680} was not primarily targeted by GSK-3β (Fig. 5*D*). Because phosphorylation of MLK3 T680A mutant was slightly lower than wild type MLK3 under *in vivo* condition, it is possible that the Thr^{680} site, which conforms to Pro-directed kinase, is a target of some unknown Pro-directed kinase. Ser^{793} has also been reported as an *in vivo* MLK3 phosphorylation site (50). However, the identity of the kinase for Ser^{793} was not known. Our data suggest that GSK-3β may be involved in the phosphorylation of Ser^{793} site. Some of the GSK3 substrates require prior phosphorylation at serine or threonine residue, located four residues C-terminal to the site of GSK3 phosphorylation (51). One interpretation of our phosphorylation data (Fig. 5) could also be that Ser^{793} is first phosphorylated by some yet to be identified "priming kinase" before GSK3β essentially phosphorylates the Ser^{789} site on MLK3.

JNK activation often contributes to cell death. Earlier, we and others have reported that MLK3 as well as other MLKs are potent activators of JNK (5, 6, 36). In addition, GSK-3β also facilitates JNK activation (22, 34). When we looked for the functional effects of MLK3 point mutations on JNK activation by GSK-3β, we found that the phosphorylation of MLK3 by GSK-3β indeed regulated JNK activation, and GSK-3β phosphorylation sites were involved in the JNK activation, albeit to different degrees (Fig. 6*A*). Interestingly, the MLK3 S793A mutant itself was not activated by GSK-3β at all (Fig. 6*B*); however, downstream JNK activation was partly blocked by S793A mutation. It is possible that GSK-3β might utilize other MLK family members to regulate downstream JNK activation. When we analyzed the sequences of other known MLK family members: MLK1, MLK2, and MLK4α and β, we found that these family members also contain either one (in MLK1) or multiple (in MLK2 and MLK4) putative GSK-3β phosphorylation sites. Whether these putative sites on other MLKs are also phosphorylated by GSK-3β is not yet known. Nonetheless, our data clearly suggested that GSK-3β indeed regulates JNK activation via MLK3 phosphorylation (Fig. 6*A*).

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Overexpression of MLK3 (9) or GSK-3*β* (16) has been shown to cause neuronal cell death. Earlier, we showed that overexpression of MLK3 also causes cell death in non-neuronal cells (12). Based on these published reports, we investigated how phosphorylation of MLK3 by GSK-3*β* might impact the cell death pathway in differentiated PC-12 cells. We showed that overexpression of GSK-3*β* or MLK3 alone in differentiated PC-12 cells caused cell death, which was synergized to substantial cell death when both GSK-3*β* and MLK3 were co-expressed (Fig. 7*A*). The MLK inhibitor CEP-11004 prevented cell death caused by overexpression of GSK-3*β* or MLK3 (Fig. 7*A*) consistent with our earlier results, indicating that GSK-3*β* was upstream of MLK3 in neuronal cell death pathway. When we looked for any functional effects of expression of MLK3 phosphorylation-deficient mutants on NGF withdrawal-induced cell death in PC-12 cells, we found that the S793A mutant significantly prevented the growth factor withdrawal-induced cell death, whereas the S789A,S793A mutant almost completely obliterated the effect of growth factor deprivation-induced cell death (Fig. 7*B*). These results support the notion that GSK-3*β* causes neuronal cell death via MLK3 phosphorylation/activation.

Based on our current data and an earlier published report where we had demonstrated that the PI3K-AKT pathway can down-regulate the MLK3-induced cell death pathway via phosphorylation of Ser^{674} on MLK3 by AKT/PKB (12), we propose a model of cell death and survival, mediated through MLK3 phosphorylation by AKT and GSK-3*β* (Fig. 8). It has already been reported that NGF deprivation inhibits the PI3K-AKT pathway (23). Thus, under NGF deprivation conditions, the attenuated AKT activation will allow GSK-3*β* activation via reduction of GSK-3*β* Ser^9 phosphorylation (53, 54). The dephosphorylated and activated GSK-3*β* will then phosphorylate MLK3 on Ser^{789} and Ser^{793}, leading to activation of MLK3, which will then trigger JNK activation and promote cell death. In our present study, we have not shown whether GSK-3*β* activation causes cell death via JNK activation; however, it is well established that MLK3 activation causes cell death via JNK activation (9). Thus, we propose that GSK-3*β*, which is known to activate JNK, may cause cell death using GSK-3*β* → MLK3 → JNK pathway. On the contrary, under conditions where growth factors (*e.g.* insulin-like growth factor 1, insulin, and NGF) are available, the PI3K-AKT pathway will be active (12, 52, 55, 56). Consequently, activated AKT will phosphorylate GSK-3*β* at Ser^9, and, at the same time, will also phosphorylate Ser^{674} on MLK3. The direct phosphorylation of GSK-3*β* and MLK3 by AKT will collectively act to down-modulate downstream JNK activation and thus favor cell survival.

In conclusion, for the first time, we provide direct biochemical evidence to support the notion that GSK-3*β* is a potent upstream activator of MLK3 in the growth factor deprivation-induced cell death pathway. We also provide evidence that MLK3 is a physiological substrate of GSK-3*β*, because MLK3 was phosphorylated by GSK-3*β* under *in vivo* conditions. Second, we have also demonstrated that in GSK-3*β*-deficient MEFs, UV, a known activator of GSK-3*β*, fails to activate MLK3. It is tempting to speculate from these observations that growth factor deprivation-induced neurodegenerative diseases might be prevented via targeting the GSK-3*β*-MLK3 pathway.

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Supplementary Material

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Acknowledgments

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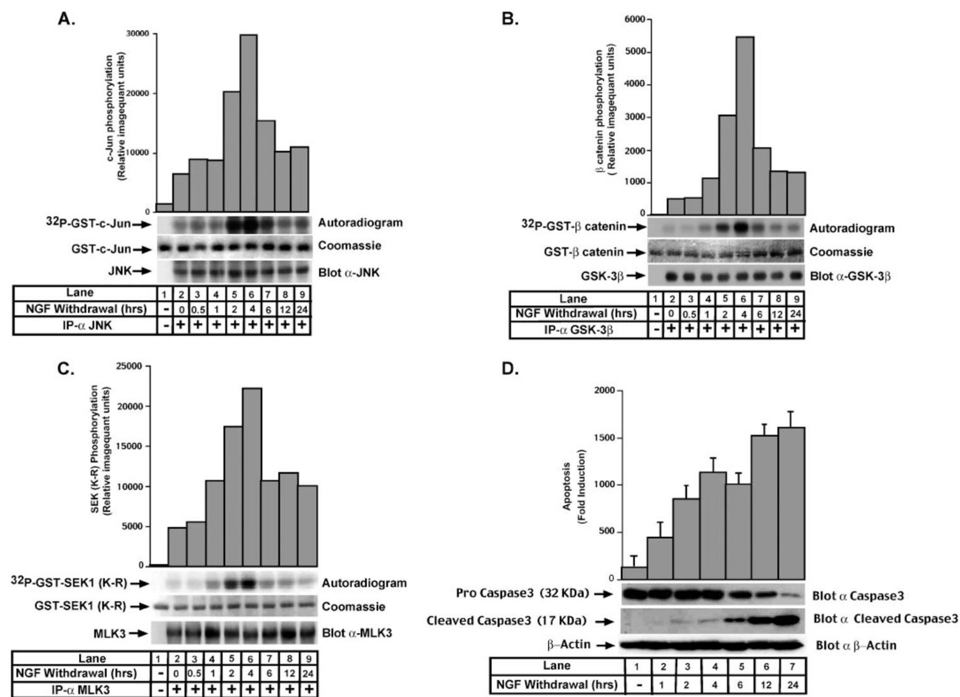

FIGURE 1. NGF withdrawal induces GSK-3β, MLK3, and JNK kinase activities and apoptosis

PC-12 cells were differentiated in low serum medium containing 2.5 S NGF (50 ng/ml) for 5–7 days. NGF withdrawal was initiated by washing the cells once with low serum medium and then placing them back in low serum medium containing anti-NGF antibody. The cells were harvested at different time intervals as indicated. *A*, NGF withdrawal activates JNK. JNK was immunoprecipitated (*IP*) from cell lysates using anti-JNK antibody, and *in vitro* kinase assay was performed with GST-c-Jun as the substrate. *Lane 1* is substrate alone, and *lane 2* represents control cells without NGF withdrawal. *B*, NGF withdrawal activates GSK-3*β*. GSK-3*β* was immunoprecipitated from cell lysates using an anti-GSK-3*β* antibody, and an *in vitro* kinase assay was performed with GST-*β*-catenin as the substrate. *C*, NGF withdrawal activates MLK3. MLK3 was immunoprecipitated from cell lysates using anti-MLK3 antibody, and an *in vitro* kinase assay was performed with GST-SEK1 (K-R) as the substrate. The *bar graphs* in *A–C* show the kinase activities presented in arbitrary PhosphorImager units, as measured from the *top panels*. The *middle panels* show the equal amounts of substrates used for each kinase measured, and the *bottom panels* show the equal expression of endogenous kinases from PC-12 cells, as indicated in each panel. The data presented in *A–C* represents one of four similar experiments. *D*, NGF withdrawal induces apoptosis in differentiated PC-12 cells. Apoptosis was measured upon NGF withdrawal in terms of mono- and oligonucleosome appearance, by using an apoptosome kit following the manufacturer's protocol. The cell extracts were also blotted for total and cleaved caspase-3 and *β*-actin. The *bar graph* shows the averages ± S.E. of three similar experiments (Roche Applied Science, Indianapolis, IN).

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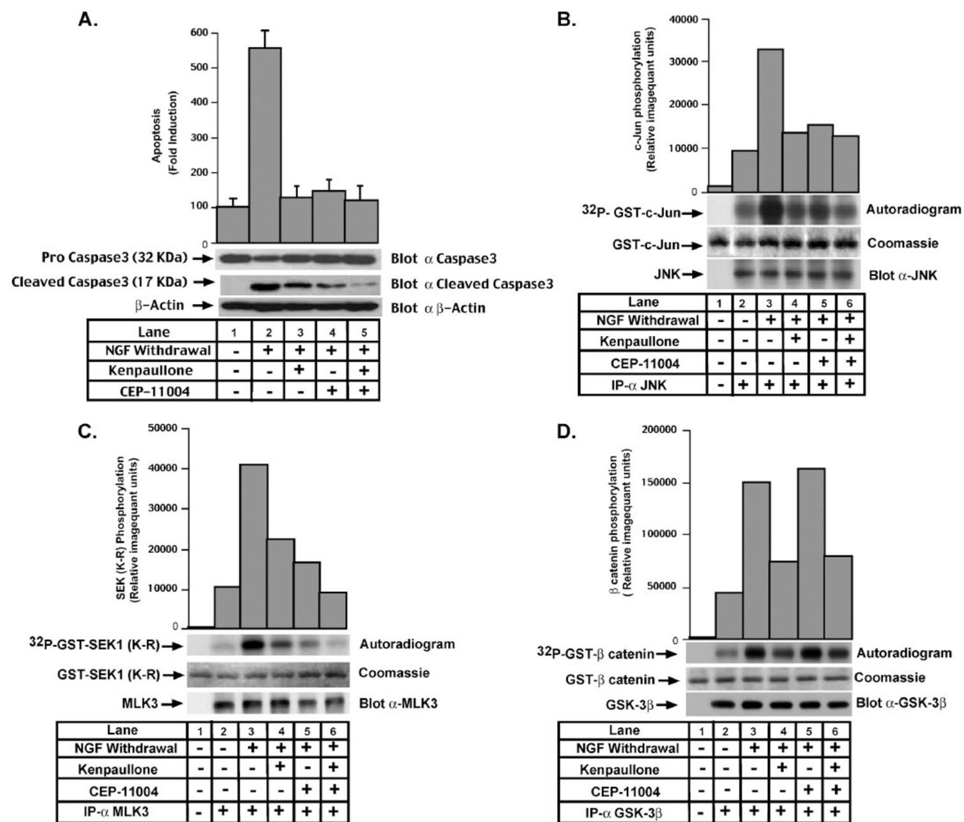

FIGURE 2. NGF withdrawal-induced apoptosis is attenuated by MLK3 or GSK-3*β* inhibitors

The differentiated PC-12 cells were treated for 12 h either with kenpaullone (5 *μ*M) or CEP-11004 (500 nM), before NGF withdrawal (for 4 h). The NGF withdrawal was carried out as described in the legend Fig. 1, except the fresh stocks of MLK3 or GSK-3 inhibitors were added in the withdrawal medium as indicated. The cells were lysed and processed for kinase and apoptosis assays. *A*, NGF withdrawal-induced apoptosis is inhibited by kenpaullone and CEP-11004. Following addition of either kenpaullone or CEP-11004, apoptosis was estimated using an apoptosome kit as described in the legend to Fig. 1. The cell extracts were also blotted for total and cleaved caspase-3 and *β*-actin. The *bar graph* shows the averages ± S.E. of four similar experiments. *B–D*, effect of kenpaullone and CEP-11004 on JNK (*B*), MLK3 (*C*), and GSK-3*β* (*D*) kinase activities. The cells were pretreated with either kenpaullone or CEP-11004 alone or together as described above, and *in vitro* kinase assays were performed to measure the kinase activities of JNK, MLK3, and GSK-3*β* in the presence of their respective substrates. The *bar graphs* in *B–D* show the kinase activities presented in arbitrary PhosphorImager units, as measured from the *top panels*. The *middle panels* show the equal amounts of substrate used for each kinase measured. The *bottom panels* show the equal expression of endogenous kinases in PC-12 cells, as indicated in each panel. The data presented in *B–D* represent one of four similar experiments. *IP*, immunoprecipitation.

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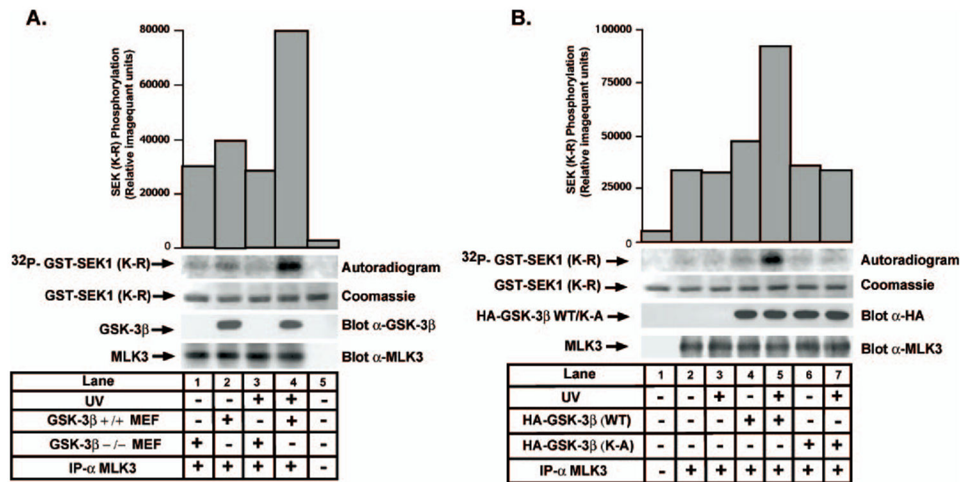

FIGURE 3. GSK-3β is an activator of MLK3

Normal (GSK-3$\beta^{+/+}$) and GSK-3β-deficient (GSK-3$\beta^{-/-}$) MEFs were irradiated with 80 J/M^2 of UV radiation. After UV irradiation, the cells were placed back in a tissue culture incubator for 30 min and then harvested. *A*, UV-induced MLK3 activation is mediated via GSK-3β. Endogenous MLK3 was immunoprecipitated from MEFs. The kinase assay of MLK3 was performed as described in the legend to Fig. 1. The *last lane* (*lane 5*) is substrate alone. *B*, reconstitution of kinase-active GSK-3β in GSK-3$\beta^{-/-}$MEFs revives UV-induced MLK3 activation. The expression plasmids of HA-GSK-3β WT or kinase-dead (K-A) were transfected into GSK-3$\beta^{-/-}$ cells. The cells were exposed to UV as indicated, and MLK3 kinase activity was measured. *Lane 1* is substrate alone, and *lane 2* is control GSK-3$\beta^{-/-}$ MEF cells. The *bar graphs* in *A* and *B* show the endogenous MLK3 kinase activities present in arbitrary PhosphorImager units, as measured from the *top panels*. The *middle panel* shows the expression of either endogenous GSK-3β (*A*) or reconstituted recombinant GSK-3β (*B*). The *bottom panels* show the equal expression of endogenous MLK3 in MEF cells. The data presented in *A* and *B* represent one of three similar experiments.

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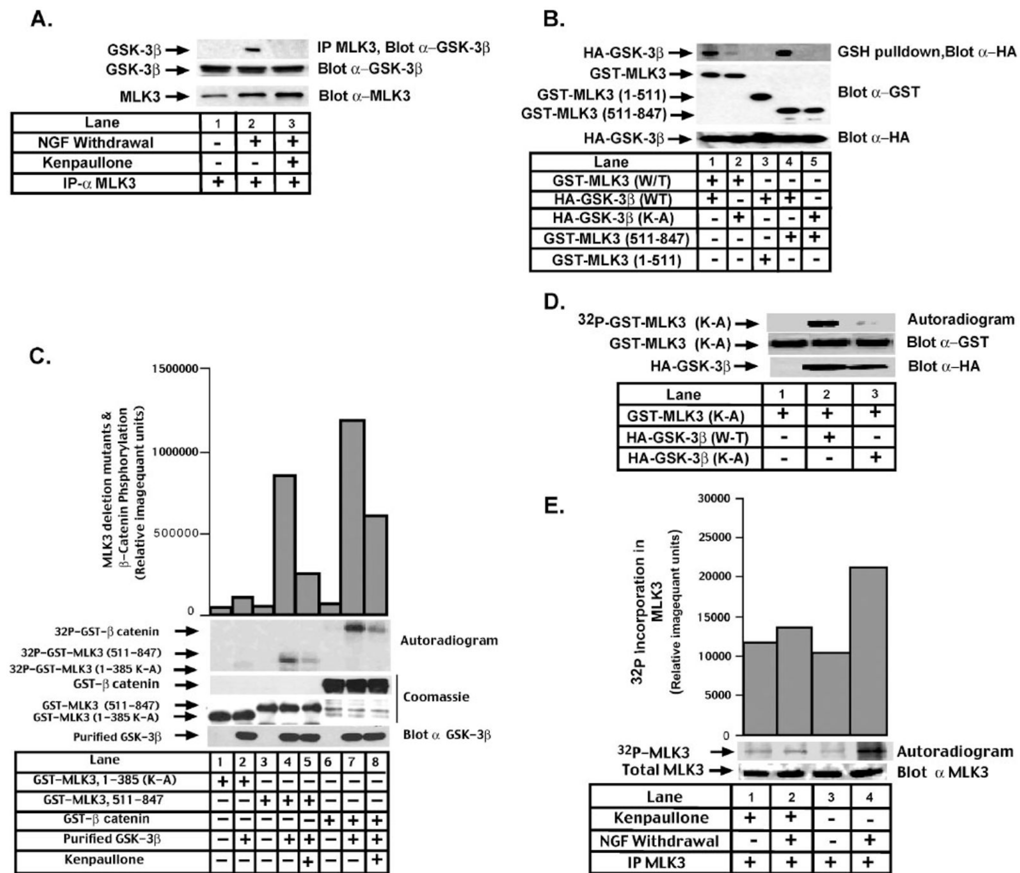

FIGURE 4. GSK-3β interacts with and phosphorylates MLK3

Endogenous interaction between MLK3 and GSK-3β was determined in PC-12 cells, whereas interactions between recombinant proteins or phosphorylation of full-length of MLK3 was performed in HEK-293 cells. *A*, NGF withdrawal induces interaction between endogenous MLK3 and GSK-3β in PC-12 cells. The differentiated PC-12 cells were pretreated with kenpaullone or vehicle for 12 h. prior to NGF withdrawal (for 4 h). Endogenous MLK3 was immunoprecipitated (*IP*) using anti-MLK3 antibody, and immunoblotting was performed by anti-GSK-3β antibody. The cell extracts were also blotted for MLK3 and GSK-3β. *B*, GSK-3β interacts with the C-terminal regulatory domain of MLK3. Different GST-MLK3 constructs, as indicated, were co-transfected with either HA-tagged, WT, or kinase-inactive (K-A) GSK-3β. MLK3 proteins were pulled down with glutathione-Sepharose beads, and any associated GSK-3β protein was detected using anti-HA antibody (12CA5). The cell lysates were also blotted with anti-HA and anti-GST antibodies to determine the expression of recombinant GSK-3β and MLK3, respectively. *C*, GSK-3β directly phosphorylates the C-terminal regulatory domain of MLK3. Different MLK3 proteins, as indicated, were expressed and purified as GST fusion proteins from bacteria. *In vitro* kinase assays were performed in the presence of purified kinase active GSK-3β, either in the presence or absence of kenpaullone as indicated. The kinase activities of purified GSK-3β proteins were determined using bacterially expressed β-catenin protein as the substrate. The polyvinylidene difluoride membrane after kinase assay was first stained with Coomassie stain and then blotted with anti-GSK-3β antibody. *D*, GSK-3β

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phosphorylates MLK3 *in vivo*. HEK-293 cells were transfected with GST-MLK3 (K-A), in combination with either WT (GSK-3β) or kinase inactive (GSK-3β, K-A) GSK-3β. Post-transfection, the cells were labeled with [^{32}P]orthophosphate, and the kinase-inactive MLK3 (GST-MLK3, K-A) proteins were precipitated using glutathione-Sepharose beads and subjected to SDS-PAGE. Phosphorylation of kinase-inactive MLK3 was detected by autoradiography. The cell extracts were also blotted with anti-GST and anti-HA antibodies to examine the expression of recombinant MLK3 and GSK-3β proteins, respectively. *E*, endogenous MLK3 is phosphorylated by NGF withdrawal-induced GSK-3β. PC-12 cells were differentiated and pretreated either with kenpaullone or vehicle as indicated for 12 h. The cells were labeled with ^{32}P and NGF was withdrawn for 4 h as described in the legend to Fig. 1. The endogenous MLK3 was immunoprecipitated, resolved, and detected by autoradiography. The data presented in *A*–*C* represent one of three similar experiments, and the data in *D* and *E* represent one of two similar experiments.

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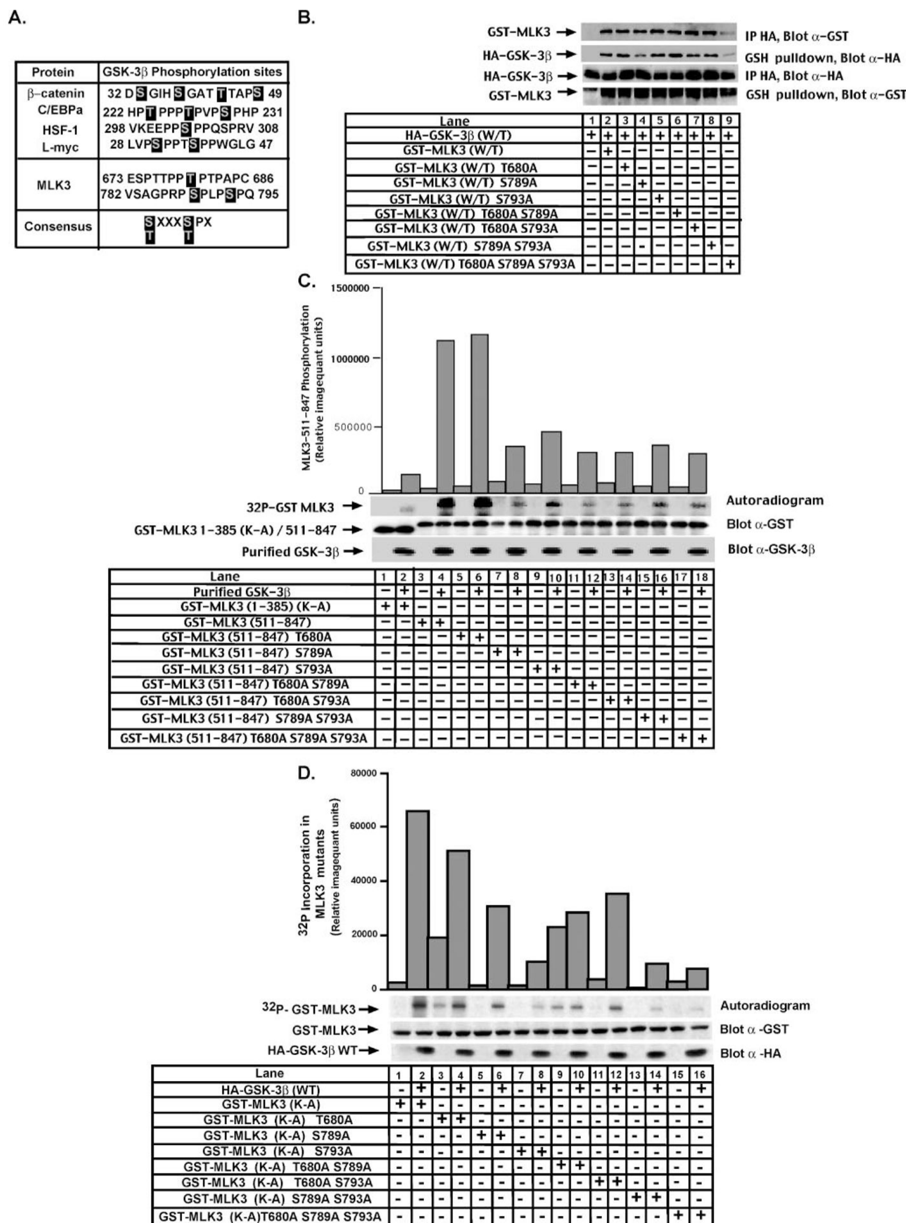

FIGURE 5. GSK-3β phosphorylates specific sites on MLK3

A, three putative GSK-3β consensus phosphorylation sites are present in the C-terminal regulatory domain of MLK3. Putative sites are highlighted in *bold type*. Several known GSK-3β substrate sequences are also shown. *B*, GSK-3β interacts with MLK3 mutants. HEK-293 cells were co-transfected with HA-GSK-3β, along with wild type or different putative GSK-3β phosphorylation site-deficient MLK3 mutants, as indicated. The recombinant GSK-3β or MLK3 were immunoprecipitated by anti-HA and glutathione-Sepharose beads, respectively. The immunoprecipitates were blotted with either anti-GST antibody for GSK-3β associated MLK3 (*top panel*) or with anti-HA antibody for MLK3 associated GSK-3β (*second panel* from *top*). Cell extracts were also blotted with anti-HA and anti-GST antibodies for examining the expression of HA-GSK-3β and GST-MLK3

proteins. *C*, the C-terminal regulatory domain of MLK3 contains specific GSK-3*β* phosphorylation sites. The putative GSK-3*β* phosphorylation sites in the C-terminal regulatory domain (MLK3, 511– 847) of MLK3 were mutated and expressed as GST fusion proteins in bacteria. *In vitro* phosphorylation reactions were carried out with different MLK3 mutant proteins and purified active GSK-3*β* protein, as indicated. *D*, GSK-3*β* phosphorylation sites on MLK3 are also targeted by GSK-3*β* under *in vivo* conditions. Different phosphorylation-deficient MLK3 mutants as described above were transfected along with GSK-3*β* in HEK-293 cells, as indicated. *In vivo* cell labeling with [^{32}P]orthophosphate was carried out as described in the legend to Fig. 4*D*. The MLK3 mutant proteins were pulled down with glutathione-Sepharose, and the phosphorylation of different MLK3 mutants by GSK-3*β* was detected and quantified by phosphorimaging. The data presented in *B* and *C* represent one of three similar experiments, and the data in *D* represents one of two similar experiments.

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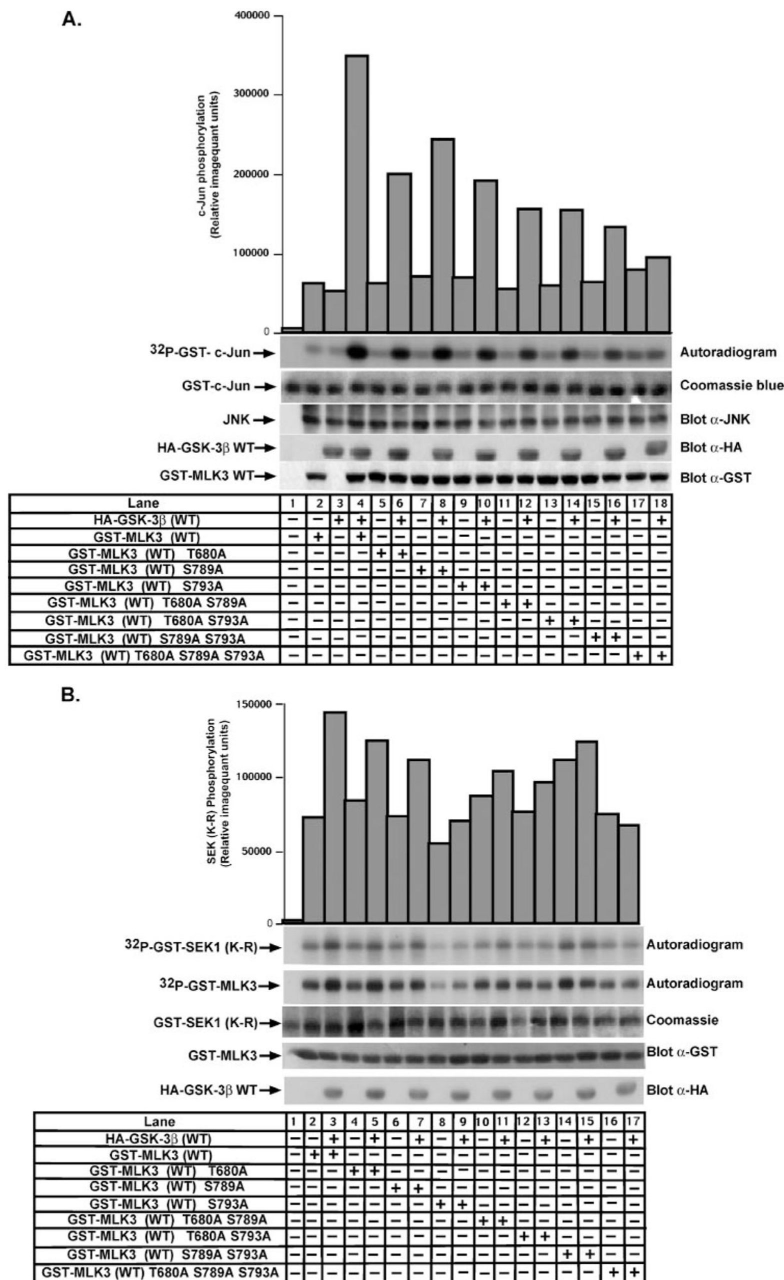

FIGURE 6. GSK-3β regulates JNK activation by phosphorylating MLK3 on specific sites in the C-terminal regulatory domain

A, GSK-3β-induced JNK activation is mediated via phosphorylation of MLK3. GSK-3β phosphorylation site-deficient MLK3 mutants, as indicated, were transfected along with GSK-3β in HEK-293 cells. Endogenous JNK was immunoprecipitated by anti-JNK antibody, and kinase assays were carried out using GST-c-Jun as the substrate. *B*, GSK-3β regulates MLK3 kinase activity via phosphorylation. Different GSK-3β phosphorylation site-deficient MLK3 mutants were transfected into HEK-293 cells along with GSK-3β, as indicated. MLK3 proteins were pulled down by glutathione-Sepharose beads, and kinase

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assays were performed using SEK1 (K-R) as the substrate. The data presented in *A* and *B* represent one of three similar experiments.

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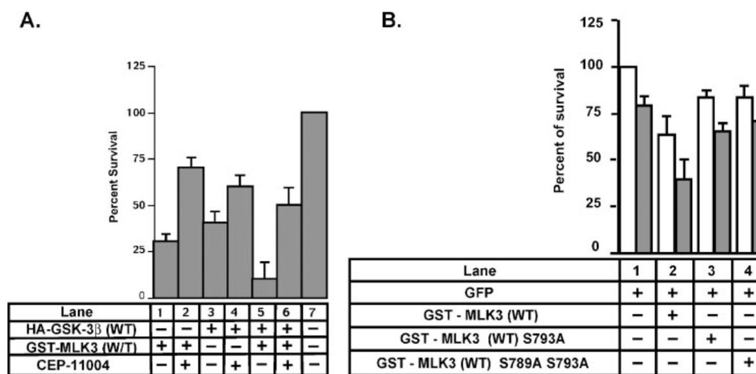

FIGURE 7. GSK-3*β*-induced cell death is attenuated by MLK3 mutants, lacking GSK-3*β* phosphorylation sites and CEP-11004

Approximately 2×10^6 NGF-differentiated PC-12 cells were transfected with indicated expression plasmids along with pEGFP-C1 (vector expressing green fluorescence protein) using an AMAXA biosystem. The cells were replated in chamber tissue culture slides, the nuclei were stained with DAPI, and GFP-positive cells were scored for the presence of apoptotic nuclei using a fluorescence microscope. *A*, GSK-3*β*-induced cell death is attenuated by the MLK3 inhibitor, CEP-11004. The differentiated PC-12 cells transfected with indicated expression plasmids were treated either with CEP-11004 or vehicle alone. Apoptotic nuclei from GFP-positive cells were scored. *B*, NGF withdrawal-induced GSK-3*β* stimulates cell death in a MLK3 phosphorylation-dependent manner. Differentiated PC-12 cells were transfected with either wild type MLK3 or MLK3 mutants: S793A or S789A,S793A (double mutant). 36 h post-transfection, NGF was withdrawn for 6 h as described in the legend to Fig. 1, and GFP-positive apoptotic nuclei were scored. The *open bars* represent the percentage of cell survival without NGF withdrawal, and *solid bars* represent the percentage of cell survival upon NGF withdrawal in transfected cells as indicated. The data presented in *A* and *B* represent one of three similar experiments.

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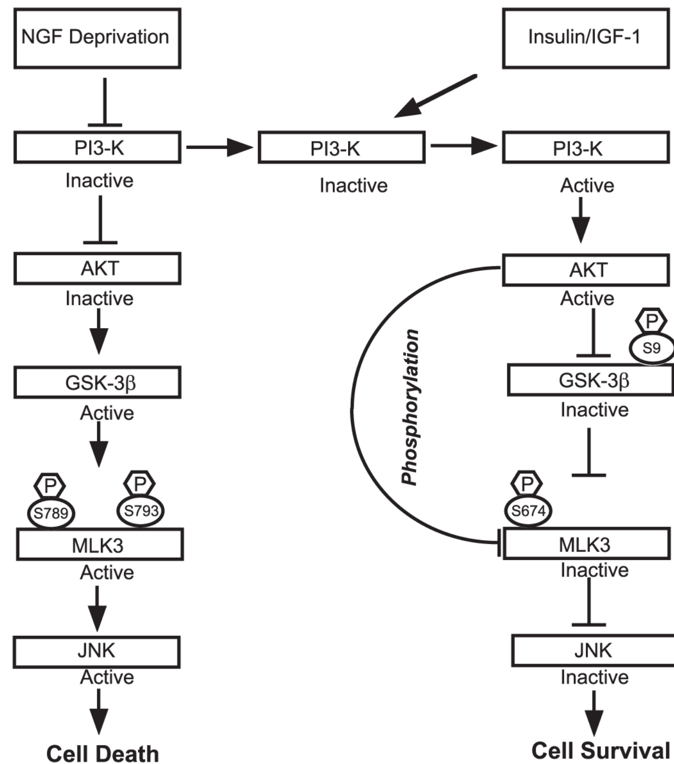

FIGURE 8. A model for the apoptotic and survival signaling pathways mediated by MLK3 phosphorylation

Removal of the survival agent, NGF, inhibits the PI3K-AKT pathway. AKT inhibition leads to activation of GSK-3*β*, which in turn phosphorylates its downstream substrate, MLK3 on Ser^{789} and Ser^{793}. This phosphorylation causes MLK3 activation, finally leading to cell death via JNK activation. On the contrary, survival agents, such as insulin and insulin-like growth factor 1, cause activation of PI3K-AKT pathway. The activated AKT phosphorylates GSK-3*β* at Ser^{9} and at the same time also phospho-rylates MLK3 at Ser^{674}. The AKT-mediated phosphorylation of GSK-3*β* and MLK3 down-regulates their kinase activities, leading to cell survival by attenuating JNK activity.