The Mixed Lineage Kinase DLK Utilizes MKK7 and Not MKK4 as Substrate*

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Mixed lineage kinases DLK (dual leucine zipper-bearing kinase) and MLK3 have been proposed to function as mitogen-activated protein kinase kinase kinases in pathways leading to stress-activated protein kinase/c-Jun NH2-terminal kinase activation. Differences in primary protein structure place these MLK (mixed lineage kinase) enzymes in separate subfamilies and suggest that they perform distinct functional roles. Both DLK and MLK3 associated with, phosphorylated, and activated MKK7 in vitro. Unlike MLK3, however, DLK did not phosphorylate or activate recombinant MKK4 in vitro. In confirmatory experiments performed in vivo, DLK both associated with and activated MKK7. The relative localization of endogenous DLK, MLK3, MKK4, and MKK7 was determined in cells of the nervous system. Distinct from MLK3, which was identified in non-neuronal cells, DLK and MKK7 were detected predominantly in neurons in sections of adult rat cortex by immunocytochemistry. Subcellular fractionation experiments of cerebral cortex identified DLK and MKK7 in similar nuclear and extranuclear subcellular compartments. Concordant with biochemical experiments, however, MKK4 occupied compartments distinct from that of DLK and MKK7. That DLK and MKK7 occupied subcellular compartments distinct from MKK4 was confirmed by immunocytochemistry in primary neuronal culture. The dissimilar cellular specificity of DLK and MLK3 and the specific substrate utilization and subcellular compartmentation of DLK suggest that specific mixed lineage kinases participate in unique signal transduction events.

A large body of work has focused on signal transduction via protein kinases generically termed mitogen-activated protein kinases (MAPK) that link a variety of extracellular signals to cellular responses as diverse as proliferation, differentiation, and apoptosis (reviewed in Refs. 1–3). Biochemical and genetic evidence has demonstrated that activation of a prototypical MAPK occurs through sequential activation of a series of upstream kinases: a serine/threonine MAPK kinase kinase (MAPKKK) phosphorylates a dual specificity protein kinase (MAPKK or MKK or MEK) that in turn phosphorylates and activates a MAPK. Three groups of mammalian MAPKs and the upstream kinases and stimuli that activate them have been studied most extensively. These include the p42/p44MAPKs (extracellular signal-regulated kinases, ERK1 and ERK2), that are generally activated by mitogens and differentiation inducing stimuli, the p46/p54SAPKs (stress-activated protein kinases, SAPKs), and the p38MAPKs. Stress-activated protein kinases were discovered as the principal c-Jun NH2-terminal kinases and therefore have also been termed JNKs. Distinct from ERK1 and ERK2, the SAPKs are predominantly activated by cell stress-inducing signals such as heat shock, ultraviolet irradiation, proinflammatory cytokines, hyperosmolality, ischemia/reperfusion, and axonal injury.

Like previously identified MAPK pathways in mammalian cells and yeast, the SAPK pathways were initially thought to lead in a linear fashion from activation of a Rho-like small GTPase through a series of intermediate protein kinases to SAPK activation. However, with the identification of two MAPKKs (MKK4/SEK1, MKK7/JNKK2) many MAPKKs (four MEKKs, five MLKs, ASK1, Tpl-2, and TAK1) and multiple additional MAPKK kinases that appear to lie in pathways proximal to the SAPKs, it is clear that the organization, regulation, and function of these protein kinase pathways remain poorly understood.

The five identified members of the mixed lineage kinase (MLK) family share two common structural features (4, 5). Each has a kinase catalytic domain whose primary structure is hybrid between those found in serine/threonine and tyrosine protein but most closely resemble MAPK kinase kinases. Second, closely juxtaposed COOH-terminal to the catalytic domain, each MLK protein has a domain that is predicted to form two leucine/isoleucine zippers separated by a short spacer region. It has been proposed that all members of the MLK family behave functionally as MAPK kinase kinases in pathways leading to activation of SAPKs (6). In support of this hypothesis, MLK2, MLK3, DLK, and LZK have all been shown to activate p46SAPK when overexpressed in cultured cells (6–12). Furthermore, MLK2 and MLK3 have been shown to associate with, phosphorylate, and activate MKK4 (6, 12, 13).

Despite the common features of the MLK family, the various members of the family are likely to have diverse biological behavior. This is predicted by comparison of structure that shows that MLK1, MLK2, and MLK3 form one closely related
Identification of a DLK Substrate

MLK subfamily while DLK and LZK form a second distinct subfamily (5, 11). MLK2 and MLK3 have highly conserved kinase catalytic and leucine zipper domains, sharing more than 70% sequence identity. Both possess an SH3 domain in their NH2-terminal region, and both have a functional CRIB domain that mediates GTP-dependent association with Rac1 and Cdc42Hs (9, 14). DLK and LZK share highly conserved kinase catalytic and leucine zipper domains that are more than 90% identical. These domains have only 36% identity to those of MLK2 and MLK3. Given the noted structural differences, DLK and LZK substrates may be distinct from those utilized by MLK2 and MLK3. Indeed, DLK and LZK substrates have not been identified. Moreover, DLK and LZK lack both CRIB motifs and SH3 domains and possess COOH-terminal regions that are structurally distinct from those of MLK2 and 3. These observations predict that protein-protein interactions unique to each protein kinase lead to distinct regulation and function by determining different subcellular compartmentalization and by providing access to different substrates.

The studies reported here provide evidence in support of this hypothesis. In vitro and complementary in vivo biochemical studies demonstrated that both DLK and MLK3 directly associate with, phosphorylate, and activate MKK7. Unlike MLK3, however, DLK did not phosphorylate or activate recombinant MKK4 in vitro. To investigate the physiological relevance of these observations, the cellular and subcellular localization of endogenous DLK, MLK3, MKK7, and MKK4 was investigated both in neuron cell culture and in the nervous system. Whereas MLK3 was identified predominantly in the periventricular ependyma and meninges, DLK and MKK7 were predominantly localized in neurons. DLK and MKK7 were identified in similar nuclear and extranuclear compartments. However, MKK4 occupied compartments distinct from that of DLK and MKK7. The dissimilar cellular and subcellular compartmentalization and differential substrate utilization of DLK and MLK3 provide evidence that specific mixed lineage kinases participate in unique signal transduction events.

MATERIALS AND METHODS

Antibodies—A rabbit polyclonal antisera directed against thecarboxyl-terminal 223 amino acids of DLK (C1) was described previously (5). Affinity purified rabbit anti-MKK7 polyclonal serum was a gift of Professor Eisuke Nihada (15). Monoclonal antibodies against the FLAG epitope (M2, Kodak/IBI), the Myc epitope (9E10, Oncogene Science), and the hemagglutinin epitope (12CA5, BMB) were obtained commercially as were rabbit polyclonal antibodies against SAPK/JNK3 (Upstate Biotechnology, Inc.), MLK3 (Santa Cruz), phospho-MKK4 (New England Biolabs), and MKK4 (Upstate Biotechnology, Inc.). Mouse monoclonal antibodies for SAPK/JNK3 (Pharmagen) were obtained commercially. In immunoblotting experiments, GST-MKK proteins were detected using the C1 polyclonal serum that was raised against a GST-DLK fusion protein.

Plasmids and Plasmid Constructions—Previously described plasmids used herein included DLK185A and DLK (16), MKK4 and MKK4-KM (Ref. 17, gifts of Dr. G. Johnson), FLAG-MLK3, and FLAG-MLK3(K144E) (Ref. 12, gifts of Dr. J. Woodgett), GST-MKK1 and GST-MKK3 (Ref. 18, gifts of Dr. K.-L. Guan), GST-MKK5 (Ref. 19, gift of Dr. J. Dixon), GST-MKK7 (Ref. 20, gift of Dr. R. Davis), Myc-p46SAPK (15), and GST-c-Jun (1–79) (7, GST-MKK7(K76A) was created by introducing point mutations into the GST-MKK7 template using sequential polymerase chain reaction steps as described (16). Synthetic oligonucleotides were used included 1) a 5′-MKK7 oligonucleotide 5′-CGCCGATC-CATGTCGGGCTCCATCA-3′, 2) a 3′-MKK7 antisense oligonucleotide 5′-CCGCAATACCATCTCAGAAAGGGCAG-3′, and 3) 5′-CATCTTCTTCCTGGTTGCGCAAATGGCGG-3′ and 4) 5′-CCGATAGCGGACACAGAATG-3′ as sense K-A mutagenesis oligonucleotides, respectively. The BamHI-EcoRI fragment of the resultant amplification product was used to substitute into the BamHI-EcoRI-digested PEX-GKT plasmid (21). FLAG-MKK7 and FLAG-MKK7-KA expression constructs were prepared using polymerase chain reaction employing Expand High-Fidelity DNA polymerase (Boehringer Mannheim), GST-MKK7 or GST-MKK7(KA) plasmid as templates, and synthetic oligonucleotides, including a 5′ oligonucleotide encoding a Kozak’s consensus and a FLAG-epitope (5′-ATAAGCTTTCCAGGCTTGAGCTCAACGACCGGATGCAACAGGGGCCTCCATACACTTGTTCA-3′). Amplified fragments were subcloned into pCR3.1 (Invitrogen) following the manufacturer’s recommendations. All new constructs were sequenced to assure their sequence fidelity.

Cell Culture and Transfections—COS 7 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Experiments involving transient transfections were carried out as described previously (7) with a total of 2 μg of the appropriate combinations of eukaryotic expression plasmids and pCMV-neo (Life Technologies, Inc.) according to the manufacturer’s protocol.

Primary embryonic cortical neuron culture was established from dissected cortices obtained from Sprague-Dawley rat fetuses at embryonic day 17. Cortical cells were dissociated in Neurobasal Medium containing B-27, GutaMax-I and AlbuMax II (Life Technologies, Inc.) and 2 × 105 cells/well of a 24-well plate were seeded on glass coverslips precoated with 100 μg/ml poly-L-lysine. Cultures were incubated for 2 weeks at 37 °C in humidified 95% air, 5% CO2. Half of the medium was replaced twice weekly. Cultures were characterized by immunocytochemistry using neuron specific antibodies against neurofilament protein (SMI31, Steinberger Meyer Immunocemicals) and astrocyte-specific antibody, glial fibrillary acidic protein (Sigma). Cultures typically were composed of 95% neurons and 5% astrocytes as described previously (22). Immunoprecipitations and Immunoblotting—Immunoprecipitations were performed as described elsewhere (7) using the indicated antibodies. For immunoblotting, immunoprecipitates or 30 μl of cell lysate were separated under reducing conditions by SDS-polyacrylamide gel electrophoresis and immunoblotted as described previously (7, 16). Expression and Purification of GST Fusion Proteins—With the exception of GST-MKK7 and GST-MKK7(KA), GST fusion proteins were expressed in Escherichia coli and were prepared, purified, analyzed, and quantified as described previously (5). Purified recombinant GST-MKK4, GST-MKK4(KA), and GST-p46SAPK were obtained commercially (Upstate Biotechnology, Inc.). Recombinant GST-MKK7 and GST-MKK7(KA) were prepared as described by Frangioni et al. (23). Briefly, following expression in culture, bacterial pellets were washed in 50 ml of ice-cold STE (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) and resuspended in STE containing 100 μg of lysozyme. After a 15-min incubation on ice, dithiothreitol (5 mM final concentration) and Sarkosyl (1.5% final concentration) were added, and the resultant suspension was pulled through several times through a 18-gauge needle. Supernatant obtained following centrifugation of the suspension at 10,000 × g for 5 min was adjusted to contain 4% Triton X-100 and purified on glutathione-Sepharose beads. Fusion proteins were eluted from beads with 10 μl reduced glutathione in a buffer containing 75 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 0.1% Triton X-100 and were concentrated by ultrafiltration using Centricon-10 (Amicon, Inc.)

In Vitro Kinase Assays—Cells were transfected as indicated in figure legends and lysates were prepared as described previously (7) in 1 ml of a buffer containing 50 mM HEPES, 7.5 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 20 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, and protease inhibitors. Protease inhibitors included 2 μM phenylmethylsulfonyl fluoride, 0.5 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 1 μg/ml aprotinin. p46SAPK immune-complex kinase assays were performed as described previously (7). Complexes were incubated for 30 min at 30 °C in 30 μl of kinase buffer (25 mM HEPES, pH 7.2, 10% glycerol, 100 mM NaCl, 20 mM MgCl2, 0.1 mM sodium vanadate, and protease inhibitors) containing 25 μM ATP, 5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham) and 2 μg of GST-c-Jun (1–79). Reactions were terminated by addition of Laemmli buffer, boiled, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and autoradiographed. Where indicated, incorporated counts were counted with a Bio-Rad phosphorimager. Equivalent expression of transfected p46SAPK was assessed by immunoblotting. DLK and MLK3 immune-complex kinase assays were performed as above except that 2 μg of GST-MKK4, GST-MKK1, GST-MKK3, GST-MKK5, or their kinase negative mutants were substituted as substrate were indicated in figure legends. MKK7 immune-complex kinase assays were carried out as above except that 2 μg of GST-SAPK was substituted as substrate.

In Vitro SAPK Activation Assays—In vitro activation of SAPK was determined essentially as described (7). FLAG epitope-tagged DLK, DLK185A, MLK3, or MLK3(K144E) were immunoprecipitated from 500 μg of transiently transfected COS 7 cell lysate. In vitro reconstituted coupled kinase assays were performed by combining indicated
MKL immune complexes with either recombinant GST-MKK4 or GST-MKK7 or their kinase negative mutants as indicated in kinase buffer containing 50 μM ATP and 5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amershams Pharmacat Biotech) and incubated at 30 °C for 60 min. Recombinant GST-SAPK and GST-c-Jun(1-69)-Sepharose beads were added and reaction mixes were incubated for an additional 15 min at 30 °C. Reactions were terminated by addition of Laemmli buffer, boiled, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and autoradiographed.

**Affinity Precipitation—FLAG-DLK, FLAG-MLK3, or vector only were expressed and labeled with [35S]methionine in a single reaction reticulocyte lysate-based *in vitro* transcription/translation system according to the directions of the manufacturer (Promega). One-tenth of each reaction mix was incubated for 30 min at room temperature with or without 10 μg of GST-MKK7 and 25 μl of glutathione-agarose in a PBST buffer containing phosphate-buffered 150 mM NaCl (pH 7.4), 1% Triton X-100, and protease inhibitors. Beads were washed five times with PBST then eluted in 5 mM reduced glutathione. Eluates were separated on SDS-PAGE under reducing conditions. Gels were fixed, treated with ENHANCE (DuPont), dried, and autoradiographed.

**Immunocytochemistry—** Adult Sprague-Dawley rats were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline. Nervous tissues were removed, washed in cold PBS, and cryoprotected in sucrose prior to freezing in liquid nitrogen. Spinal cords were cut in 6-μm sections on a cryostat; 40-μm sections were obtained with a sliding microtome of brain and cerebellum. Free-floating sections of brain and cerebellum were used for immunocytochemistry as described (24). Primary antibody was detected with species-appropriate horseradish peroxidase-conjugated anti-IgG secondary antibodies and developed with nickel enhanced 3,3'-diaminobenzidine tetrahydrochloride using the Vector Elite detection kit (Vector Laboratories). Indirect immunofluorescence studies utilized complementary secondary antibodies conjugated to Cy2 or Cy3 (The Jackson Laboratory) as indicated in the legends to Figs. 6–9. In studies of primary cortical neurons in culture, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. After exposure to the primary antibody at 4 °C overnight, the bound antibody was detected using a secondary antibody conjugated to Cy3 or Cy2.

**Subcellular Fractionation Studies**—The preparation of intact nuclei from adult rat cortex and from primary cortical neurons was carried out by isopycnic banding at the 30–35% iodixanol interface, in order to cause the least possible disruption of the nucleoprotein complexes within them, following the manufacturers protocol (OptiPrep).

**Rat Model of Sciatic Nerve Injury—** Adult male Sprague-Dawley rats weighing 175–225 g were anesthetized with chloral hydrate. The sciatic nerve was exposed in the gluteal region and cut. Control rats were anesthetized and the sciatic nerve was exposed but not cut. At 5 min, 30 min, and 1 day after axotomy, animals were perfused in cold PBS, cryoprotected in sucrose, and frozen in liquid nitrogen. Three animals at each time point were employed. A minimum of three sections per spinal cord were analyzed by immunofluorescence.

**RESULTS**

**Distinct from MLK3, DLK Neither Phosphorylates Nor Activates MKK4 in Vitro—** Preliminary experiments demonstrated that catalytically inactive MKK4 attenuated the ability of DLK to activate p46SAPK when overexpressed in co-transfected COS 7 cells (data not shown and Ref. 6). To more closely examine the potential epistatic relationship between DLK and MKK4, the ability of DLK to phosphorylate MKK4 *in vitro* was investigated. Immunoprecipitated overexpressed DLK, MLK3, or their inactive mutants were incubated with catalytically inactive recombinant GST-MKK4-KR in a buffer containing radioactive ATP and magnesium. As reported by others (10, 12), MLK3, but not its inactive mutant, phosphorylated GST-MKK4-KR *in vitro* (Fig. 1A). Contrary to expectation, DLK did not phosphorylate GST-MKK4-KR. In similar experiments, the capacity of immunoprecipitated DLK to phosphorylate in *vitro* catalytically inactive mutants of GST fusion proteins of MKK1, MKK3, or MKK5 was also tested (Fig. 1B). DLK did not phosphorylate recombinant MKK1, MKK3, or MKK5 in *vitro*.

These unexpected results were confirmed by studying the ability of immunoprecipitated DLK to activate MKK4 in an *in vitro* reconstitution experiment. Immunoprecipitated overexpressed DLK, MLK3, or their catalytically inactive mutants were sequentially combined in the presence of ATP and magnesium with recombinant MKK4 (or its kinase negative mutant) and SAPK. SAPK activation was assayed by capture of SAPK onto GST-c-Jun-agarose beads followed by kinase assay in the presence of [γ-32P]ATP. As reported previously, MLK3 but not its kinase negative mutant activated MKK4 and ultimately SAPK (Fig. 2) (10, 12). However, in three repeated experiments, DLK did not activate MKK4 in *vitro*.

**DLK Phosphorylates and Activates MKK7 in Vitro—** By analysis of primary structure, MLK2 and MLK3 form a closely related group structurally distant from DLK. Therefore, it was hypothesized that DLK utilizes substrate or substrates distinct from MKK4. The recently identified MAP kinase kinase MKK7/ JNK2 shown to specifically activate SAPK was considered a potential candidate substrate (15, 25, 26). To test this possibility, *in vitro* kinase assays were repeated as above. Indeed, immunoprecipitated DLK phosphorylated a recombinant catalytically inactive GST-MKK7 fusion protein *in vitro*; immunoprecipitated MLK3 behaved in a similar fashion (Fig. 3A). In control experiments, neither immunoprecipitated catalytically inactive DLK (K185A) nor catalytically inactive MLK3 (K144E) phosphorylated GST-MKK7-KA.

In *vitro* reconstitution coupled kinase assays were used to confirm and extend these observations. As reported previously by others (15, 26), bacterially expressed and purified wild type GST-MKK7 activated rSAPK *in vitro* without known prior activation by a MAPKKK. To confirm that MKK7 catalytic
activity was required for SAPK activation in these experiments, a mutant GST-MKK7 was created in which the invariant lysine in subdomain II (Lys76, known to stabilize ATP in the ATP binding site in other kinases) was replaced with alanine. GST-MKK7(KA) mutant failed to activate rSAPK in vitro. Given these results, we examined whether overexpressed and immunoprecipitated DLK or MLK3 would potentiate the activation of GST-MKK7 in vitro. Immunoprecipitated DLK and MLK3, but not their catalytically inactive mutants, further activated recombinant GST-MKK7 and ultimately SAPK (Fig. 3B). Substitution of the mutant GST-MKK7-KA in reconstituted experiments blocked the ability of immunoprecipitated DLK and MLK3 to activate SAPK.

**DLK Activates MKK7 in Vivo**—Experiments were performed to confirm that DLK activates MKK7 in vivo. COS 7 cells were transiently co-transfected with plasmids encoding FLAG-tagged MKK7 and either Myc-tagged DLK or catalytically inactive Myc-DLK (K185A). Activation of immunoprecipitated MKK7 was evaluated in an in vitro coupled kinase assay using recombinant SAPK and GST-c-Jun (1–79) as substrate (Fig. 4). Expression of DLK but not catalytically inactive K185A resulted in the activation of MKK7. In control experiments, when DLK was co-expressed with catalytically inactive MKK7-KA, immunoprecipitated MKK7-KA failed to phosphorylate rSAPK in vitro. These results demonstrate that overexpressed DLK can activate overexpressed MKK7 in vivo.

**DLK Associates with MKK7 in Vivo and in Vitro**—To examine whether overexpressed DLK interacts in vivo with overexpressed MKK7, myc-tagged DLK was co-expressed with FLAG-tagged MKK7 in COS 7 cells. As shown, FLAG-tagged MKK7 co-immunoprecipitated with myc-tagged DLK (Fig. 5A). To confirm a direct interaction between DLK and MKK7, metabolically labeled DLK expressed in a reticulocyte lysate system was incubated with bacterially expressed and purified GST-MKK7 control. As shown, DLK co-purified in the presence of GST-MKK7 when isolated by glutathione-agarose affinity chromatography (Fig. 5B). MLK3 behaved in a similar fashion. Taken together with the results of the experiments described above, these results provide evidence that MKK7 may serve as a necessary intermediate in a pathway between DLK or MLK3 and SAPK activation in vivo.

**Distribution of DLK, MLK3, and MKK7 Proteins in Normal Adult Rat Nervous System**—To obtain evidence supporting the epistatic relationship between endogenous DLK and MLK3 and their potential substrates in brain, the subcellular localization
of DLK, MLK3, and MKK7 proteins was examined by immunohistochemistry (Fig. 6). Using the previously characterized C1 polyclonal serum generated against a COOH-terminal DLK-GST fusion protein (5), DLK immunoreactivity was detected in neurons of the central nervous system (Fig. 6, A–D). This finding was consistent with previous observations using in situ RNA hybridization in which DLK transcript was widely detected in neurons of the central and peripheral nervous system (16). In pyramidal cells of the cerebral cortex and in Purkinje cells of the cerebellum, strong DLK immunoreactivity was also detected in apical dendrites. MLK3 immunoreactivity could not be distinguished above background in neurons. Instead, MLK3 protein was identified primarily in cells of the periventricular ependyma, choroid plexus, and meninges. (Fig. 6, E and F and not shown). Using a previously characterized MKK7 rabbit antiserum (24), MKK7, like DLK, was identified predominantly in neurons of the central nervous system (Fig. 6, G–J). Whereas DLK and MKK7 immunoreactivity was identified in the same cells of the cerebral cortex and cerebellum, regional differences were detected in the intensity of MKK7 and DLK staining (compare granule cells of the dentate gyrus of hippocampus, Fig. 6, D, I, and J).

Identification of DLK, MKK7, and MKK4 in Cellular Subfractions—Several experiments were undertaken to study the subcellular localization of DLK, MKK7, and MKK4. Normal rat cerebral cortex was disrupted and cells were subfractionated by isopycnic banding. Nuclear, cytosolic, and microsomal fractions were identified by immunoblotting using subfraction specific markers (Fig. 7A). The purity of isolated nuclear preparations was demonstrated as shown in Fig. 7, B and C. Following separation of subfraction lysates on SDS-PAGE and immunoblotting, DLK was identified most abundantly in the microsomal fraction but was also prominent in the cytosol. Like MKK4, MKK7 was particularly enriched in the cytosolic fraction. A small but detectable quantity of DLK and MKK7 were identified within the nuclear fraction (Fig. 7A). Neither antibodies directed against phosphorylated-MKK4 (New England Biolabs) nor phosphorylation-independent antibodies directed against MKK4 (Sigma and Santa Cruz) recognized MKK4 in the nuclear fraction.

Fig. 5. DLK associates with MKK7. A, COS 7 cells were cotransfected with indicated combinations of plasmid encoding Myc-tagged DLK (0.5 μg), FLAG-tagged MKK7 (0.5 μg), FLAG-MKK7-RA (0.5 μg), or empty vector (to 2 μg total). Cell lysates were immunoprecipitated as indicated with anti-Myc (9E10) or anti-FLAG (M2) antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, then immunoblotted as indicated with anti-DLK polyclonal serum (C1) or anti-FLAG (M2) antibody. Twenty μg of whole cell lysate obtained from Myc-DLK and FLAG-MKK7 transfected cells were run in the left lanes and after immunoblotting with the indicated antibodies served as mobility markers. B, DLK and MLK3 or vector-only control were in vitro transcribed and translated in the presence of [35S]methionine. Samples were either mixed or not mixed with GST-MKK7 as indicated. Following incubation with glutathione-agarose, samples were washed and eluted with reduced glutathione. Eluates were separated by SDS-PAGE and visualized by autoradiography.

Fig. 6. Indirect immunohistochemistry demonstrating localization of DLK, MLK3, and MKK7 in normal adult rat nervous system. DLK (A–D) and MKK7 protein (G–J) co-localized within neurons of the central and peripheral nervous system including those of the cerebral cortex (compare A and G) and cerebellum (B, H). MLK3 protein was identified in the ependyma (E, F) but could not be identified in neurons even by immunofluorescence (F). Note DLK and MKK7 immunoreactivity within dendritic processes and accentuated staining in a perinuclear and nuclear distribution (C, H). Within the neurons of the hippocampus, MKK7 immunoreactivity was most prominent within the neurons of the lower leaflet of the dentate gyrus (I, J), whereas DLK staining was more uniformly distributed (compare D and I, J). Purkinje cells of the cerebellum and their apical dendrites stained intensely for DLK and MKK7 (B, H). Note differences in staining intensity between DLK and MKK7 within the granular cell layer (B, H).
To assure that the nuclear localization of DLK and MKK7 suggested by the immunoblotting experiments above was not due to contamination of nuclear fractions with associated microsomal membranes, isolated nuclei from rat cerebral cortex were fixed and analyzed by indirect immunofluorescence. Confocal microscopy demonstrated homogeneous distribution of DLK and MKK7 protein throughout the nucleus (Fig. 7, D, E, and insets). Immunofluorescence and confocal microscopy were used to analyze additional aliquots for the presence of DLK (D) and MKK7 (E). Insets represent reconstructions of confocal images in the z plane. Control experiments lacking primary antibody or using primary antibody preadsorbed with a molar excess of appropriate fusion protein failed to stain isolated nuclei (data not shown).

**Fig. 7.** Localization of DLK, MKK7, and MKK4 in subfractionated cerebral cortex. A, cerebral cortex was homogenized and cells were subfractionated as described under “Materials and Methods.” Twenty-μg aliquots of nuclear (N), microsomal (M), and cytosolic (C) fractions were separated on SDS-PAGE, transferred to nitrocellulose, then immunoblotted as indicated with antibodies directed against DLK, MKK7, MKK4, and phosphorylated MKK4. Fractions were also immunoblotted with antibodies specific for α3 subunit of sodium-potassium ATPase (alpha3), synaptophysin (synapt), and histone 1 to demonstrate suitability of fractionation. In order to demonstrate the purity of the nuclear preparation, an aliquot was stained with DAPI indicating the presence of DNA. Shown is an identical field visualized both by phase (B) and immunofluorescence microscopy (C). Immunofluorescence and confocal microscopy were used to analyze additional aliquots for the presence of DLK (D) and MKK7 (E). Insets represent reconstructions of confocal images in the z plane. Control experiments lacking primary antibody or using primary antibody preadsorbed with a molar excess of appropriate fusion protein failed to stain isolated nuclei (data not shown).

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**DLK Translocates to the Nucleus in an in Vivo Model of Neuronal Injury**—Several components of the various MAP kinase pathways have been demonstrated to translocate within cells following appropriate stimulation. For this reason, the subcellular localization of DLK was also examined by immunofluorescence microscopy in an in vivo model of neuronal injury. Axotomy of the rat sciatic nerve led to translocation of DLK from a predominantly non-nuclear cell compartment to a distinctly nuclear localization within motor neurons of the lumbar spinal cord beginning within 5 min of axonal injury (Fig. 8). Strong nuclear DLK immunoreactivity was maintained at 30 min and at 24 h following injury. Three animals at each time point were examined and a minimum of three sections per spinal cord was evaluated by indirect immunofluorescence for each animal studied. Sham-operated animals were used as controls at each time point studied. In these control animals,
the intensity of DLK immunoreactivity in the nucleus was never observed to be above that observed in the cytoplasm. However, in sections from injured animals, from 2 to 14 cells per section exhibited dramatically increased nuclear localization of DLK. Phosphorylated MKK4 was not identified in the cell nucleus either in control animals or following axotomy at any time point studied (data not shown). However, MKK7, like DLK, accumulated within the nucleus following axotomy.

**DLK Immunoreactivity Co-localizes with That of MKK7 in Neuronal Culture**—To investigate the subcellular localization of DLK, MKK7, and MKK4 proteins in an additional system, embryonic rat cortical neurons in primary culture were examined by immunocytochemistry. DLK immunoreactivity was detected in a prominent perinuclear and intranuclear distribution in neurons (Fig. 9, A, G, I, and K). DLK was also detected within all neuronal processes and was prominently associated with plasma membrane (Fig. 9, A, G, I, K, and Fig. 9A, inset). This was consistent with our published observations that DLK was associated with plasma membrane fractions isolated from synaptosomal preparations (16). Like DLK, MKK7 immunoreactivity was identified in perinuclear and intranuclear compartments as well as in cell processes (Fig. 9, B, H, J, L). Using three distinct commercially obtained antibodies, MKK4 immunoreactivity was detected in neuron cell processes and in a non-nuclear distribution within the neuron cell body that was distinct from that of DLK and MKK7 (Fig. 9, C and D).

The observations that DLK and MKK7 occupied compartments distinct from MKK4 suggested the hypothesis that spatially and functionally distinct SAPK pathways composed of unique components exist within individual cells. To examine this hypothesis further, the subcellular distribution of SAPK/β-JNK3 and SAPKγ-JNK1 were investigated by immunohistochemistry in primary neuronal culture. SAPKβ/γ-JNK3 immunoreactivity was identified exclusively in the neuronal nuclei (Fig. 9F). In contrast, SAPKγ/JNK1 immunoreactivity was extranuclear and localized predominantly to the neuronal processes (Fig. 9E). Therefore, different SAPK species and different MKK species occupy distinct compartments within neurons.

**DISCUSSION**

This paper establishes DLK as a MAPK kinase kinase capable of associating with, phosphorylating, and activating MKK7. That DLK functions in this role in a physiologic setting is supported by observations that endogenous DLK and MKK7 are present in the same compartments within neurons. Moreover, both biochemical evidence and analysis of subcellular localization indicate that DLK does not use MKK4 as an intermediate in SAPK activation.

MAPKKK substrate specificity appears to be defined by the combination of several factors that include (but may not be limited to) the biochemical affinity of the kinase for its substrate, cell type-specific expression, and differential targeting of unique MAPKKKs to specific subcellular compartments. The results presented herein provide an example of each of these determinants. In a fashion reminiscent of the specificity of MKK3 and MKK6 for p38MAPKs (27–29) and of MKK7 for the p46/p54JNKs (15, 25, 26, 30), DLK appears to possess enzymatic specificity for MKK7. Conversely, our results demonstrate that MKK7 may serve as a substrate for several MAPKKKs, including MLK3 and MEKK1. Precedence from the SAPK pathway literature suggests that this is not an unexpected paradox since MKK4 appears to behave as a substrate for multiple MAPKKKs at least in biochemical assays (reviewed in Ref. 2). However, by imposing a number of additional determinants of interaction specificity, nature appears to have restricted interactions between specific kinases and substrates in the SAPK-like pathways.

That DLK and MLK3 are expressed in different cell types in the nervous system provides one example by which interactions between members of the MLK family and specific MAPK kinases may be restricted. Specification of subcellular compartmentation within individual cells may provide a second mechanism determining these interactions. Recognition that various MAPK kinases and MAPK kinase kinases are targeted to distinct subcellular compartments supports this hypothesis. The subcellular localization of endogenous DLK within neurons is complex. In the nerve terminal, endogenous DLK is present both in cytosol and in subcellular fractions enriched in plasma.
membrane (16). In the cell body, DLK occupies compartments that include the plasma membrane, cytosol, and nucleus. Recently, the distinct subcellular compartmentation of other MAPKKKs has also been described. Endogenous MEKK family members occupy distinct subcellular compartments in COS cells including Golgi, post-Golgi, and nuclear compartments (31), and the mixed lineage kinase MLK2 co-localizes with phosphorylated JNK1/2 along microtubules in injected Swiss 3T3 cells (9). Our results show that like the MAPKKKs, endogenous MKK4 and MKK7 also occupy distinct neuronal subcellular compartments.

Our intent in examining the subcellular localization of DLK and its potential MAPK kinase substrates was to provide evidence supporting the biochemical data suggesting that endogenous DLK uses MKK7 and not MKK4 as substrate. Consistent with the biochemical data, the subcellular compartmentation of DLK and MKK7 are similar in neurons; the subcellular compartmentation of MKK4 is distinct from that of DLK and MKK7. Because DLK and MKK7 have overlapping but not identical subcellular compartmentation in neurons, genetic and other experimental approaches will be necessary to further validate the hypothesis that endogenous DLK specifically uses MKK7 as substrate.

SAPK isoforms also appear to be differentially compartmentalized within neurons. Ten splice isoforms derived from three SAPK/JNK genes have been isolated from brain (32). Although in most cases the specific splice isoform has not been studied, SAPK species have been identified associated with several cellular compartments, including cytosol, microtubules (9), and nuclei (13, 33–36). Our observation that endogenous SAPK/JNK1 and SAPK/JNK3 occupy distinct subcellular compartments in the same cell extends these observations. Beyond differences in subcellular compartmentation, limited evidence presently exists to support the hypothesis that discretely localized SAPK species within the same cell have dissociated function. The most direct evidence presently available in this regard derives from the observation that genetic deletion of JNK3, but not deletion of JNK1 or 2, protects mice from hippocampal neuron injury and apoptotic cell death associated with chronic administration of a glutamate receptor agonist (37). That SAPK/JNK3 and SAPK/JNK1 are differentially compartmentalized within individual neurons is consistent with the results of these JNK null-mutant experiments. Together, these observations suggest the existence of spatially and functionally discrete SAPK species within the same cell.

Translocation between subcellular compartments may provide an additional mechanism by which MLK protein kinases gain access to their specific substrates. This report documents that DLK and MKK7 can translocate to the cell nucleus. The mechanism and functional significance of translocation of DLK and MKK7 to the neuronal nucleus following axonal injury will require additional detailed study. Each of these protein kinases is present in neuronal nuclei in vivo under basal conditions, yet each accumulates following axotomy. That nuclear accumulation of DLK occurs rapidly suggests that this kinase translocates from extranuclear pools of pre-existing protein. The nuclear translocation of MAP kinase kinase kinases following a cellular stimulus has not been observed previously, although MEKK1 has been identified in the nucleus of COS cells under basal conditions (7). It is not known whether stimulus-induced nuclear translocation is a response common to other MAPKKK proteins.

Activation of SAPK (particularly SAPK/JNK3) has been implicated in the neuronal response to various types of cellular injury (19, 21, 35). SAPK-activating and -interfering mutant overexpression studies performed in PC12 cells first implicated SAPK in the neuronal apoptotic response to nerve growth factor withdrawal (34). Recent studies demonstrated that SAPK/JNK3 translocates to the nucleus of hippocampal neurons following hypoxic injury in humans (37). Indeed, deletion of SAPK/JNK3 protects mice from hippocampal neuron injury and programmed cell death associated with chronic administration of a glutamate receptor agonist (35). Finally, it was recently reported that sciatic nerve axotomy induces sustained SAPK, c-Jun, and AP-1 activation in lumbar dorsal root ganglion axons (19). Rapid and sustained translocation of DLK corresponds temporally with this activation. Combined with these observations, the results reported herein provide provocative preliminary evidence suggesting that DLK participates in a cellular response to axonal injury mediated by MKK7 and SAPK/JNK3.

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REFERENCES
1. Fanger, G. R., Gerwins, P., Widmann, C., Jarpe, M. B., and Johnson, G. L. (1997) Curr. Opin. Genet. Dev. 7, 67–74
2. Kyriakis, J. M., and Avruch, J. (1996) J. Biol. Chem. 271, 24313–24316
3. Treisman, R. (1996) Curr. Opin. Cell Biol. 8, 205–215
4. Dorow, D. S., Deverreux, L., Dietzsch, E., and De Kreter, T. (1993) Eur. J. Biochem. 213, 701–710
5. Holzman, L. B., Merritt, S. E., and Fan, G. (1994) J. Biol. Chem. 269, 30808–30817
6. Hirai, S. I., Katoch, M., Terada, M., Kyriakis, J. M., Zon, L. I., Rana, A., Avruch, J., and Ohno, S. (1997) J. Biol. Chem. 272, 15167–15173
7. Fan, G., Merritt, S. E., Kortenjann, M., Shaw, P. E., and Holzman, L. B. (1996) J. Biol. Chem. 271, 24788–24793
8. Hirai, S., Izawa, M., Oeada, S., Spyrou, G., and Ohno, S. (1996) Oncogene 12, 641–650
9. Nagata, K. I., Pals, A., Futter, C., Aspenson, P., Schaefer, E., Nakata, T., Hirakawa, N., and Hall, A. (1995) EMBO J. 14, 149–158
10. Rana, A., Gallo, K., Godowski, P., Hirai, S., Ohno, S., Zon, L. I., Kyriakis, J. M., and Avruch, J. (1996) J. Biol. Chem. 271, 19025–19028
11. Sakuma, H., Ikeda, A., Oka, S., Kosztuzsny, Y., Zanetta, J. P., and Kawasaki, T. (1995) J. Biol. Chem. 270, 28622–28629
12. Tibbles, L. A., Ing, Y. L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J. R., and Lassam, N. J. (1996) EMBO J. 15, 7026–7035
13. Cavigelli, M., Dolfi, P., Clarret, F. X., and Karin, M. (1995) EMBO J. 14, 5967–5970
14. Teramoto, H., Kosu, O. A., Miyata, I., Ighishi, T., Miki, T., and Guttkind, J. S. (1995) J. Biol. Chem. 270, 27225–27228
15. Hirai, S., Izawa, M., Oeada, S., Spyrou, G., and Ohno, S. (1996) Oncogene 12, 641–650
16. Chen, Y. R., Meyer, C. F., and Tan, T. H. (1996) J. Biol. Chem. 271, 16883–16896
17. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) J. Biol. Chem. 272, 8288–8295
18. Zheng, C. F., and Guan, K. L. (1993) J. Biol. Chem. 268, 11435–11439
19. Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) J. Biol. Chem. 270, 12665–12669
20. Kenney, A. M., and Kocsis, J. D. (1998) J. Neurosci. 18, 1318–1328
21. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
22. Brewer, G. J., Torricelli, J. R., Evege, E. K., and Price, P. J. (1999) J. Neurosci. Res. 55, 567–576
23. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7337–7342
24. Ensen, H., Raingeaud, J., and Davis, R. J. (1998) J. Biol. Chem. 273, 1741–1748
25. Han, J., Lee, J. D., Jiang, Y. I., Feng, F., and Ulevitch, R. J. (1996) J. Biol. Chem. 271, 2886–2891
26. Raingeaud, J., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1996) EMBO J. 15, 4961–4972
27. Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) EMBO J. 15, 2760–2770
28. Chen, Y. R., Meyer, C. F., and Tan, T. H. (1996) J. Biol. Chem. 271, 631–634
29. Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., Greenberg, M. E., Sawyers, C. L., and Davis, R. J. (1997) Science 271, 693–696
30. Mizukami, Y., Yoshioka, K., Morimoto, S., and Yoshida, K. (1997) J. Biol. Chem. 272, 16657–16662
31. Zhang, Y., Zhou, L., and Miller, C. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2586–2591
32. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rimon, M., Zheng, T. S., Davis, R. J., Rakie, P., and Flavell, R. A. (1997) Nature 389, 865–870