Characterization of Dual Leucine Zipper-bearing Kinase, a Mixed Lineage Kinase Present in Synaptic Terminals Whose Phosphorylation State Is Regulated by Membrane Depolarization via Calcineurin*

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Marina Matać, Steven E. Merritts, Guang Fanš, Geng Geng Yu†, and Lawrence B. Holzman§†

From the †Department of Neurology, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania 15261, ‡Department of Veteran Affairs, Ann Arbor, Michigan 48105, and the §Division of Nephrology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109-0676.

The biochemistry and regulation of dual leucine zipper-bearing kinase (DLK), a member of the mixed lineage kinase or MLK subfamily of protein kinases, was examined in the nervous system. DLK transcript expression in the nervous system was predominantly neuronal. DLK protein was present in synaptic terminals where it was associated with both plasma membrane and cytosol fractions. Within these two fractions, DLK had differing characteristics. Cytosolic DLK existed in both a phosphorylated and dephosphorylated state; DLK associated with plasma membrane existed in the dephosphorylated state only. On nonreducing SDS-polyacrylamide gel electrophoresis, cytosolic DLK migrated at 130 kDa, while membrane associated DLK migrated with an apparent Mr, 260,000. Similarly, DLK transiently expressed in COS 7 cells autophosphorylated in vivo and migrated at approximately 260 kDa when separated by nonreducing SDS-polyacrylamide gel electrophoresis. In cotransfection experiments, FLAG-tagged DLK or a FLAG-tagged truncated DLK mutant (F-Δ520) was coimmunoprecipitated with Myc-tagged DLK and formed complexes under nonreducing conditions consistent with the conclusion that DLK formed covalently associated homodimers in overexpressing COS 7 cells. In aggregating neuronal-glial cultures, depolarization of plasma membrane lead to dephosphorylation of DLK. Treatment of aggregates with 5 nM or 200 nM okadaic acid lead to a shift in electrophoretic mobility consistent with phosphorylation of DLK. Treatment with cyclosporin A, a specific inhibitor of the calcium/calmodulin-dependent protein phosphatase 2B (calcineurin), had no effect on DLK phosphorylation under basal conditions. However, cyclosporin A completely inhibited DLK dephosphorylation upon membrane depolarization.

Depolarization of nerve terminal plasma membrane results in activation of voltage-gated calcium channels, calcium influx, and activation of a complex mechanism that governs and modulates synaptic vesicle trafficking and neurotransmitter release (reviewed in Ref. 1). Phosphorylation-mediated signal transduction has been implicated as critical in these processes (reviewed in Ref. 2). Membrane depolarization is associated with increased phosphorylation of a number of neuronal proteins through several mechanisms including activation of calcium/calmodulin-independent protein kinase II and activation of protein kinase C (reviewed in Ref. 3). In contrast, several nerve terminal proteins, including dynamin I, p145, and p170, exist in a phosphorylated state in resting nerve terminals and become rapidly dephosphorylated following membrane depolarization (4–6).

Evidence that dephosphorylation events are important in synaptic vesicle trafficking derives primarily from studies of dynamin I. By virtue of its homology to the Drosophila gene product shibire, dynamin I has been recognized as important in synaptic vesicle endocytosis (reviewed in Ref. 7). A mutation in shibire leads to endocytic block (8, 9), defective synaptic vesicle reformation (10), and attenuation of neurotransmitter release (11, 12). Dynamin I exhibits intrinsic GTPase activity that is stimulated by several factors (13–15) including phosphorylation by protein kinase C (16). Following membrane depolarization, calcium/calmodulin-dependent protein phosphatase 2B (calcineurin) dephosphorylates dynamin I (6, 16–19) resulting in attenuation of dynamin’s GTPase activity (19). Therefore, dynamin I may serve as a molecular switch activated by calcineurin that is capable of regulating synaptic vesicle endocytosis (7, 19).

Additional work suggests that signal transduction mediated via calcineurin may play a central role in nerve terminal function. Calcineurin represents approximately 1% of total brain protein (20, 21) and is enriched in nerve terminals (22). By either a direct or indirect mechanism, calcineurin is necessary for membrane depolarization-induced dephosphorylation of p145 (6), a Grb2-binding protein of unknown function that colocalizes with dynamin I in the nerve terminal (23). Treatment with specific inhibitors of calcineurin activation results in the augmentation of glutamate release from intact synaptosomes stimulated with the potassium channel blocker 4-aminopyridine, an agent that mimics physiologic stimulation of nerve terminals (6). Moreover, electrophysiological studies have provided evidence that calcineurin may participate in regulating synaptic plasticity by modulating both pre- and postsynaptic processes (24–26). Despite the considerable progress made in these areas, little is known about the molecular nature of the signal transduction pathway(s) through which calcineurin exerts its effect in the neuron.

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†To whom correspondence should be addressed: Division of Nephrology, Dept. of Internal Medicine, University of Michigan Medical School, Rm. 1560, Medical Science Research Building II, Ann Arbor, MI 48109-0676. Tel.: 313-936-4812; Fax: 313-763-0982; E-mail: lbholzman@uv1.im.ed.unich.edu.
The mixed lineage kinase or MLK\(^1\) subfamily of protein kinases are a recently described subfamily of protein kinases that share two common structural features (27, 28). Each has a distinctive kinase catalytic domain whose primary structure is hybrid between those found in serine/threonine and tyrosine protein kinases. Second, closely juxtaposed COOH-terminal to the catalytic domain, each MLK protein has a domain that would be predicted to form two leucine/isoleucine zipper protein-protein interaction domains. Despite the hybrid structure of the catalytic domains, two members of the family have been shown to exhibit serine/threonine-specific kinase autocatalytic activity in vitro (28, 29). Little more has been reported about the biochemistry or functional role of any member of this subfamily of protein kinases.

Dual leucine zipper bearing kinase, or DLK, has been identified as a member of the MLK subfamily (28). A previously reported survey of adult and embryonic mouse tissues demonstrated that DLK transcript expression was most abundant in nervous tissue where it is predominantly found in neurons and in higher concentrations in the peripheral nervous system compared to the central nervous system (30). The localization of DLK in nervous tissue led to the characterization of DLK in brain tissue.

Characterization of DLK in Brain

Characterization of DLK in brain tissue was first preformed in vitro with 25 \(\mu\)M ATP and 10 \(\mu\)Ci of \((^3\text{P})\)ATP (3000 Ci/mmol, Amerham) for 20 min at 30°C. For artificial substrate phosphorylation, complexes were incubated in 50 \(\mu\)M of kinase buffer containing 25 \(\mu\)M ATP, 10 \(\mu\)Ci of \((^3\text{P})\)ATP (3000 Ci/mmol, Amerham), and 2 \(\mu\)g of either \(\beta\)-casein (Sigma), myelin basic protein (MBP) (Sigma), histone 1 (Sigma), bovine serum albumin (Sigma, raytide (Oncogene Science), or poly(glycine) (G)-3.1 (Sigma) for 20 min at 30°C. Reactions were terminated by addition of Laemmli buffer, boiled, resolved by SDS-PAGE, transferred to PVDF membranes, and autoradiographed. For experiments aimed at determining DLK catalytic activity as a function of MgCl\(_2\) and MnCl\(_2\) concentration, reactions were carried out in kinase buffer containing MgCl\(_2\) or MnCl\(_2\) at concentrations varying between 0.5-25 \(\mu\)M. For phosphoaminoacid analysis, phosphoaminoacid standards were visualized by ninhydrin staining, and radioactivity was detected by phosphorimaging (Bio-Rad).

In Situ Hybridization—In situ hybridization was performed on rat and mouse nervous tissue with similar results. Rodents were perfused with 4% paraformaldehyde-buffered saline, and central and peripheral nervous tissue was dissected and cryoprotected with sucrose. Forty to sixty-micron sections of brain, spinal cord, and dorsal root ganglia were used for floating in situ hybridization (31) and four-micron paraffin embedded sections of brain, brain stem, cerebellum, spinal cord, and dorsal root ganglia were used for slide-mounted in situ hybridization performed as described previously (32). Digoxigenin-labeled sense and antisense riboprobes were reverse transcribed according to the protocol of Boehringer Mannheim, and then hybridization was performed in a hybridization buffer with 4 \(\mu\)g of bovine serum albumin, 1 mM EDTA, 50% deionized formamide, and 50% formalin. After hybridization, the sections were washed and then developed with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Vector laboratories) according to the manufacturer's instructions. For Western analysis, immunoprecipitates or protein G-Sepharose were analyzed by SDS-PAGE and transferred to nitrocellulose or PVDF membranes, and autoradiographed as described previously.

The abbreviations used are: MLK, mixed lineage kinase; DLK, dual leucine zipper bearing kinase; PAGE, polyacrylamide gel electrophoresis; CsA, cyclosporin A; CaM, calmodulin; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; GST, glutathione S-transferase; PVD, polyvinylidene difluoride; MBP, myelin basic protein; MES, 2-(N-morpholino)ethanesulfonic acid.

\(^1\) The abbreviations used are: MLK, mixed lineage kinase; DLK, dual leucine zipper bearing kinase; PAGE, polyacrylamide gel electrophoresis; CsA, cyclosporin A; CaM, calmodulin; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; GST, glutathione S-transferase; PVD, polyvinylidene difluoride; MBP, myelin basic protein; MES, 2-(N-morpholino)ethanesulfonic acid.
prepared by sucrose gradient centrifugation as described by Ueda et al. (33) with the following modifications. Adult rat cortex was homogenized in solution A containing 0.3 M sucrose, 1 mM magnesium acetate, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM NaHCO₃, pH 7.4. The brain was centrifuged at 1000 × gₛ for 10 min. The supernatant was collected and centrifuged at 27,000 × gₛ for 15 min, and the pellets obtained were homogenized in solution A. An equal amount of 1.28 M sucrose solution was added to achieve a 0.8 M sucrose suspension which was then centrifuged at 270,000 × gₛ for 45 min. The supernatant and floating myelin bands were carefully removed. The pellet comprising the crude synapticosomal fraction was lysed in 6 mM Tris maleate buffer containing 0.2 mM phenylmethylsulfonyl fluoride, pH 8.3, homogenized, and incubated for 45 min. The lysed synaptosomes underwent further fractionation through a discontinuous gradient of 0.4 M and 0.6 M sucrose solution to obtain the synaptic vesicle and the synaptic membrane fraction. Alternatively, synapticosomal fractions were prepared from rat brain exactly as described by Huttner (34). To assess the association of DLK with membranes, a volume containing 180 µg of total protein of lysed crude synapticosomal fraction was pelleted at 25,000 × gₛ in a TLA-100 rotor (Beckmann) for 20 min. Pellet (LP1) fractions were resuspended in 60 µl of 10 mM HEPES, pH 7.4, or 60 µl of this buffer containing either 0.2 mM EGTA, 0.2 mM KCl, 1.0 mM KCl, or 1% SDS. Additional samples were resuspended in 60 µl of 100 mM Na₂CO₃, pH 11.5. Samples were incubated for 15 min at 4°C, then pelleted at 25,000 × gₛ for 20 min. Pellets were solubilized in Laemmli buffer containing 8 M urea, boiled, and processed for Western analysis. Phase partitioning of LP1 membrane-associated components with Triton X-114 was performed as described by Bordier (35).

Phosphatase Experiments—Immunoprecipitated F-WT complexes obtained as above, or LS2 fractions containing 25 µg of total protein were digested for 1 h at 37°C with 0.5 unit of protein acid phosphatase Sigma) in a buffer containing 20 mM MES, pH 5.5, 0.1 mM MgCl₂, and 0.1 mM dithiothreitol. Reactions were terminated with Laemmli buffer and were processed for Western analysis.

Aggregating Neuronal-Glial Cell Cultures— Cultures were prepared from 15-day rat embryonic telencephalon according to previously published procedures (36, 37). After 33 days in culture, either 20 µM veratridine (Sigma), 5 or 200 µM okadaic acid (Sigma), or 30 µM cycloheximide (gift of Sandoz) was added to the culture media alone or in combination as indicated for 4 h. At the end of the treatment period, cells were homogenized in a buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM NaCl₂, 2 mM EDTA, 2 mM EGTA, 20 µg/ml each antipain, pepstatin, and leupeptin, and 200 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, and 60 mM NaF (all Sigma). Following determination of protein concentrations, 25 µg of total protein per sample was analyzed by Western analysis with C1 anti-DLK antiserum diluted 1:2000 in TBS containing 1% Triton X-100.

RESULTS

DLK Kinase Catalytic Activity—DLK autophosphorylates in vitro on serine and threonine but not tyrosine (28). To confirm the substrate specificity of DLK and to establish an in vitro assay for kinase catalytic activity, DLK was assayed for its ability to phosphorylate a variety of proteins and peptides known to be substrates for other protein kinases. DLK phosphorylated β-casein, histone 1, and myelin basic protein. Bovine serum albumin, raytide, and poly(Arg:Ser) were not substrates for DLK under the specified assay conditions. Phosphoamid acid analysis of β-casein and MBP that had been phosphorylated by DLK in vitro confirmed that phosphorylation occurred on serine and threonine (Fig. 1D). Using β-casein as a substrate, assays were performed to establish the optimal in vitro reaction buffer conditions for immunoprecipitated DLK. DLK catalytic activity was linear over at least 30 min. The kinase demonstrated a broad concentration requirement for MgCl₂ with the greatest enzyme activity occurring at concentrations greater than or equal to 10 mM. MnCl₂ could not substitute for MgCl₂ and did not augment DLK catalytic activity in the in vitro buffer.

Published alignments of multiple serine/threonine protein kinase catalytic domains indicate the presence of an invariant Glu that defines subdomain III. Previously published crystallographic work and structure-function analysis of other protein kinases indicate that this Glu typically participates with an invariant lysine in subdomain II to stabilize ATP in the ATP binding site. In DLK, a single Glu (Glu-192) falls between the invariant lysine in subdomain II (Lys-185) and the conserved sequence of subdomain IV (28). Glu-192 is located in a distinctly unusual location, only 7 residues COOH-terminal to the invariant lysine in subdomain II. To test the functional significance of Lys-185 and Glu-192, Lys → Ala and Glu → Ala point mutations were created. When transfected into COS 7 cells and assayed in vitro, the K185A mutant had undetectable catalytic activity and failed to detectably phosphorylate β-casein (Fig. 1, A and C). However, the E192A mutant possessed catalytic activity equivalent to that of wild type DLK in both assays. Therefore, DLK appears to coordinate ATP in a fashion alternative to prototypical serine/threonine protein kinases.

Localization of DLK in Brain—As previously published (28), DLK transcript is most abundantly expressed in the brain. In situ RNA hybridization using digoxigenin-labeled antisense probe for mouse LDK was performed on brains from 1- to 12-week-old mice.
andsenseriboprobesreverse-transcribedfromDLKcDNAtem-
plates was used to examine DLK transcript localization in the
nervoussystem.AsshowninFig.2,controldigoxigenin-labeled
sense riboprobes showed no detectable hybridization signal.
However, antisense DLK riboprobes showed considerable neu-
ron-specific hybridization within all regions of the central nerv-
ous system examined. To confirm that DLK protein was pres-
ent in the same distribution within the brain, protein extracts
were obtained from gray matter regions including rat brain
cortex, cerebellum, brain stem, spinal cord, and dorsal root

**Fig. 2. In situ RNA hybridization of DLK in nervous system.** a, in situ hybridization using digoxigenin-labeled antisense (A, C, and E) and sense (B, D, and F) riboprobes to DLK on 40-μm cryosections of brain showed considerable hybridization within neurons of the cortical layers (C), dentate gyrus (DG), and pyramidal neurons of the hippocampus (Py). Also labeled were neurons within the basal ganglia, thalamus (not shown), and temporal cortex (TC). There was small hybridization signal within the white matter as seen in the corpus callosum (CC). DLK message was also found within the neurons of the brain stem nuclei (trigeminal nucleus V and facial nucleus VII) (E). Digoxigenin-labeled sense riboprobes showed no detectable hybridization signal (B, D, and F). b, in situ hybridization using digoxigenin-labeled antisense (A, B, D, E, and F) and sense (C) riboprobes on 4-μm paraffin sections showed intense hybridization in the Purkinje cells of the cerebellum (P) and in neurons near the cerebellar peduncles and vestibular nuclei (VN). In the spinal cord (D), intense hybridization was seen within the neurons of the gray matter and especially within the anterior horn neurons (E). A low level hybridization signal was seen within the glial cells of the descending white matter tracts of the spinal cord. F shows DLK transcript in large and small neurons of the dorsal root ganglion. No detectable hybridization was seen when using digoxigenin-labeled sense probes (C).
Characterization of DLK in Brain

**Fig. 3. Subcellular distribution of DLK in synaptosomal preparations.** Crude synaptosomes were prepared from adult rat cortex. Following hypotonic lysis, crude synaptosomes were further fractionated using two methods. A, lysed crude synaptosomes were further fractionated as described (33). Twenty-five μg of protein from lysed synaptosomal fraction (Sy), synaptic vesicle fraction (SV), synaptic plasma membrane fraction (SPM), and myelin were separated by 4–15% gradient SDS-PAGE under reducing conditions and immunoblotted with antibodies against DLK. SV and SPM fractions were also immunoblotted with the indicated synaptic vesicle-specific (choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), synaptophysin) and nerve terminal plasma membrane-specific (Na⁺-K⁺ ATPase, α3 catalytic subunit) markers. B, hypoosmotically lysed crude synaptosomes were fractionated by differential centrifugation according to the method of Huttner et al. (34) into fractions enriched in synaptic plasma membranes (LP1), in synaptic vesicles (LP2), and in cytosol (LS2). Equal aliquots of the indicated subcellular fractions were separated under reducing conditions on 7.5% SDS-PAGE and immunoblotted with the indicated antisera. Relative molecular mass markers are shown at left (×10⁶). Experiments were repeated three times with similar results.

ganglion, as well as from white matter regions including corpus callosum and optic nerve. These were reduced and immunoblotted using the previously characterized DLK polyclonal antisemur (CL (28)). DLK was detected as a 130-kDa immunoreactive band within all regions of the brain examined (data not shown). DLK detected in corpus callosum and optic nerve may derive from axons in passage. In immunoblotting experiments not shown, DLK protein was detected in extracts of neuron-like, rat adrenal chromaffin PC-12 cells in culture but not in mixed glial cell cultures.

**DLK Protein Is Enriched in Synaptic Terminals—**Initial immunohistochemistry experiments using a DLK polyclonal antisemur indicated that DLK protein was localized within neurites in brain sections and in monolayer cell cultures (not shown). To examine this observation more closely, subcellular fractionation of rat cerebral cortex was undertaken using two well characterized methods. Using the method of Ueda et al. (33), DLK was identified in the synaptosomal fraction where it was enriched in the nerve terminal plasma membrane and was minimally detectable in fractions enriched in synaptic vesicles (Fig. 3A). DLK was not detected in the myelin fraction. An alternative subcellular fractionation protocol was employed to confirm these observations (Huttner et al. (34)). Here, DLK was not detectable in the fraction enriched in synaptic vesicles (LP2) and other slowly sedimenting synaptosomal organelles, but was present in the synaptosomal plasma membrane-enriched fraction (LP1) and the synaptosomal cytosolic fraction (LS2) (Fig. 3B).

DLK identified from the cytosolic (LS2) fraction separated as a doublet on SDS-PAGE under reducing conditions. However, DLK isolated from the synaptosomal plasma membrane-enriched fraction (LP1) fraction was identified as a single band with electrophoretic mobility identical to the faster migrating DLK species noted in the cytosol. Additional, more rapidly migrating immunoreactive forms of the protein were consistently detected only in the plasma membrane fraction. In experiments not shown, these more rapidly migrating immunoreactive forms were detected only faintly in whole brain lysates but were enriched in whole synaptosome lysates. Moreover, both the N2 antiserum (that recognizes the NH₂-terminal region of DLK) and the C1 antiserum (that recognizes the COOH-terminal region of DLK) recognized the more rapidly migrating immunoreactive forms. Therefore, these more rapidly migrating forms are unlikely to represent nonspecific, cross-reacting proteins. After digestion with potato acid phosphatase, DLK isolated from the cytosol ran only as the more rapidly migrating species (Fig. 4B). Like other phosphoproteins, the DLK species obtained from the cytosol exhibiting slower electrophoretic mobility represents a phosphorylated form of DLK.

When separated by SDS-PAGE under nonreducing conditions, DLK present within the synaptosome cytosolic fraction (LS2) migrated with an apparent Mr of 130,000; that within the synaptic plasma membrane-enriched fraction (LP1) ran with significantly reduced electrophoretic mobility (broad band in the range of 260–400 kDa) (Fig. 4A).

To examine the characteristics of the association of DLK with the membrane, the LP1 fraction was extracted under the indicated conditions, and the protein remaining with the membrane was separated on SDS-PAGE and immunoblotted (Fig. 5, left). DLK was not extractable by buffers of increased ionic strength, pH 11.5 carbonate buffer, or 1% Triton X-100 (not shown). Since DLK was extractable only in buffers containing 1% SDS and other strong denaturing agents (not shown), it behaved in a fashion similar to intrinsic membrane proteins.

DLK was only minimally extractable from the plasma membrane by 1% Triton X-114 (Bordier (35)) where it partitioned with the aqueous phase (Fig. 5, right). Since DLK does not possess a hydrophobic transmembrane domain and was not significantly extractable by 1% Triton X-114, it may be associated through a disulfide linkage with an integral membrane protein or macromolecular complex.

**DLK Forms Disulfide-linked Complexes When Overexpressed in COS 7 Cells—**To examine the characteristics of DLK in an...
alternative system, DLK was overexpressed in transiently transfected COS 7 cells. Like DLK found in the nerve terminal LP1 fraction, DLK formed a complex that migrated at approximately 260 kDa on nonreducing SDS-PAGE (Fig. 6A). Unlike DLK isolated from synaptosomes, DLK expressed in COS cells ran as a ≥260-kDa complex in both cytosolic and particulate fractions of a 100,000 × g spin on nonreducing SDS-PAGE (data not shown). This complex formation was not dependent on the kinase catalytic activity of DLK since the K185A mutant, a catalytically inactive mutant, when expressed in the same system had similar electrophoretic mobility (Fig. 6B). That DLK ran with an apparent molecular mass on nonreducing SDS-PAGE that was twice that observed on reducing SDS-PAGE suggested that it could form disulfide-linked homodimers in this system.

To test this possibility, expression vectors were constructed that allowed the expression of DLK tagged with either of two different epitope tags, FLAG-tagged wild type DLK and Myc-tagged wild type DLK (Fig. 7A). These were coexpressed in COS 7 cells. As shown in Fig. 7B, cytosolic FLAG-tagged DLK immunoprecipitated with Myc-tagged DLK indicating that, in this system, DLK formed homo-elves. To confirm this observation, FLAG-tagged DLK was truncated by insertion of a stop codon immediately COOH-terminal to the leucine zipper domain (F-Δ520). Note in the schematic diagram (Fig. 7A) that the C1 antisemur should recognize only the wild type DLK and not the truncation mutant whereas a second, N2 antisemur, should recognize both forms of the protein. This was confirmed experimentally (Fig. 7C). As predicted, when the truncation mutant was expressed in COS 7 cells, a 68-kDa product was identified by immunoblotting after reduction and separation by SDS-PAGE. Cell lysates were prepared in which the truncation mutant was coexpressed with the Myc-tagged wild type DLK. These lysates were immunoprecipitated with the C1 antibody. Precipitates were separated on SDS-PAGE, transferred, and Western-blotted with the M2 antibody that recognized only the truncated form of DLK. As demonstrated (Fig. 7C, lane 5), the truncated form of DLK immunoprecipitated with the wild type.

In order to examine whether DLK forms disulfide-linked homodimers in this system, coprecipitated F-Δ520 and M-WT were resolved using nonreducing conditions on SDS-PAGE (Fig. 7D). As seen in lane 4, a 200-kDa band consistent with the dimerization of the 68-kDa truncated form and 130-kDa wild type form was observed. Moreover, in lane 5, the predicted 68-kDa and a 140-kDa form of the mutant was identified when protein extract obtained from COS 7 cells expressing the truncated form of DLK alone was separated by SDS-PAGE under nonreducing conditions. Therefore, in this system, DLK formed disulfide-linked homodimers independent of the DLK domain COOH-terminal to the leucine zipper motifs.

DLK May Autophosphorylate in Vivo—Like DLK found in the nerve terminal cytosol, DLK derived from transiently transfected COS 7 cells overexpressing DLK migrated as a doublet on reducing SDS-PAGE (Fig. 8). To demonstrate that DLK exists in part as a phosphoprotein in this system, immunoprecipitated DLK was digested with potato acid phosphatase. This digestion eliminated the more slowly migrating form within the DLK doublet. DLK catalytic activity is required for phosphorylation in vivo since the catalytically inactive K185A mutant expressed in COS 7 cells migrated with the dephosphorylated form of DLK on SDS-PAGE. This result suggests that DLK autophosphorylates in vivo.

Regulation of DLK Phosphorylation State in Aggregating Neuronal-Glial Cultures—Fetal forebrain aggregating mixed neuronal-glial cultures were used to examine regulation of the phosphorylation state of DLK in nervous tissue (36, 37). Brain aggregates grown in defined media develop multiple synaptic contacts and mature CNS myelin over time. After 33 days in culture, cell aggregates were treated as indicated in Fig. 9. Cell lysates were reduced, separated by SDS-PAGE, and immunoblotted with DLK antiserum. In untreated neuroaggregate cultures, DLK migrated as a doublet with electrophoretic mobility similar to DLK found in the synaptosomal cytosol. To test whether membrane depolarization resulted in an alteration in DLK phosphorylation, aggregating cultures were treated with veratridine, which maintains the sodium channel in the open position and leads to sodium influx and membrane depolarization (38). Treatment with veratridine for 4 h lead to a shift in electrophoretic mobility of DLK consistent with dephosphorylation of DLK upon membrane depolarization. As shown, DLK electrophoretic mobility after membrane depolarization was similar to that seen in preparations of the synaptic plasma membrane fraction (Fig. 9A) and postsynaptic density membrane fractions (data not shown).

Calcium/calmodulin-dependent protein phosphatase 2B (calcineurin) mediates the membrane depolarization-induced dephosphorylation of several cytoskeletal (46) and nerve terminal phosphoproteins, including dynamin and p145 (6, 19). Treatment of aggregating cultures with cyclosporin A (CsA) alone, which when bound to cyclophilin A specifically inhibits the
activity of calcineurin (39), did not have any effect on the electrophoretic mobility of DLK. However, CsA completely inhibited veratridine-induced DLK dephosphorylation in this system. These results suggest that increased intracellular calcium caused by membrane depolarization mediates the dephosphorylation of DLK via a pathway that is dependent upon the activation of calcineurin (Fig. 9B).

**DISCUSSION**

This report characterizes a novel serine/threonine protein kinase that, within the nervous system, is expressed predominantly in neurons and is enriched in synaptic terminals. That DLK plays a role in nerve terminal function is suggested by the
observation that within aggregating neuronal-glial cultures, DLK undergoes dephosphorylation following plasma membrane depolarization. Moreover, like other identified nerve terminal proteins that become dephosphorylated following membrane depolarization, phosphorylation of DLK is blocked by cyclosporin A.

Subcellular compartmentalization appears to be a common cellular mechanism that provides specificity in the regulation and action of protein kinases (reviewed in Ref. 40). Following cell stimulation, several protein kinases have been shown to be translocated to new sites within the cell where they bind to specific anchoring proteins, gain access to their substrates, and affect their physiological functions. DLK is present both in the cytosol and in subcellular fractions enriched in nerve terminal plasma membrane. In each of these compartments, DLK has distinctly different biochemical characteristics. In the synaptic plasma membrane-enriched fraction, DLK is not phosphorylated, forms a high molecular weight complex, and is strongly associated with the membrane. In contrast, in the cytosolic compartment, DLK exists in a phosphorylated form and separates as a monomer on SDS-PAGE run under nonreducing conditions. (DLK may also exist in an unphosphorylated state in the synaptosomal cytosol; however, the unphosphorylated form observed in the synaptic plasma membrane fraction may represent dephosphorylation that occurs during subcellular fractionation.) These differences suggest that DLK is catalytically active in only one compartment and may be associated with a membrane anchoring complex in the synaptic plasma membrane compartment. Further study is required to determine whether DLK translocates between these compartments.

Given their unique structure, it has been hypothesized that MLK proteins form homo- or hetero-oligomers, associated through coiled-coil interactions involving their conserved leucine zipper domains (27-29). Consistent with this hypothesis, DLK expressed in COS 7 cells did form homodimers. The possibility that DLK formed higher order oligomers in this system cannot be excluded especially since the protein appeared to be a component of a high molecular weight complex present in the L1 portion of synaptosomal preparations from brain. Given our expectation that DLK would form oligomers through leucine zipper interactions, we were surprised that DLK formed homodimers that were associated through intermolecular disulfide bonds in the COS cell system, especially within the reducing environment of the cytosol. However, this property allowed the direct demonstration of DLK's ability to form homodimers on SDS-PAGE run under nonreducing conditions. In contrast, DLK isolated from the cytosol of the synaptosomes did not form disulfide-linked oligomers, although non-disulfide-linked oligomerization of DLK within this compartment remains possible. That DLK formed disulfide-linked oligomers may be an unusual artifact. Johnston and Sudhof (41) have described a phenomenon in which closely juxtaposed but non-disulfide-linked oligomers of synaptophysin became oxidized in the presence of detergent and upon reduction formed intermolecular linkages with properly positioned cysteines of juxtaposed polypeptide chains. This possibility was tested in the present system. In contrast to that observed by Johnston and Sudhof (41), preparation of cell lysates without detergent had no effect on disulfide-linked oligomerization.

The observation that DLK's catalytic activity is required for phosphorylation under basal conditions in COS 7 cells suggests that the protein undergoes autophosphorylation in vivo. In DLK, phosphorylation may occur by an intramolecular event, by an intermolecular interaction within a DLK oligomer in a manner similar to activated receptor tyrosine kinases (42), or indirectly by activation of another protein kinase. This result also implies that DLK is catalytically active under basal conditions in the cytosol of nerve terminals, in COS 7 cells overexpression wild type DLK, and in aggregating neuronal-glial culture; in each of these situations, DLK separates as a doublet on SDS-PAGE under reducing conditions. In vitro kinase catalytic assays of DLK immunoprecipitated from unstimulated COS 7 cells support this contention.

The phosphorylation state of DLK is governed by the activity of several serine/threonine protein phosphatases in aggregating neuronal-glial cultures. Experiments in this system are summarized and interpreted as follows. Veratridine blocks the closing of sodium channels, leading to membrane depolarization and to increased intracellular calcium via voltage-gated calcium channels (38). When bound to calcium, calmodulin activates the serine/threonine protein phosphatase calcineurin (43). Cyclosporin A serves as a useful pharmacological inhibitor of calcineurin activity. When complexed with its specific immunophilin, cyclophilin, cyclosporin A inhibits calcineurin activity in a specific fashion (39). DLK existed in both phosphorylated and unphosphorylated forms in untreated neuroaggregate cultures. Treatment of these cultures with veratridine shifted the electrophoretic mobility of DLK, consistent with dephosphorylation of the DLK molecule upon membrane depolarization. Cyclosporin A treatment alone did not have an effect on the electrophoretic mobility of DLK. However, CsA completely blocked veratridine-induced dephosphorylation of DLK. These results suggest that calcium entry caused by membrane depolarization mediated the dephosphorylation of DLK directly by calcineurin or by a pathway dependent upon calcineurin activity.

Several examples of depolarization-induced, calcineurin-mediated protein dephosphorylation events have been recognized in the neuron. In addition to those described in the introduction above, a similar pathway of depolarization-induced, calcineurin-dependent dephosphorylation of neurofilament subunits NF-H and NF-M and the microtubule-associated protein $\tau$ has recently been demonstrated (46).

At rest, serine/threonine protein phosphatase PP2A and possibly PP1 also participate in regulating the phosphorylation state of DLK since treatment of unstimulated neuroaggregate cultures with okadaic acid resulted in increased abundance of phosphorylated DLK. The relative abundance of phosphorylated and unphosphorylated DLK at steady state may be governed directly by the activity of PP1 and PP2A on constitutively auto-phosphorylating DLK. Alternatively, the steady state phosphorylation of DLK may be governed indirectly by incident protein kinase pathways whose activity is modulated by PP1 and/or PP2A.

The possibility that DLK functions in a calcineurin-dependent fashion to regulate nerve terminal function is attractive and merits further study. It will be immediately important to define the rate at which membrane depolarization affects DLK's phosphorylation state both in culture and in synaptosome preparations and to determine whether DLK is a direct substrate for calcineurin. Also critical to understanding the function of DLK in the nerve terminal will be identification of physiological substrates for DLK. When aligned to protein sequence data bases, DLK catalytic subdomains I through VII are most similar to those of the serine/threonine family of mitogen-activated protein kinase kinase kinases including CTR-1,

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2. G. Fan and L. B. Holzman, unpublished observation.
Characterization of DLK in Brain

STE11, and Byr2 (28). Recently, multiple putative Rac and/or Cdc42 binding proteins were identified in a search of GenBank™ using the sequence of a minimal Cdc42 binding domain in a murine p65PAK isoform (44). Several MLK proteins including DLK were identified by this search, and MLK3 was demonstrated to bind weakly to Rac and Cdc42 but not Rho in a GTP-dependent fashion in a filter binding assay. Therefore, DLK may function as an intermediate in a small GTPase-dependent mitogen-activated protein kinase-like pathway such as the stress-activated protein kinase pathway or a still unidentified MAP kinase pathway.

No protein kinase that serves as a substrate of calcineurin has been previously identified in any cell type. Indeed, the only known example of a protein kinase pathway that is influenced by the activity of calcineurin is the stress-activated protein kinase pathway in T-cells (45). The mechanism by which this effect is transduced has not been described. Therefore, the observation that DLK is a component of a calcineurin-regulated signal transduction pathway in neurons may have broad implications for understanding neuronal function as well as calcineurin’s function in other systems.

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