Identification of Structural and Functional Domains in Mixed Lineage Kinase Dual Leucine Zipper-bearing Kinase Required for Complex Formation and Stress-activated Protein Kinase Activation*

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Accumulating evidence suggests that mitogen-activated protein kinase signaling pathways form modular signaling complexes. Because the mixed lineage kinase dual leucine zipper-bearing kinase (DLK) is a large modular protein, structure-function analysis was undertaken to examine the role of DLK domains in macromolecular complex formation. DLK mutants were used to demonstrate that a DLK leucine zipper-leucine zipper interaction is necessary for DLK dimerization and to show that DLK dimerization mediated by the leucine zipper domain is prerequisite for DLK activity and subsequent activation of stress-activated protein kinase (SAPK). Heterologous mixed lineage kinase family members can be co-immunoprecipitated. However, the DLK leucine zipper domain interacted specifically only with the DLK leucine zipper domain; in contrast, DLK NH2-terminal region was sufficient to co-immunoprecipitate leucine zipper kinase and DLK. DLK has been shown to associate with the putative scaffold protein JIP1. This association occurred through the DLK NH2-terminal region and occurred independently of DLK catalytic activity. Although the DLK NH2-terminal region associated directly with JIP-1, this region did not interact directly with either DLK or leucine zipper kinase. Therefore, DLK may interact with heterologous mixed lineage kinase proteins via intermediary proteins. The NH2-terminal region of overexpressed DLK was required for activation of SAPK. These results provide evidence that protein complex formation is required for signal transduction from DLK to SAPK.

Signal transduction via protein kinases generically termed mitogen-activated protein kinases (MAPKs) link a variety of extracellular signals to cellular responses as diverse as proliferation, differentiation, and apoptosis (reviewed in Refs. 1–4).

Among the MAPKs, the stress-activated protein kinases (SAPKs or JNKs) are predominantly activated by cell stress-inducing signals, such as heat shock, ultraviolet irradiation, proinflammatory cytokines, hyperosmolarity, ischemia/reperfusion, and axonal injury. 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 (MAPK kinase (also known as MKK or MEK)) that in turn phosphorylates and activates a MAPK.

Like the prototypical MAPK pathway, the mammalian 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 MAPK kinases (MKK4/SEK1 and MKK7/JNKK2) many MAPKKKs (four mitogen-activated protein kinase/extracellular signal regulated kinase kinase kinases: a serine/threonine MAPK kinase kinase (MAPKKK) many MAPKKKs (four mitogen-activated protein kinase/extracellular signal regulated kinase kinase kinases, ASK1, Tpl2, and TAK1) and multiple additional MAPKKKs 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 incompletely understood.

Distinct MAPK pathways that respond to specific stimuli and effect unique cellular responses may employ subsets of identical protein kinases within the same cell. For these reasons, cells have evolved mechanisms that afford pathway specificity (5). Studies in yeast have provided evidence that MAP kinase pathways are assembled from a unique combination of protein kinases into distinct protein complexes or modules (6, 7). The components of these modules interact via direct protein-protein interactions and/or are tethered to scaffolding proteins (8–12). Importantly, assembly of MAPK modules appears to allow segregation of MAPK signaling components into units that are responsive to independent stimuli and that can regulate functionally distinct substrates.

Identification of JIP-1 as a scaffold protein that interacts in a specific fashion with members of the MLK family of MAPKKKs, with MKK7 but not MKK4, and with JNKs first established that mammalian cells organize SAPK pathways into modules in a fashion similar to yeast (13). Yet little is known about mechanisms that regulate the assembly or activity of these modules.

The five MLKs function as MAPK kinase kinases and share two structural features (14–18). Each has a kinase catalytic domain, the primary structure of which most closely resembles MAPK kinase kinases. Second, closely juxtaposed COOH-terminal to the catalytic domain, each MLK protein has two leucine/isoleucine zippers separated by a short spacer region. Despite these common features, MLK family members are likely to have diverse biological behavior. This is predicted by the comparison of structure that shows that MLKs 1, 2, and 3 form...
one closely related MLK subfamily, whereas DLK and LZK form a second distinct subfamily (15, 19). MLKs 2 and 3 share more than 70% sequence identity within their kinase catalytic and leucine zipper domains. Both possess an Src homology 3 domain in their NH2-terminal region and both have a functional CRIB domain that mediates GTP-dependent association with Rac1 and Cdc42Hs (20, 21). DLK and LZK share kinase catalytic and leucine zipper domains that are more than 90% identical, whereas these domains have only 36% identity to those of MLKs 2 and 3. Moreover, DLK and LZK lack both CRIB motifs and Src homology 3 domains and possess COOH-terminal regions that are structurally distinct from each other and from those of MLKs 2 and 3. These observations suggest that protein-protein interactions unique to each protein kinase may participate in specifying SAPK module assembly and may play a role in regulating the activity and subcellular localization of these modules.

Structure-function analysis of DLK was undertaken to begin to understand the mechanisms that regulate the assembly and activity of DLK-dependent SAPK modules.

MATERIALS AND METHODS

Reagents—Polyclonal antibodies to the COOH-terminal 223 amino acids amino to MLK 250 amino acids of DLK were described previously (22, 23), GST-NH2-terminal DLK fusion protein was prepared as described previously (22). Anti-FLAG epitope monoclonal antibody (M2, Kodak/IBI) and the anti-Myc epitope monoclonal antibody (9E10, Oncogene Science) were obtained commercially. All other reagents used were of the highest purity available.

Eukaryotic Expression Constructs—A schematic diagram of various DLK expression constructs used in this study is shown in Fig. 1. Construction and characterization of mammalian expression constructs encoding FLAG-DLK, Myc-DLK, FLAG-DLK (K185A), and FLAG-DLK (1–520) were described previously (23). The pCDNA3 expression construct expressing Myc-epitope tagged JIP-1 was a gift of Dr. Benjamin Margolis (30). Point mutations to create pFLAG-DLK-PP (L437P/L463P) were introduced in pFLAG-DLK using mutant synthetic oligonucleotides and sequential PCR steps as described (22). Synthetic oligonucleotides used included: 1) the FLAG construct sense primer (28), 2) a wild type antisense oligonucleotide 5'-GACAGGGCGGCCGCTCT-3', 3) 5'-GACATCCTTGAGGGCGCTCT-3', and 4) 5'-TGCCTGGTC-3' for the antisense and sense K185A mutagenesis oligonucleotides, respectively, and 5) 5'-ATCCGACACTGGCTACATA-3' and 6) 5'-TCCTCAAGGCGACTGACAT-3' for the antisense and sense E192A mutagenesis oligonucleotides, respectively. HindIII-EcoRI fragments of the resultant amplification products were subcloned into the HindIII-EcoRI prepared pFLAG-DLK plasmid. The mammalian expression constructs pFLAG-DLK (405–520) and pMyc-DLK (405–520) were generated by PCR using pCDNA3-FLAG-DLK (1–520) as template, sense primer 5'-ataaagcttccagaggccatggactacaaggacgacgatgaca-3' and 5'-ataaagcttccagaggccatggagcagaag-3' for the antisense and sense in vivo methods. pCDNA3-FLAG-DLK (1–520) were generated by PCR using pCDNA3-FLAG-DLK (1–520) as template and the sense primer 5'-ataaagcttccagaggccatggactacaaggacgacgatgaca-3' and 5'-ataaagcttccagaggccatggagcagaag-3' for the antisense and sense E192A mutagenesis oligonucleotides, respectively. HindIII-EcoRI fragments of the resultant amplification products were subcloned into the HindIII-EcoRI prepared pFLAG-DLK plasmid. The mammalian expression constructs pFLAG-DLK (405–520) and pMyc-DLK (405–520) were generated by PCR using pCDNA3-FLAG-DLK (1–520) as template, sense primer 5'-ataaagcttccagaggccatggactacaaggacgacgatgaca-3' and 5'-ataaagcttccagaggccatggagcagaag-3' for the antisense and sense in vivo methods.

RESULTS

Leucine Zipper Are Necessary for DLK Homodimerization—We have previously shown that DLK can form disulfide-linked homodimers (22). To determine whether the leucine zipper domain present in DLK is necessary for oligomerization of the DLK peptide, expression constructs encoding a series of DLK truncation and point mutants were created (Fig. 1). A plasmid encoding a Myc epitope-tagged DLK leucine zipper domain (m-LZ) was co-expressed by transient transfection with 3* L. B. Holzman, manuscript in preparation.

3* L. B. Holzman and Kovari, I., unpublished data.


**DLK Complex Formation**

**Fig. 2. Leucine zipper domain is sufficient for the oligomerization of DLK.** The plasmid encoding m-LZ was transiently cotransfected into 293T cells with plasmids encoding indicated DLK constructs. Immunoblots of cell lysates from indicated experiments (shown in left panels of A–C) demonstrate expression of DLK constructs. W.B., Western blot; I.P., immunoprecipitation; m, Myc; F, FLAG. Immunoprecipitations from corresponding cell lysates were performed using anti-Myc antibody (Ab) (9E10). Immunoprecipitates were separated by SDS-PAGE and immunoblotted as indicated using either COOH-terminal DLK antibody (A, right panel) or anti-FLAG antibody (B and C, right panels). D, immunoblot demonstrating expression of Myc-leucine zipper construct (m-LZ) in each cell lysate.

F-DLK, F-DLK(1–520), F-DLK(1–418), DLK(521–888), and F-DLK(405–520) in 293T cells. As shown in Fig. 2, m-LZ co-immunoprecipitated F-DLK, F-DLK(1–520), and F-DLK(405–520). However, m-LZ did not co-immunoprecipitate DLK mutants lacking the leucine zipper domain (DLK(521–888) or F-DLK(1–418); Fig. 2, A and B). That m-LZ did not co-immunoprecipitate DLK mutants lacking the leucine zipper domain suggested that the LZ was necessary for DLK oligomer formation. This possibility was examined further by creating a FLAG-tagged DLK point mutant in which the α-helical structure of the two DLK leucine zipper motifs was disrupted. Leucine residues at positions 437 and 463 were replaced by proline residues to create F-DLK-PP. m-LZ was co-expressed with wild type F-DLK or F-DLK-PP in 293T cells. m-LZ co-immunoprecipitated wild type F-DLK but not F-DLK-PP (Fig. 2A).

To confirm a direct interaction between the DLK leucine zipper domain and itself, DLK leucine zipper domain (m-LZ) was co-expressed with F-DLK, F-DLK-PP or F-LZ in a reticulocyte lysate system. As shown, the Myc-tagged leucine zipper domain of DLK co-immunoprecipitated F-LZ, wild type F-DLK, but not F-DLK-PP (Fig. 3). Collectively, these results provide evidence that the DLK leucine zipper-leucine zipper interaction is necessary for DLK dimerization.

Leucine Zippers Are Necessary for DLK Autophosphorylation and SAPK/JNK Activation—Overexpression of wild type DLK by transient transfection results in DLK autophosphorylation and SAPK/JNK activation (17, 22). We sought to examine the hypothesis that DLK homodimerization is necessary for these activities. Myc-p46SAPK was co-expressed by transient transfection with F-DLK, F-K185A, F-DLK(1–520), F-DLK(1–418), or F-DLK-PP in 293T cells. Following immunoprecipitation of DLK or its mutants, autophosphorylation was assessed *in vitro* after incubation in a kinase buffer containing [³²P]ATP. As shown in Fig. 4, only F-DLK and F-DLK(1–520) became phosphorylated under these conditions. DLK mutants that lacked the leucine zipper domain or that possessed disrupted α-helices failed to become phosphorylated. To examine the capacity of the various overexpressed DLK mutants to activate SAPK/JNK in this system, Myc-p46SAPK was co-expressed by transient transfection with F-DLK, F-K185A, F-DLK(1–520), F-DLK(1–418), or F-DLK-PP in 293T cells. Following immunoprecipitation of DLK or its mutants, autophosphorylation was assessed *in vitro* after incubation in a kinase buffer containing [³²P]ATP. As shown in Fig. 4, only F-DLK and F-DLK(1–520) became phosphorylated under these conditions. DLK mutants that lacked the leucine zipper domain or that possessed disrupted α-helices failed to become phosphorylated. To examine the capacity of the various overexpressed DLK mutants to activate SAPK/JNK in this system, Myc-p46SAPK was co-expressed by transient transfection with F-DLK, F-K185A, F-DLK(1–520), F-DLK(1–418), or F-DLK-PP in 293T cells. Following immunoprecipitation of DLK or its mutants, autophosphorylation was assessed *in vitro* after incubation in a kinase buffer containing [³²P]ATP. As shown in Fig. 4, only F-DLK and F-DLK(1–520) became phosphorylated under these conditions. DLK mutants that lacked the leucine zipper domain or that possessed disrupted α-helices failed to become phosphorylated.

**Fig. 3.** DLK leucine zipper directly interacts with itself. Myc-LZ and the indicated DLK constructs were co-expressed in the presence of [³²P]methionine in an *in vitro* coupled transcription-translation reticulocyte lysate system. A, expression of indicated constructs was demonstrated by autoradiography after lysates were separated by SDS-PAGE (left panel). I.P., immunoprecipitation. To examine potential association of expressed proteins, the indicated lysates were immunoprecipitated using anti-FLAG antibody, separated by SDS-PAGE, and autoradiographed (right panel). B, left panel, expression of indicated constructs in lysates after separation by SDS-PAGE and autoradiography. W.B., Western blot. As shown in the right panel, the corresponding lysates were immunoprecipitated with anti-Myc antibody, separated by SDS-PAGE, and immunoblotted with anti-FLAG antibody.
kinase buffer in the presence of [32P]ATP, and then analyzed by appropriate anti-FLAG or COOH-terminal DLK antibodies, incubated in constructs. A, DLK or its mutants were immunoprecipitated with appropriate anti-FLAG or COOH-terminal DLK antibodies, incubated in kinase buffer in the presence of [32P]ATP, and then analyzed by autoradiography after separation by SDS-PAGE. B, p46SAPK was immunoprecipitated from the same cell lysate and analyzed for catalytic activity. C, cell lysates from corresponding experiments were immunoblotted using anti-Myc antibody to demonstrate equivalent expression of p46SAPK. D, a combination of anti-FLAG and anti-C-terminal DLK antibody was used to demonstrate the expression of the various DLK constructs.

Ref. 24) and that MLK3 can co-immunoprecipitate co-expressed MLK2 (26). In a similar experiment, F-DLK was co-expressed with HA-tagged LZK by transient transfection in 293T cells. F-DLK co-immunoprecipitated HA-LZK (see Fig. 7B). Therefore, overexpressed DLK can form complexes with either MLK3 or LZK despite their structurally distinct leucine zipper domains.

To examine whether heterogeneous MLK complex formation is mediated via a leucine zipper-leucine zipper interaction, Myc-tagged DLK leucine zipper domain (m-LZ) was co-expressed with F-MLK3, HA-LZK, or F-JIP1 in 293T cells (Fig. 5). m-LZ was immunoprecipitated from each set of cell lysates, and immunoprecipitates were analyzed for co-immunoprecipitation of MLK3, LZK, or JIP1. Although m-LZ co-immunoprecipitated F-DLK, the DLK leucine zipper domain did not co-immunoprecipitate MLK3, LZK, or JIP1. This result indicates that the DLK leucine zipper domain interacts specifically only with the DLK leucine zipper and not with leucine zipper domains of other structurally similar MLK proteins. Moreover, these results suggest that the interactions of DLK with heterologous MLK protein kinases may be mediated via other DLK domains.

The observations that the leucine zipper domain of DLK interacted specifically with DLK and was necessary for SAPK activation suggested that co-expression of the DLK leucine zipper domain with various MLK proteins would result in selective attenuation of SAPK activation induced by overexpression of the various MLK proteins. To test this prediction, plasmids encoding SAPK, MLK proteins (including DLK, MLK3, and LZK), and variable amounts of DLK leucine zipper domain were co-expressed in 293T cells. SAPK was immunoprecipitated from each cell lysate and assayed for catalytic activity. DLK-induced SAPK activation was attenuated in a fashion directly proportionate to the quantity of DLK leucine zipper domain expressed (Fig. 6, top panel). However, MLK3-mediated SAPK activation was unaffected by co-expression of even a relatively large quantity of DLK leucine zipper domain (Fig. 6, middle panel). Co-expression of DLK leucine zipper domain did attenuate LZK-mediated SAPK activation, but attenuation was observed only when competed with a relatively large quantity of DLK leucine zipper domain (Fig. 6, bottom panel). Together with the results of experiments described above, these observations suggest the DLK leucine zipper domain has a high affinity for itself, a significantly lower affinity for the LZK leucine zipper domain, and no significant affinity for the MLK3 leucine zipper domain.

**NH2-Terminal Region of DLK Mediates Co-immunoprecipitation of LZK, DLK, and JIP1**—A series of co-immunoprecipitation experiments with DLK deletion mutants was undertaken to identify DLK domains capable of forming complexes with LZK and JIP1. Wild type HA-tagged LZK was co-expressed by transient transfection with F-DLK, F-DLK (1–520), F-DLK (1–418), or F-DLK (250–418) (Fig. 7B). Overexpressed LZK co-immunoprecipitated with F-DLK, F-DLK (1–520), and F-DLK (1–418), but not F-DLK (250–418). This observation confirmed that the DLK leucine zipper domain was not necessary for LZK-DLK association in this system and suggested that the NH2-terminal region of DLK mediated LZK-DLK co-immunoprecipitation. To corroborate this result, HA-LZK was co-expressed with F-DLK or a mutant lacking the entire DLK region NH2-terminal to its catalytic domain (F-DLK (135–888)) (Fig. 7B). As expected, overexpressed LZK co-immunoprecipitated wild type DLK but not F-DLK (135–888).

Given the previous results, we also considered the possibility that DLK might associate with additional DLK peptides via its NH2-terminal region. In similar co-immunoprecipitation experiments, Myc-tagged DLK was co-expressed in 293T cells with F-DLK (1–520), and F-DLK (1–418), F-DLK (250–418), or DLK (135–888) (Fig. 7C). Wild type DLK was co-immunoprecipitated with F-DLK (1–520) and F-DLK (1–418) but not F-DLK (250–418) or DLK (135–888). Therefore, in a fashion similar to LZK, overexpressed DLK can be co-immunoprecipitated with DLK via its NH2-terminal region. Of note, this...
co-immunoprecipitation occurred independently of the DLK leucine zipper domain.

DLK and MLK3 have been shown to directly associate with JIP1 (13). Additional co-immunoprecipitation experiments were carried out using DLK deletion mutants to locate the JIP1 binding region within DLK. Following overexpression in 293T cells, Myc-tagged JIP1 could be co-immunoprecipitated with F-DLK, F-DLK (1–520), and catalytically inactive DLK, F-K185A (Fig. 7A). However, JIP1 could not be co-immunoprecipitated with DLK (521–888) or F-DLK (135–888). These results imply that the DLK NH2-terminal domain is necessary for the association of DLK and JIP1. Importantly, the association of JIP1 and DLK occurs independently of DLK catalytic activity.

NH2 Terminus of DLK Binds Directly to JIP-1 but Not to DLK or LZK—We next examined whether the association of the NH2-terminal region of DLK with LZK, DLK, and JIP1 occurs via a direct interaction. Metabolically labeled LZK, DLK, and JIP1 expressed in a reticulocyte lysate system were incubated individually with bacterially expressed and purified

Fig. 6. Leucine zipper domain of DLK specifically inhibits DLK activation of SAPK. Plasmids encoding Myc-SAPK (0.4 μg), indicated FLAG-tagged MLKs (0.4 μg), indicated quantity of FLAG-tagged DLK leucine zipper domain (DLK (405–520)), and vector (to 2 μg) were transiently cotransfected into 293T cells. W.B., Western blot; Ab, antibody. SAPK was immunoprecipitated from the cell lysate and analyzed for catalytic activity in vitro using GST-c-Jun as substrate. Immunoblots from corresponding experiments were used to evaluate the relative expression of SAPK and indicated MLK proteins. Also indicated (% stim) are the levels of SAPK activity relative to that seen in cells coexpressing MLK and SAPK only.

Fig. 7. NH2-terminal region of DLK participates in binding of DLK to JIP1, LZK, and DLK. 293T cells were co-transfected with plasmids encoding the indicated proteins or their mutants. In each set of experiments, cell lysates were immunoprecipitated with the indicated antibody. Immunoprecipitates were separated by SDS-PAGE and then immunoblotted with the antibody indicated in each panel. In each experiment, expression of the various expression constructs was assessed with immunoblots of corresponding cell lysates. A, DLK NH2-terminal domain is required for co-immunoprecipitation of LZK. W.B., Western blot; I.P., immunoprecipitation; Ab, antibody. B, DLK leucine zipper domain is not required for co-immunoprecipitation of DLK. The DLK NH2-terminal domain co-immunoprecipitated DLK. C, DLK NH2-terminal domain is required for co-immunoprecipitation of JIP-1.
directly with either DLK or LZK despite the observation that co-expressed with Myc-tagged p46 SAPK in 293T cells (Fig. 4). The activation of immunoprecipitated Myc-p46SAPK was evaluated in mammalian cells.

**DLK NH2-terminal Domain Is Necessary for SAPK/JNK Activation**—To test the functional necessity of the DLK NH2-terminal domain in SAPK/JNK activation, FLAG-tagged DLK deleted of the NH2-terminal region (F-DLK (135–888)) was co-expressed with Myc-tagged p46SAPK in 293T cells (Fig. 4). Activation of immunoprecipitated Myc-p46SAPK was evaluated in a kinase assay using recombinant GST-c-Jun (1–79) as substrate. Unlike overexpressed wild type DLK, which activated SAPK, F-DLK (135–888) did not activate SAPK above control. These results suggest that the NH2-terminal region of overexpressed DLK is required for activation of SAPK. As a means of testing whether the catalytic activity of F-DLK (135–888) was preserved, this mutant was overexpressed, immunoprecipitated, and assayed for its ability to phosphorylate a nonspecific substrate in vitro (Fig. 9). Although one cannot exclude the possibility that the immunoprecipitate containing F-DLK (135–888) also possesses kinase catalytic activity not associated with F-DLK (135–888), immunoprecipitate containing F-DLK (135–888) appeared to retain catalytic activity above that containing the catalytically inactive DLK(K185A).

**DISCUSSION**

The results presented herein provide preliminary insight into the mechanism governing DLK activation. Our previously published work demonstrated that overexpressed DLK forms covalently linked homodimers (22). As reported herein, homodimerization of DLK is mediated by a direct interaction requiring its leucine zipper domain. Leucine zipper domain dimerization is prerequisite for DLK phosphorylation and subsequent activation of SAPK. These observations support the hypothesis that—in a fashion similar to the MAPKKK Raf (27, 28)—generic receptor tyrosine kinases (29)—mixed lineage kinase dimerization results in subsequent transphosphorylation and MLK activation (15, 26). In support of this model, co-expression of the isolated DLK leucine zipper domain effect- ively attenuates the ability of DLK to activate SAPK activity, presumably by competitively inhibiting DLK dimerization and subsequent DLK transphosphorylation and activation. However, the mechanism governing DLK dimerization remains undetermined. DLK dimerization does not require DLK phosphorylation or catalytic activity because a catalytically inactive and unphosphorylated form of DLK (K185A) forms homodimers (22). Further, the isolated DLK leucine zipper domain readily associates with itself and appears to associate with any truncated form of DLK as long as these mutants (functional or not) contain the leucine zipper domain. Collectively, these observations suggest that the DLK leucine zipper domain-leucine zipper domain interaction is of high affinity and that unchecked, DLK seeks its most favorable configuration by forming stable homodimers. Presuming that DLK homodimerization precedes DLK activation, these observations suggest that a mechanism must exist that regulates the assembly of DLK homodimers. The nature of this mechanism requires further investigation.

It has been speculated that mixed lineage kinase leucine zipper motifs also mediate formation of hetero-oligomeric complexes between members of the mixed lineage kinase family (15, 25, 26). Formation of these hetero-oligomers was predicted based principally on the observation of strong structural similarity among the leucine zipper domains of the MLK family members, particularly between members of the subfamily comprising MLKs 1, 2, and 3 and between members of the DLK/ Lzk subfamily. Indeed, as noted herein, co-immunoprecipitation experiments demonstrate that overexpressed DLK can be co-immunoprecipitated with overexpressed LZK and with the structurally more distant MLK3. Others have noted that overexpressed MLK3 can be co-immunoprecipitated with overexpressed LZK and with the structurally more distant MLK2 (26). Contrary to expectation, our experiments indicate that the leucine zipper domain of DLK interacts exclusively with that of DLK and not with the leucine zipper domain of the structurally similar LZK or the more distant MLK3. Therefore, the unique DLK-DLK leucine zipper domain interaction—and more generally, unique DLK-MLK leucine zipper domain interactions—may provide an important determinant of signaling specificity by limiting functional interactions between proximally localized heterologous MLK peptides. That DLK leucine zipper domains interact to form only homooligomers suggests that the interactions detected by co-immunoprecipitation of DLK with heterologous MLK protein kinases must be mediated by another DLK domain. Indeed, co-immunoprecipitation of DLK with both DLK and heterologous MLK proteins occurred independently of the leucine zipper domain of DLK and was mediated by the NH2-terminal region of DLK. These interactions appeared to occur via an undefined intermediary protein because a direct interaction between the NH2-terminal domain of DLK and DLK or LZK.
could not be demonstrated. As discussed below, because the scaffold protein JIP-1 does associate directly with the NH₂-terminal region of DLK, it is possible that JIP-1 serves as this intermediary. However, it is also possible that a yet unidentified protein serves in this capacity. If JIP-1 serves as the intermediary protein involved in these interactions, then JIP-1 should possess at least two MLK binding domains or should homodimerize. In preliminary data supporting the later possibility, overexpressed Myc- and FLAG-tagged JIP-1 do co-immunoprecipitate. The functional significance of indirect interaction between the NH₂-terminal region of DLK and other MLK proteins has been suggested by experiments in which overexpressed catalytically inactive DLK (K185A) attenuated overexpressed MLK3-induced SAPK activation (24). The true physiological relevance of these observations remains unclear.

Emerging evidence suggests that mammalian SAPK pathway components may be organized into signaling complexes or modules. As suggested by our understanding of MAPK signaling in yeast, these complexes may be specified by direct protein-protein interactions between pathway components and/or by assembly of signaling molecules on scaffold proteins (6–12). The recent identification of JIP-1—a scaffold protein that interacts in a specific fashion with members of the MLK family of MAPKKKs, with MKK7 but not MKK4, and with JNKs—has focused attention on complex formation mediated by a scaffold protein as one determinant of SAPK pathway signaling specificity (13). The results reported herein confirm the direct association of DLK with JIP-1 and map the JIP-1 binding domain in DLK to the region NH₂-terminal to its catalytic domain. Importantly, the DLK NH₂-terminal region is required for SAPK activation induced by DLK overexpression. This result suggests that protein-protein interactions mediated through the NH₂-terminal domain of DLK (and independent of DLK dimerization via its leucine zipper domain) are required for DLK-mediated SAPK activation. It remains to be determined whether disrupting DLK-JIP-1 interaction specifically is sufficient to interrupt signaling to SAPK because, as discussed above, the DLK-NH₂-terminal region may participate in relevant protein-protein interactions independent of JIP-1. However, these results are consistent with the demonstration by Whitmarsh et al. (13) that co-expression of JIP-1 with MLK3 resulted in augmentation of overexpressed MLK3-induced SAPK activation and support the hypothesis that JIP-1-mediated SAPK module formation is necessary for DLK-dependent activation of SAPK.

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