Dimerization via Tandem Leucine Zippers Is Essential for the Activation of the Mitogen-activated Protein Kinase Kinase Kinase, MLK-3*

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Mixed lineage kinase-3 (MLK-3) is a mitogen-activated kinase kinase kinase that mediates stress-activating protein kinase (SAPK)/c-Jun NH2-terminal kinase activation. MLK-3 and other MLK family kinases are characterized by the presence of multiple protein-protein interaction domains including a tandem leucine/isoleucine zipper (LZs) motif. Leucine zippers are known to mediate protein dimerization raising the possibility that the tandem leucine/isoleucine zippers may function as a dimerization motif of MLK-3. Using both co-immunoprecipitation and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we demonstrated that MLK-3 forms disulfide bridged homo-dimers and that the LZs motif is sufficient for MLK-3 homodimerization. We next asked whether MLK-3 utilizes a dimerization-based activation mechanism analogous to that of receptor tyrosine kinases. We found that dimerization via the LZs motif is a prerequisite for MLK-3 autophosphorylation. We then demonstrated that co-expression of Cdc42 lead to a substantial increase in MLK-3 dimerization, indicating that binding by this GTPase may induce MLK-3 dimerization. Moreover, the LZs minus form of MLK-3 failed to activate the downstream target SAPK, and expression of a MLK-3 LZs polypeptide was found to block SAPK activation by wild type MLK-3. Taken together, these findings indicate that dimerization plays a pivotal role in MLK-3 activation.

The mixed lineage kinase (MLK) family comprises a group of highly related serine/threonine kinases that function as MAPKKKs (1–9). Several members of this kinase family have been found to activate the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) stress-signaling pathway (via SEK1) and at least two MLK kinases also up-regulate the p38/activation kinase stress-signaling pathway. MLKs are in turn activated by a number of upstream agonists. For example, MLK-3 has been found to mediate signals from the Ste20 homologues germinal center kinase, and hematopoietic kinase, the guanine-nucleotide exchange protein C3G, and is a target of the small GTPases Cdc42 and Rac (1, 2, 4, 7). Although it is clear that the MLKs play important roles in transmitting a variety of different signals to the stress-signaling pathway, the mechanisms that directly regulate MLK activity are not well understood.

The MLK family consists of two subgroups: the highly related MLK-1 (10), MLK-2/MST (11, 12), and MLK-3/SPRK/PTK (13–15) and the DLK/MUK/ZPK (9, 16–18) and LZK (19) subfamily. MLK-2 and MLK-3 bear a number of protein-protein interaction domains including an NH2-terminal SH3 domain, a basic region, a Cdc42 and Rac interactive binding (CRIB) (20) region, and a COOH-terminal proline-rich region (see Fig. 1). MLK-1 likely bears the same array of motifs, but to date, only a partial amino acid sequence has been reported for this kinase (10). DLK/MUK/ZPK and LZK display somewhat more homology to each other than to MLK-1, MLK-2, and MLK-3 but share the characteristic feature of this group of kinases, namely, two leucine zippers carboxyl-terminal to the catalytic domain. The tandem LZs of MLK-1, MLK-2, and MLK-3 are 68% identical and are separated by a highly conserved spacer region of 13 amino acids (this region is designated as the “leucine zipper-spacer-leucine zipper” region). The LZs of DLK/MUK/ZPK and LZK show even greater homology and are separated by a larger spacer region of 31 amino acids. The fact that all MLK family members bear highly related LZs implies a common role for these motifs in the functioning of these kinases.

Leucine zippers are generally defined as a row of 4–7 heptad leucine repeats, although in some cases, the canonical leucine residues are replaced by isoleucine or valine (as in the case of MLK-3) (21). X-ray diffraction and NMR spectroscopy studies have demonstrated that leucine zippers form parallel coiled-coil α-helices, which wrap around one another via the hydrophobic interaction of the leucine side chains (22, 23). Leucine zipper motifs were initially identified in transcription factors and shown to mediate homo- or heterodimerization and play a role in DNA binding (21). Leucine zipper motifs have been found subsequently in a wide array of proteins and found to play pivotal roles in regulating the activity of these proteins (24–27). However, interactions between leucine zippers are relatively restricted; each leucine zipper binds only one or at most a few such partners (21). Secondary structure analyses of the leucine zipper-spacer-leucine zipper region within MLK-3 indicates that this domain forms a “α-helix-loop-α-helix” structure (10), which appears to be a hybrid of leucine zipper and helix-loop-helix motifs (10, 28). Because both leucine zippers and helix-loop-helix motifs have been found to mediate dimerization of some transcription factors, we first asked whether the MLK-3 leucine zipper domain functioned as a dimerization motif. We found that MLK-3 does, in fact, form homodimers via the tandem leucine/isoleu-
To address whether the activation of MLK-3 is mediated via an analogous dimerization-based mechanism. We show that a constitutively active form of Cdc42 promotes MLK-3 homodimerization. We initially employed a co-immunoprecipitation approach. First, a 3'-9 and 3'-EcoRI sequences flanking both sides of the tandem leucine zipper region and the sequences encoding both the catalytic domain (amino acids 124–366), two leucine/isoleucine zippers (amino acids 403–462); MLK-3 lacking the tandem leucine zipper region (amino acids 403–462); MLK-3D lacking the amino acids 591–849; the MLK-3LZs expression construct encodes only the leucine zipper region of MLK-3 (amino acids 397–471).

**Fig. 1. A** schematic of MLK-3 protein interaction domains. Beginning at the amino terminus, MLK-3 protein interaction domains include an SH3 domain (amino acids 48–100), which is followed by the catalytic domain (amino acids 124–366), two leucine/isoleucine zippers (amino acids 403–459), a basic region, a Cdc42 and Rac interactive binding (CRIB) motif (amino acids 490–507), and a proline-rich region (amino acids 663–820). B, expression constructs used in this study. MLK-3LZs expression construct contains an MLK-3 cDNA sequence lacking the tandem leucine zipper region (amino acids 403–462); MLK-3LZs was generated using a polymerase chain reaction using primers that flank the MLK-3 vector. The construct was then digested with Pml 1 and BamHI and subcloned into the EcoRI and BamHI restriction sites amino-terminal to the Flag DNA sequence of the pCMV5b Flag vector. The construct was then digested with Pml 1 and BamHI, the BamHI site was “filled-in” using Klenow fragment, re-ligated, and sequenced.

**Transient Transfections**—All transfection assays were carried out using LipofectAMINE (Life Technologies, Inc.) and 293 cells. 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 250 ng/ml fumonizone (Bristol-Myers Squibb, Montreal, QC), 200 units/ml penicillin, and 100 units/ml streptomycin (ICN Pharmaceuticals, Costa Mesa, CA). In brief, 100-mm culture dishes containing subconfluent 293 cells were transfected with 5–10 μg of plasmid DNA and 15–40 μg of LipofectAMINE according to the manufacturer’s instructions. After a 5-h incubation with the DNA-LipofectAMINE mix in OPTIMEM (Life Technologies, Inc.), the medium was then replaced with fresh Dulbecco’s modified Eagle’s medium and harvested as described below.

**Immunoprecipitation and Affinity Purification**—Either 2 or 3 days (see “Results”) after transfection, the 293 cells were lysed in 1 ml of ice-cold buffer containing 1.5 mM MgCl₂, 1% Triton X-100, 50 mM Hepes, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 10% glycerol supplemented with several protease inhibitors (50 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml apronin (ICN Pharmaceuticals)) together with various phosphatase inhibitors (1 mM orthovanadate, 1 mM fluoride, and 1 mM β-glycerophosphate). The lysates were passed three times through a 25G needle, and cellular debris was removed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatants were then used for immunoprecipitation or subjected to affinity purification. For immunoprecipitations, mouse monoclonal M2 anti-Flag antibody (Eastman Kodak Co.) or anti-HA antibody (ascitic fluid prepared from 12CA5 cells using standard methods) was added to the lysates for 1 h at 4 °C, and the resulting immune complexes were collected by adding 30 μl of 0.1 mg/ml protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. Tagged MLK-3 or SAPK proteins were affinity purified by incubating the lysates with 100 μl of 50% glutathione 4B (Amersham Pharmacia Biotech) for 1 h at 4 °C. The proteins bound to either protein A or glutathione-Sepharose were then washed three times with lysis buffer prior to SDS-PAGE or in vitro kinase assay.

**SDS-PAGE and Western Blotting**—30 μl of 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) were incubated with 30 μg of plasmid DNA and 15–40 μl of kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM EDTA, and 10 μM ATP) for 20 min at 30 °C. The reactions were stopped by adding 30 μl of 2× SDS sample buffer containing 20% EDTA. The samples were then boiled and separated by SDS-PAGE. The polyacrylamide gels were then dried and subjected to autoradiography. After boiling for 3 min, the samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10% bovine serum albumin for 1 h at room temperature. In addition to the anti-Flag and anti-HA antibodies, in some experiments, rabbit polyclonal antisera directed against the MLK-3 SH3 domain2 and anti-phosphotyrosine antibody (PY20; Santa Cruz Biotechnology, Santa Cruz, California) were also used as primary antibody. The membranes were then washed twice for 10 min in TBS (25 mM Tris-HCl, pH 7.4, 0.13 mM NaCl, and 2.7 mM KC1) for 30 min and then incubated with the primary antibody in 5% bovine serum albumin for 1 h at room temperature. After two 10-min washes, the membranes were incubated with Supersignal Chemiluminescent Substrates (Pierce) for 5 min and then subjected to autoradiography.

**In Vitro Kinase Assay**—Affinity purified GST-HA-MLK-3 proteins were incubated with 30 μl of kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM EDTA) and 10 μCi of [γ-32P]ATP for 20 min at 30 °C. The reactions were stopped by adding 30 μl of 2× SDS sample buffer containing 20 mM EDTA. The samples were then boiled and separated by SDS-PAGE. The polyacrylamide gels were then dried and subjected to autoradiography.

**RESULTS**

**MLK-3 Forms Oligomers**—To address whether MLK-3 forms homodimers (or oligomers), we initially employed a co-immunoprecipitation approach. For these studies, two different...
epitope-tagged (Flag and HA) MLK-3 expression constructs were transfected either alone or together into 293 cells. Anti-HA antibody was used to immunoprecipitate the putative HA-MLK-3-Flag-MLK-3 complexes, and following SDS-PAGE, Western blot analyses were carried out with an anti-Flag monoclonal antibody to detect any Flag-MLK-3 bound to HA-MLK-3. As shown in Fig. 2A, the Flag-tagged form of MLK-3 was detected only when co-expressed with HA-MLK-3, indicating that these two different epitope-tagged forms of MLK-3 do, in fact, co-precipitate. As a control, the same immunoprecipitates were also subjected to Western blotting with anti-HA antibody to demonstrate that HA-MLK-3 was expressed at similar levels in both the single and double transfactions (second and third lanes). C, as an additional control, these lysates were also subjected to immunoprecipitation and Western blotting with the anti-Flag antibody to show that Flag-MLK-3 was expressed at similar levels in both the single and double transfecteds (first and second lanes).

**Fig. 2.** MLK-3 forms oligomers when over-expressed in 293 cells. Flag- and HA epitope-tagged forms of MLK-3 were transiently expressed in 293 cells either alone or together as indicated. Anti-HA antibody (Ab) was used for immunoprecipitation (Ip) and anti-Flag antibody for Western blotting (WB). A, as shown in the second lane, when HA- and Flag-MLK-3 were co-expressed, HA-MLK-3 bound to the Flag-tagged form of MLK-3. B, the same cell lysates were subjected to immunoprecipitation and Western blotting with anti-HA antibody to demonstrate that HA-MLK-3 was expressed at similar levels in both the single and double transfactions (second and third lanes). C, as an additional control, these lysates were also subjected to immunoprecipitation and Western blotting with the anti-Flag antibody to show that Flag-MLK-3 was expressed at similar levels in both the single and double transfecteds (first and second lanes).

SDS-PAGE in either the presence or the absence of the reducing agent DTT. As shown in Fig. 3A, in the presence of DTT, MLK-3 migrated solely as a monomer of 105 kDa. However, in the absence of DTT, both monomeric and dimeric forms (of approximately 220 kDa) of MLK-3 were observed. Although these data imply that a substantial fraction of MLK-3 protein, when over-expressed in cells, exists in the form of dimers stabilized by disulfide bridges, it remained possible that MLK-3 formed heterodimers with an unknown protein of similar size and thus migrated as an apparent homodimer. To examine this possibility, an expression construct encoding a COOH-terminal truncated form of MLK-3, containing the LZs and CRIB regions but lacking amino acids 591–847 (designated as Flag-MLK-3ΔC591 (Fig. 1) was generated and used in similar nonreducing SDS-PAGE experiments. If, in fact, MLK-3 forms homodimers in *vivo*, it follows that the truncated form of MLK-3 will form dimers twice the size of the monomeric form. However, if MLK-3 heterodimerizes with another protein similar in size to the wild type MLK-3, the resulting dimer would be of intermediate size. As shown in Fig. 3B, under nonreducing conditions, this truncated form of MLK-3 migrated only as monomer of approximately 65 kDa. However, under nonreducing conditions, MLK-3ΔC591 migrated both as a monomer and as a dimer of approximately 130 kDa (i.e., twice the size of the monomeric form). These data indicate that MLK-3 does, in fact, form disulfide-bridged homodimers and that the COOH terminus (amino acids 591–847) of MLK-3 is not required for either dimerization or disulfide bond-mediated interactions.

**The LZs Are Responsible for MLK-3 Homodimerization—**
Having demonstrated that MLK-3 forms homodimers, we next investigated whether MLK-3 homodimerizes via the LZs motif. For these studies, we used polymerase chain reaction-based mutagenesis to generate an MLK-3 mutant lacking just the LZs region (ΔLZs) MLK-3 as indicated. A, when anti-HA antibody (Ab) was used for immunoprecipitation (Ip) and anti-Flag antibody for Western blotting (WB), the wild type form of HA-MLK-3 co-immunoprecipitated with Flag-MLK-3 (third lane). In contrast, the LZs minus form of HA-MLK-3 failed to bind to the Flag-tagged form of MLK-3 (fourth lane). B, as a control, the same cell lysates were subjected to immunoprecipitation and Western blotting with anti-Flag antibody to show that HA-MLK-3 and HA-MLK-3ΔLZs (third and fourth lanes) were expressed at equivalent levels in these transfectants. C, as an additional control, the same cell lysates were also subjected to immunoprecipitation and Western blotting with anti-Flag antibody to demonstrate that Flag-MLK-3 was expressed in both single and double transfections at equivalent levels (second, third, and fourth lanes).

Different epitope-tagged (Flag and HA) MLK-3 kinase dead (K144R) expression constructs were generated and used in co-immunoprecipitation studies. If catalytic activity is a prerequisite for MLK-3 dimerization, the kinase dead version of MLK-3 would fail to complex with the catalytically inactive form of MLK-3; alternatively, if MLK-3 dimerization occurs prior to autophosphorylation, the kinase inactive MLK-3 will be dimerize and co-immunoprecipitate. The HA-tagged K144R version of MLK-3 was expressed either alone or together with either the Flag-tagged wild type or kinase dead forms of MLK-3. As shown in Fig. 6A, HA-MLK-3LZs co-precipitated with both kinase dead and wild type Flag-MLK-3. The fact that kinase inactive forms of MLK-3 can readily dimerize implies that dimerization of MLK-3 takes place prior to autophosphorylation.

**Dimerization via the LZs Is Required for MLK-3 Autophosphorylation**—Because MLK-3 kinase activity does not appear to be required for dimerization, we hypothesized that dimerization of MLK-3 might be an obligatory prerequisite for MLK-3 autophosphorylation activity. To test this hypothesis, we used GST-tagged wild type and leucine-zippers deleted forms of MLK-3 kinase in a series of in vitro kinase assays. In brief, if MLK-3 dimerization leads to autophosphorylation activity, the LZs region minus form of MLK-3 will exhibit no autophosphorylation activity; alternatively, if dimerization is not required for MLK-3 autophosphorylation, then MLK-3 protein lacking the LZs motif should display autophosphorylation activity. After transfection into 293 cells, the resulting GST fusion proteins were affinity purified by glutathione-Sepharose and incubated with γ-[32P]ATP and Mg2+. As shown in Fig. 7, although both the leucine-zipper minus and wild type forms of MLK-3 were expressed at similar levels, the wild type MLK-3 displayed substantially more autophosphorylation activity, indicating that the LZs motif is, in fact, required for this activity.

**Over-expression of Cdc42v12 but Not Cdc42n17 Enhances Dimerization of MLK-3**—The findings described above indicate
that MLK-3 homodimerizes via the LZs motif and that dimerization is a prerequisite for autophosphorylation activity. We next studied the upstream events leading to MLK-3 dimerization. Burbelo et al. (20) identified a binding motif (CRIB) within the MLK-3 sequence that interacts with Cdc42 but not RhoA. Because MLK-3 is known to be a downstream target of Cdc42, it seemed reasonable to propose that Cdc42 could induce MLK-3 dimerization. Accordingly, MLK-3 was expressed either alone or together with either a constitutively active (V12) or a dominant negative (N17) form of Cdc42. Two days after transfection, cell lysates were prepared and immunoprecipitated with anti-flag antisera and separated using SDS-PAGE under nonreducing conditions. Western blotting was performed using anti-MLK-3 SH3 antibody. Substantially more of the dimeric form of MLK-3 was observed in the transfectants containing Cdc42 v12 (third lane), relative to that observed when MLK-3 was expressed either alone or with the dominant negative n17 form of Cdc42 (second and fourth lanes). B, to confirm that Cdc42 induces MLK-3 homodimerization, the carboxyl-terminal truncation ΔC591MLK-3 expression construct was transiently expressed either alone or together with the Cdc42 v12 and processed the same manner. More ΔC591MLK-3 dimers were observed in the double transfectants (third lane) relative to the single transfectants (second lane). The molecular mass is shown at the left of each panel in kDa.
together with the Cdc42 V12. As shown in Fig. 8B, in the presence of Cdc42V12, there was an increase in the Flag-MLK-3ΔC591 dimers corresponding to twice the monomeric size, indicating that constitutively active Cdc42 induces MLK-3 homodimerization.

**MLK-3 Dimerization via LZs Is Crucial for Downstream Phosphorylation of SAPK**—In the experiments just described, we demonstrated that expression of Cdc42, an activator of the stress-responsive pathways upstream of MLK-3, resulted in increased MLK-3 dimerization. These findings are consistent with the notion that dimerization is a crucial step leading to MLK-3 activation. Previous studies by others and ourselves have demonstrated that MLK-3 activates the SAPK pathway by phosphorylating SEK1. To verify that dimerization via LZs is a prerequisite for MLK-3-mediated SAPK activation, two different co-expression studies were conducted. Firstly, we sought to demonstrate that the LZs minus form of MLK-3 was incapable of inducing SAPK phosphorylation. Secondly, we examined whether expression of a polypeptide (MLK-3-LZs), corresponding to just the LZs region of MLK-3, would block SAPK phosphorylation by competitively inhibiting the dimerization of the wild type MLK-3.

In the first set of experiments, GST-SAPK was expressed either alone or together with the wild type or LZs minus forms of HA-MLK-3. Two days after transfection, affinity purified GST-SAPK was Western blotted using either anti-phosphotyrosine antibody to identify phosphorylated SAPK or anti-GST antibody to evaluate the expression levels of SAPK. As shown in Fig. 9A, SAPK was phosphorylated on tyrosine residue(s) when co-expressed with wild type MLK-3. However, in the presence of the LZs minus form of MLK-3, we could find no evidence of SAPK tyrosine phosphorylation. Thus, the MLK-3 LZs motif is required for MLK-3 activation of SAPK.

In the second set of experiments, we again employed co-expression assays. The GST-SAPK, HA-MLK-3, and Flag-MLK-3ΔLZs expression constructs were co-transfected into 293 cells, and the resulting GST-tagged SAPK was affinity purified and Western blotted using either anti-phospho-tyrosine antibody or anti-GST antibody. As shown in Fig. 10A, SAPK was phosphorylated only when co-expressed with wild type MLK-3. Notably, however, the MLK-3-LZs polypeptide completely blocked SAPK phosphorylation induced by the wild type MLK-3. Because MLK-3 LZs polypeptide heterodimerizes with the full-length MLK-3 (as detected with anti-HA antiserum) in the double and triple transfections was also equivalent (third and fourth lane). D, the same cell lysates were also immunoblotted with anti-flag antibody to show that the MLK-3 LZs was, in fact, expressed in the triple transfectants (fourth lane).

**DISCUSSION**

In this report, we sought to define the mechanism of MLK-3 activation using the dimerization model of receptor tyrosine kinases as our prototype. Although MLK-3 is a nonreceptor type serine/threonine kinase, it contains an unusual tandem leucine/isoleucine zipper region, which is a hybrid of two dimerization motifs (namely the leucine zipper and helix-loop-helix motifs). Accordingly, we first asked whether this tandem leucine/isoleucine zipper region served as a dimerization motif. We found that MLK-3 LZs polypeptide completely blocked SAPK phosphorylation induced by the wild type MLK-3. Because MLK-3 LZs polypeptide heterodimerizes with the full-length form of MLK-3 (Fig. 5), taken together these observations indicate that the MLK-3-LZs polypeptide inhibits the full-length MLK-3 from forming active homodimers and thereby blocking activation of SAPK.
Monomeric MLK-3 homodimerizes via the tandem leucine zipper motif, which in turn leads to MLK-3 autophosphorylation and activation. Once activated, MLK-3 phosphorylates SEK1, resulting in activation of SAPK.

subsequent activation. We present here several lines of evidence indicating that MLK-3 activation is mediated in this manner: 1) MLK-3 bears a GTPase-interacting (CRIB) binding motif and has been shown to bind Cdc42 (but not Rho) in a GTP-dependent manner, indicating that MLK-3 is a downstream target of this GTPase (4, 20). We found co-expression of a constitutively active form of Cdc42 but not dominant negative Cdc42 or constitutively active RhoA, to increase markedly MLK-3 dimerization. 2) To address whether autophosphorylation of MLK-3 occurs before or after dimerization, we evaluated a catalytically inactive form of MLK-3 and found this variant to form dimers as readily as the wild type MLK-3, suggesting that autophosphorylation of MLK-3 occurs after dimerization. In agreement with these findings, we were also able to show that the leucine zipper minus form of MLK-3 lacks autophosphorylation activity. Moreover, it is likely that MLK-3 autophosphorylation is trans (rather than cis), because hetero-duplexes comprised of wild type MLK-3 and the polypeptide corresponding to the MLK-3 LZs region failed to activate SAPK via SEK1. 3) Because MLK-3 is a potent activator of the SAPK/JNK pathway, we used the leucine zipper minus form of MLK-3 to establish whether dimerization of MLK-3 is required for MLK-3-induced tyrosine phosphorylation of SAPK/JNK. In contrast with the wild type form of MLK-3, we found no evidence of SAPK/JNK phosphotyrosine in the presence of the MLK-3 variant lacking the leucine zipper region.

On the basis of the data shown here, we propose a model for MLK-3 activation (illustrated schematically in Fig. 11) that parallels the mechanism of receptor tyrosine kinase activation. Upstream activators such as Cdc42 induce MLK-3 homodimerization. This brings about the juxtaposition of the two catalytic domains and subsequent autophosphorylation. The active MLK-3 dimer in turn phosphorylates SEK1, ultimately leading to the activation of SAPK/JNK.

MLK1 and MLK2 also bear tandem leucine zippers that are closely related to the MLK-3 LZs, and thus it is likely that these MLK kinase family members also form homodimers. Because LZs may also mediate heterodimerization between two dissimilar proteins, it is also possible that MLK-3 forms heterodimers with MLK-1 and/or MLK-2. In this regard, we have used co-immunoprecipitation assays and found that MLK-2 and MLK-3 will, in fact, form heterodimers (data not shown). Although the expression of MLK-1 and MLK-2 is more restricted than that of MLK-3, some tissues are likely to contain at least two, and in some cases all three, of these kinases. Accordingly, heterodimerization between different MLK family members might result in the production of various dimeric forms with distinct functions and may be one means by which a relatively small number of MLK kinases could mediate signals from a wide variety of different signaling cascades. Finally, it should be noted that like MLK-3, MLK-1,3 and MLK-2 also bear a CRIB domain, and recently, Nagata et al. have reported that MLK-2 interacts with the GTP bound forms of both Cdc42 and Rac1 (33). It is therefore possible that small GTPase-induced dimerization may also regulate the activity of MLK-1 and MLK-2.

Among the MLK subfamily members, DLK has been shown to form disulfide-linked homodimers (32). Moreover, Tanaka and Hanafusa (7) have recently demonstrated that DLK will form complexes with MLK-3 and that a kinase inactive form of DLK will suppress MLK-3-mediated activation of JNK/SAPK. Although the mechanism underlying the interaction between MLK-3 and DLK was not established by Tanaka and Hanafusa, we speculate that DLK and MLK-3 heterodimerize via their respective LZs motif and that heterodimers comprised of a catalytically inactive form of DLK bound to MLK-3 are inactive; that is, in the absence of trans-phosphorylation, MLK-3 will not activate downstream targets.

Dimerization also appears to play a role in the activation of another serine/threonine MAPKKK, namely Raf. Following activation by Ras, Raf clusters at the plasma membrane and thus becomes accessible to other membrane kinases (34, 35). Whether Raf is activated via autophosphorylation or phosphorylation by other membrane kinases is not clear. However, both Farrar et al. (36) and Luo et al. (37) have demonstrated that artificially induced oligomerization of Raf is sufficient to induce its activation. Because both MLK-3 and Raf can be activated by small GTPases, it is possible that Cdc42 also recruits MLK-3 to the plasma membrane and initiates MLK-3 dimerization either by inducing conformational change or by increasing the local concentration of MLK-3 protein. Indeed, it is tempting to speculate that dimerization following membrane localization might serve as a common means of MAPKKK activation.

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