The Kinase Activation Loop Is the Key to Mixed Lineage Kinase-3 Activation via Both Autophosphorylation and Hematopoietic Progenitor Kinase 1 Phosphorylation*

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We have demonstrated previously that Cdc42 induced MLK-3 homodimerization leads to both autophosphorylation and activation of MLK-3 and postulated that autophosphorylation is an intermediate step of MLK-3 activation following its dimerization. In this report we sought to refine further the mechanism of MLK-3 activation and study the role of the putative kinase activation loop in MLK-3 activation. First, we mutated the three potential phosphorylation sites in MLK-3 putative activation loop to alanine in an effort to abrogate MLK-3 autophosphorylation. Mutant T277A displayed almost no autophosphorylation activity and was nearly nonfunctional; mutant S281A, that displayed a low level of autophosphorylation, only slightly activated its downstream targets, whereas the T278A mutant, that exhibited autophosphorylation comparable to that of the wild type, was almost fully functional. Thus, these residues within the activation loop are critical for MLK-3 autophosphorylation and activation. In addition, when the Thr277 and Ser281 residues were mutated to negatively charged glutamic acid to mimic phosphorylated serine/threonine residues, the resulting mutants were fully functional, implying that these two residues may serve as the autophosphorylation sites. Interestingly, HPK1 also phosphorylated MLK-3 activation loop in vitro, and Ser281 was found to be the major phosphorylation site, indicating that HPK1 also activates MLK-3 via phosphorylation of the kinase activation loop.

To date, five members of the mixed lineage kinase (MLK)1 family have been identified: MLK1 (1), MLK2 (MST) (2, 3), and MLK-3 (SPRK/PTK) (4–6) and the less highly conserved DLK (MUKZPK) (7–11) and LZK (12). With the exception of MLK1, all MLK family members have been shown to function as mitogen-activated kinase kinase kinases (MAPKKKs), which predominantly activate the SAPK (JNK) stress-signaling pathway (9, 12–15). Although MLK-3 and MLK2 can activate SAPK via both SEK1 (MKK4) and MKK7, MLK2 shows preferential association with MKK7 (16) and DLK activates SAPK via MKK7 only (17). This MKK7 specific SAPK activation can be mediated by the recently identified scaffold proteins JNK-interacting proteins JIP1 and JIP2 (18, 19). These scaffold proteins interact specifically with MLK2/MLK-3/DLK, SAPK, and MKK7 but not MKK4. In addition to the SAPK signaling pathway, MLK-3 also activates the p38 (RK/HOG) signaling pathway via MKK3 and MKK6 (13). Moreover, MLK2 and DLK have been found to slightly activate p38 and MLK2 also appears to activate ERK weakly (9, 15). Besides functioning as a MAPKKK, MLK-3 also acts as an IkB kinase kinase and mediates activation of the transcription factor NF-κB, which targets genes such as those involved in immune and inflammatory responses (20).

A number of upstream molecules utilize MLK-3 in SAPK activation. Dominant negative forms of MLK-3 have been shown to block SAPK activation mediated through the Ste20 homologues germinal center kinase (13) and hematopoietic progenitor kinase 1 (HPK1) (21), the small GTPases Cdc42 and Rac (22), and the guanine-nucleotide exchange protein CRK SH3-binding GNFPP (C3G) (23) and Lbc’s first cousin (Lfc) (24), suggesting that MLK-3 is downstream of these proteins in the SAPK signaling pathway. In Jurkat T cells, MLK-3 is activated upon costimulation with CD3 and CD28, and Cdc42 and Rac also activate NF-κB via MLK-3 (20, 25). Among these proteins, only HPK1, Cdc42, and Rac have been shown to directly interact with MLK-3. HPK1 binds to the MLK-3 SH3 domain and phosphorylates MLK-3 (21), whereas Cdc42 binds to the Cdc42 and Rac interactive binding region (26) and induces MLK-3 homodimerization (27). While these two events are crucial for MLK-3 activation, their underlying mechanisms are not fully understood.

Many kinases are phosphorylated in the kinase activation loop located between the conserved sequence DFG of subdomain VII and APE of subdomain VIII (28). The activation loop plays a crucial role in substrate recognition, and in many cases; phosphorylation in this segment is required to allow correct alignment of the substrates to the catalytic site (28–30). This segment can be regulated either through autophosphorylation or phosphorylation by other kinases. For example, cyclic AMP-dependent protein kinase autophosphorylates the Thr197 located in its activation loop (31), whereas ERK2 is phosphorylated on Thr183 and Tyr185 by MEK in the corresponding activation loop (32). In both cases, phosphorylation in the activation loop is essential for the activation of these kinases. The putative kinase activation loop of MLK-3 contains three potential phosphorylation sites at residues Thr277, Thr278, and Ser281 that may play a role in MLK-3 activation.

It is clear that MLK-3 plays a central role in integrating and
Abundishes—Anti-phospho-SAPK, anti-phospho-p38, and anti-p38 antibodies were gifts from Dr. J. McGlade. Anti-SAPK and anti-phospho-SEK1 antibodies were purchased from New England Biolabs. Mouse monoclonal anti-Flag antibody was obtained from Eastern Kodak, Rochester, NY, and ascitic fluid containing anti-HA antibody was prepared from 12CA5 hybridoma cells using standard methods.

Cell Culture and Transient Transfections—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal bovine serum (Life Technologies, Inc.), 250 ng/ml Fungizone (Bristol-Myers Squibb), 200 unit/ml penicillin, and 100 unit/ml streptomycin (ICN Pharmaceuticals). In most of the experiments, HeLa cells were transiently transfected using a vaccinia virus-based transfection method as described in Tibbles et al. (13). For the experiments involving the endogenous SAPK and p38, HeLa cells were transfected using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. 293 cells were cultured and transfected as described previously (27).

Immunoprecipitation and Affinity Purification—Transfected HeLa and 293 cells were harvested and purified as described previously (27) with the following modifications. Pre-chilled Nonidet P-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, and 1% Nonidet P-40) supplemented with proteinase inhibitors together with phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM β-glycerophosphate) was used to lyse the transfected cells. For immunoprecipitation, antibody together with 0.1 mg/ml protein A-Sepharose (Amersham Pharmacia Biotech), was added simultaneously to the lysates for 1 h at 4 °C. For affinity purification, GST-tagged MLK-3 proteins were incubated with 25–50 μl of 50% glutathione 4B-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. Finally, the proteins bound either to protein A or glutathione-Sepharose were then washed 3 times with the Nonidet P-40 lysis buffer.

Western Blotting—Western blots using anti-Flag, anti-HA, and anti-MLK-3 SH3 antibodies were carried out as described previously (27). Western blotting using anti-phospho-SAPK, anti-SAPK, anti-phospho-p38, and anti-p38 antibodies was carried out as described by the manufacturer’s instructions.

In Vitro Kinase Assays—The immunopurified Flag-MLK-3 proteins were incubated with 30 μl of kinase buffer I (50 mM Tris, pH 7.4, 10 mM MgCl2, 0.1 mM EGTA) and 2.5 μCi (γ-32P)ATP for 30 min at 30 °C.

RESULTS

Mutations in the Kinase Activation Loop of MLK-3 Affect MLK-3 Autophosphorylation Activity—Many kinases are phosphorylated in the activation loop between catalytic subdomains VII and VIII. For example, mitogen-activated kinases such as ERK2 and SAPK are phosphorylated and activated by a protein-catalyzed kinase which has the TXY motif (31, 33). The MAPKKK C-Raf is phosphorylated by PKC, whereas MEKK1 is activated by autophosphorylation in the activation loop (34–36) (Fig. 1A). Within the putative MLK-3 activation loop between the DGβ (subdomain VII) and APE (subdomain VIII) residues, there are 3 serine/threonine residues (Thr277, Thr278, and Ser281) that might serve as potential phosphorylation sites.
rified samples were subjected to using glutathione-Sepharose 4B (GMK3/6 (13). To establish whether autophosphorylation is required for MLK-3 activation, we first tested the ability of the various MLK-3 activation loop mutants to phosphorylate SEK1 using a gel mobility shift assay and Western blotting using phospho-specific SEK1 (Thr223) antibody. If autophosphorylation is required for MLK-3 activation, a mutant lacking autophosphorylation activity will be inactive and will not phosphorylate SEK1 (and thus will not induce an electrophoretic shift in SEK1). For these studies, HA-SEK1 was transfected either alone or together with the Flag-tagged wild type T277A, T278A, or S281A forms of MLK-3. Half of the resulting cell lysates were immunoprecipitated and Western blotted using anti-Flag Ab. The same blot was then probed with anti-phospho-SEK1 (Thr223) antibody to detect the phosphorylated form of SEK1. To detect the phosphorylated form of SEK1, the balance of the lysates were subjected to immunoprecipitation and Western blotting using anti-HA Ab to detect the HA-SEK1 proteins. The same blot was then probed with anti-phospho-SEK1 (Thr223) antibody to detect the phosphorylated form of SEK1. To detect the various forms of MLK-3, the balance of the samples were subjected to in vitro kinase assay, and the resulting autoradiograph is shown in the upper panel. The balance of the samples were Western blotted using anti-MLK-3 SH3 Ab and are shown in the lower panel. "p-GST-HA MLK-3" denotes the phosphorylated MLK-3 protein. Similar results were obtained when Flag-tagged MLK-3 expression constructs or 293 cells were used (data not shown).

To investigate whether any of these amino acids play a role in MLK-3 autophosphorylation, each one was independently mutated to alanine (T277A, T278A, and S281A) using PCR-based site-directed mutagenesis. The mutant kinases were assayed to determine whether they retained autophosphorylation activity. In these experiments, GST-HA-tagged MLK-3 expression constructs containing the above mutations were transfected into cells, purified using glutathione-Sepharose 4B (GS4B). Two-thirds of each of the purified samples were subjected to in vitro kinase assay, and the resulting autoradiograph is shown in the upper panel. The balance of the samples was Western blotted using anti-MLK-3 SH3 Ab and are shown in the lower panel. "p-GST-HA MLK-3" denotes the phosphorylated MLK-3 protein. Similar results were obtained when Flag-tagged MLK-3 expression constructs or 293 cells were used (data not shown).

A. VII Activation loop VIII

MAPK DFG-LARVADDPSHATGFLY.T.Y.VTTRVRK.APE
SAPK DFG-LRAGTSTPM...T.Y.VTTRVRK.APE
C-Raf DFG-LATVKESSG...S.QVQEPFTGVSVLM.APE
MEKK1 DFG-FAAARLASKQ.T.GAGEFQQQLQSTIAFM.APE
MLK-3 DFG-LAREMVK....TCGMS.NAGTYAWN.APE

T277, T278, S281

Fig. 1. Serine/threonine to alanine mutations in MLK-3 putative kinase activation loop affect MLK-3 autophosphorylation. Panel A is a schematic diagram that illustrates the amino acid sequence of the (putative) kinase activation loop between the catalytic subdomains VII and VIII of the MAPK (32), SAPK (33), c-Raf (35), Mekk1 (36), and MLK-3 (13). The serine and threonine residues of MAPK, SAPK, c-Raf, and Mekk1 that are phosphorylated are depicted in bold, as are the potential phosphorylatable serine and threonine residues in MLK-3. Panel B, GST-HA MLK-3 expression constructs bearing the mutations T277A, T278A, or S281A were phosphorylated (and thus will not induce an electrophoretic shift in SEK1). As shown in Fig. 2, panel A, in the presence of the wild type MLK-3, the electrophoretic mobility of SEK1 was retarded; whereas mutation of MLK-3 Thr277 to alanine completely abolished the SEK1 mobility shift. The MLK-3 T278A mutant induced a SEK1 mobility shift similar to that of the wild type MLK-3, whereas the S281A mutant induced an intermediate shift. Reprobing of panel A with anti-phospho-SEK1 antibody revealed that T278A (which exhibited relatively high level of autophosphorylation activity) phosphorylated SEK1 to the highest level among the three mutants. Notably, however, T278A activity was lower than wild type in this experiment. While this may due to experimental variation, we cannot rule out that mutation of this residue has an effect on MLK-3 activity toward SEK1. Mutant T277A that had very low level of autophosphorylation activity only weakly phosphorylated SEK1. Although S281A caused an intermediate shift of SEK1, the level of phospho-SEK1 detected was lower than anticipated for MLK-3 function and a correlation between the level of MLK-3 autophosphorylation activity and its ability to phosphorylate SEK1.

Autoactivation Is Required for MLK-3-36-mediated Activation of the Stress Signaling Pathways—To corroborate these findings, we next asked if activation of endogenous SAPK and

Fig. 2. Serine/threonine to alanine mutations in MLK-3 putative kinase activation loop affect MLK-3 phosphorylation of its substrate SEK1. HA-tagged SEK1 protein was expressed alone or together with the Flag-tagged WT, T277A, T278A, or S281A proteins. Half of the resulting cell lysates were subjected to immunoprecipitation (Ip) and Western blot (WB) using anti-HA Ab and the electrophoretic mobility of the HA-SEK1 proteins are shown in the panel A. The same blot was then Western blotted with anti-phospho-SEK1 (Thr223) antibody to detect the phosphorylated form of SEK1 (panel B). Panel C shows the expression level of the various forms of MLK-3 as detected by Ip and WB using anti-Flag Ab. (Note: the MLK-3 proteins were separated on an 8% polyacrylamide gel to show the expression level but not to resolve the mobility shift of MLK-3.)
p38 is also dependent on MLK-3 autophosphorylation activity. For these experiments, GST-HA-tagged WT, K144R, T277A, T278A, and S281A MLK-3 expression constructs were transfected into HeLa cells. Part of the resulting cell lysates were separated on four polyacrylamide gels and Western blotted with anti-phospho-SAPK, anti-SAPK, anti-phospho-p38, and anti-p38 antibodies, and the resulting autoradiographs are shown in panels A-D as indicated. One-third of each cell lysate was affinity purified using glutathione-Sepharose 4B (GS4B) and Western blotted with anti-MLK-3 SH3 Ab, and the expression level of the various MLK-3 forms is shown in the bottom panel. Similar results were obtained when 293 cells were used (data not shown).

As shown in Fig. 3, similar levels of phospho-SAPK were detected following the expression of the wild type or T278A mutant. No phosphorylation of SAPK was observed with either the kinase-dead or the T277A mutant, whereas only low levels of phospho-SAPK were detected in the presence of the S281A mutant. Very similar results were observed with p38. We consistently observed the highest levels of phospho-p38 in the presence of the wild type and T278A relative to the S281A and T277A mutants (although a basal level of the phospho-p38 was detected in the nontransfected HeLa cells). Notably, T278A mutant, which displayed a reduced ability to phosphorylate SEK1 (Fig. 2), appeared to activate SAPK as efficiently as wild type. The fact that MLK-3 can activate SAPK via other signaling molecules, for example, SEK4, may account for this difference. These data suggest that the amount of activated SAPK and p38 is dependent on the level of MLK-3 autophosphorylation activity. Collectively these data indicate that autophosphorylation is crucial for MLK-3 activation and the subsequent phosphorylation and activation of two separate stress signaling pathways.

Both Thr<sup>277</sup> and Ser<sup>281</sup> Residues May Serve as the MLK-3 Autophosphorylation Sites—We demonstrated that substitution of the activation loop residues Thr<sup>277</sup> and Ser<sup>281</sup> with alanine either abolished or diminished the autophosphorylation activity of MLK-5. Since phosphorylation of the kinase activation loop is a prerequisite for substrate recognition of some kinases, it seemed reasonable to propose that the Thr<sup>277</sup> and Ser<sup>281</sup> residues would be essential for MLK-3 substrate recognition. To examine this possibility, we mutated these two residues into negatively charged glutamic acid residues, either independently or together (so as to mimic the phosphorylation state), and tested whether the resulting mutants were capable of activating downstream signaling leading to SAPK phosphorylation. Wild type, T277D, S281D, and T277D/S281D MLK-3 expression constructs were transfected into 293 cells and the resulting lysates analyzed for the presence of the activated endogenous SAPK using anti-phospho-SAPK antibody. As shown in Fig. 4, phospho-SAPK was detected in all of the lysates (except for the nontransfected control), suggesting that all the mutants were able to activate endogenous SAPK. These results imply that these negatively charged residues could functionally replace the Thr<sup>277</sup> and Ser<sup>281</sup> residues and allow normal substrate recognition and kinase activity. The Thr<sup>277</sup> and Ser<sup>281</sup> residues may therefore act as the MLK-3 (autophosphorylation sites. If so, because the mutation of the Thr<sup>277</sup> residue to alanine almost completely abolished MLK-3 autophosphorylation activity, whereas the S281A mutation only reduced MLK-3 autophosphorylation activity, Thr<sup>277</sup> is likely to be the major autophosphorylation site of MLK-3.

HPK1 Phosphorylates the Activation Loop of MLK-3—As mentioned above, some kinases such as the mitogen-activated kinases ERK and SAPK are phosphorylated by other kinases in their activation loop thereby leading to their activation. HPK1 has also been shown to activate MLK-3 via phosphorylation (21); however, the precise site of HPK1 phosphorylation has not yet been identified. Accordingly, we investigated the possibility that HPK1 phosphorylates MLK-3 within the activation loop. We first tested whether HPK1 could phosphorylate a peptide containing the MLK-3 activation loop amino acid sequence. A DNA fragment corresponding to the activation loop was amplified via PCR and subcloned into a bacterial expression construct bearing an N-terminal GST tag. The peptide was then cleaved specifically from the purified GST fusion protein using thrombin protease and further purified. Mass spectrometry analysis indicated that the molecular mass of the resulting peptide was 2167.55 daltons, which matched the amino acid sequence “GLAREWHKTQMSAAGTYA,” this corresponds to the expected size of the MLK-3 activation loop, except for the first 2 amino acids resulting from the thrombin cleavage site of the GST protein. HA-HPK1 was then expressed in HeLa cells, immunopurified using anti-HA Ab and incubated with the purified peptide in the presence of ATP and MgCl<sub>2</sub>. The resulting peak of 2247.71 daltons (Fig. 5). The additional 80 daltons corresponds exactly to the anticipated replacement of a hydroxyl group with a phosphate group. As a negative control, the

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**Fig. 3.** Serine/threonine to alanine mutations in MLK-3 putative activation loop affect MLK-3 mediated activation of SAPK and p38. The various GST-HA-MLK-3 expression constructs including the WT, K144R, T277A, T228A, and S281A were transfected into HeLa cells and harvested after 2 days. Aliquots of each lysate were separated on four polyacrylamide gels and Western blotted with anti-phospho-SAPK, anti-SAPK, anti-phospho-p38, and anti-p38 antibodies, and the resulting autoradiographs are shown in panels A-D as indicated. One-third of each cell lysate was affinity purified using glutathione-Sepharose 4B (GS4B) and Western blotted with anti-MLK-3 SH3 Ab, and the expression level of the various MLK-3 forms is shown in the bottom panel. Similar results were obtained when 293 cells were used (data not shown).

**Fig. 4.** Mutation of Thr<sup>277</sup> and Ser<sup>281</sup> to glutamic acid in MLK-3 putative activation loop does not affect MLK-3-mediated SAPK activation. The GST-HA T277D, S281D, and T277D/S281D expression constructs were generated as described under "Experimental Procedures" and transfected into 293 cells (The WT MLK-3 expression construct was included as a control.) The cells were harvested after 2 days and aliquots of the resulting cell lysates were subjected to Western blots (WB) using anti-phospho-SAPK and anti-SAPK antibodies. The results of the WB analyses are shown in the first and second panel as indicated. Half of each cell lysate was also affinity purified using glutathione-Sepharose 4B (GS4B) and Western blotted with anti-MLK-3 SH3 Ab to detect the various forms of MLK-3 protein (bottom panel). Similar experiments were done in HeLa cells with the same results (data not shown).
same peptide was also incubated with a kinase-dead form of HPK1, but no additional peak was observed (data not shown). Taken together, these observations indicate that HPK1 phosphorylated one residue in the MLK-3 activation loop peptide in vitro.

HPK1 Phosphorylates the Ser281 Residue of the MLK-3 Activation Loop—To determine whether Thr277, Thr278, or Ser281 residue(s) was phosphorylated by HPK1, we next examined whether HPK1 could phosphorylate GST fusion proteins containing the same region of the MLK-3 activation loop with only one of the three potential residues unchanged. Accordingly, double mutants T277A/T278A, T277A/S281A, and T278A/S281A were generated. (The C-terminal sequences together with that of the wild type and the vector are listed in Fig. 6A.) Affinity purified GST fusion proteins were incubated with immunopurified HA-HPK1 in an in vitro kinase. As shown in Fig. 6B, in the presence of equivalent amounts of GST fusion proteins, HPK1 specifically phosphorylated the GST fusion proteins bearing the wild type or the T277A/T278A double mutant, but not the T277A/S281A or T278A/S281A double mutants or the GST control. When the GST wild type activation loop fusion protein was incubated with purified activated SAPK and ERK2, it was not phosphorylated by either kinase (data not shown) confirming that the HPK1 phosphorylation is specific.

These results indicate that Ser281 is the HPK1 phosphorylation site since HPK1 phosphorylated the fusion protein only when the Ser281 residue was preserved, as in the case of the wild type and the T277A/T278A double mutant. Therefore, Ser281 not only serves as potential MLK-3 autophosphorylation site but is also a HPK1 phosphorylation site.

To determine whether HPK1 phosphorylates only the Ser281 residue within the entire MLK-3 protein, a kinase-dead mutant containing the S281A mutation was generated and the ability of HPK1 to phosphorylate this mutant was tested using an in vitro kinase assay. GST-HA-tagged K144R or K144R S281A constructs were transfected alone or together with HA-tagged HPK1. The expressed proteins were immunoprecipitated using anti-HA antibody; one-third of the precipitates were subjected to Western blotting and the balance of the samples were evaluated via an in vitro kinase assay. As shown in Fig. 7, both K144R and K144R/S281A mutants exhibited undetectable levels of kinase activity. HPK1 was able to phosphorylate the K144R form of MLK-3, however, only a faint band (upon prolonged exposure) of the phosphorylated K144R/S281A form was observed. The Ser281 residue therefore acts as the major HPK1 phosphorylation site. Notably, the autophosphorylation activity of HPK1 was greatly reduced in the presence of the K144R/S281A MLK-3 mutant and also slightly reduced in the presence of the K144R mutant, suggesting the MLK-3 Ser281 phospho-
Fig. 7. **The Ser^{281} residue is the major HPK1 phosphorylation site.** A GST-HA-tagged kinase-dead MLK-3 mutant containing the S281A mutation was generated as described under “Experimental Procedures.” GST-HA tagged forms of MLK-3 K144R, K144R/S281A, and HA-HPK1 expression constructs were transfected alone or together into HeLa cells. The resulting cell lysates were subjected to immunoprecipitation (Ip) using anti-HA Ab to isolate both the MLK-3 and HPK1 proteins. The HA immunoprecipitates were then subjected to either in vitro kinase assay or WB. The upper panel shows the phosphorylated HPK1 and MLK-3, whereas the lower panel shows the expression level of HPK1 and the various MLK-3 proteins.

**DISCUSSION**

The kinase activation loop, defined as the region spanning between the subdomains VII and VIII, has been shown in a number of kinases to regulate catalytic activity (28). In this study, we examined the role of kinase activation loop in MLK-3 activation. First, we mutated the three potential phosphorylation residues in the kinase activation loop to investigate whether these residues are required for MLK-3 autophosphorylation activity. We found that mutating residue Thr^{277} to alanine significantly reduced MLK-3 autophosphorylation activity. This same MLK-3 mutant only had negligible phosphorylation activity toward its substrate SEK1 and did not activate either JNK/SAPK or p38/RK. The S281A mutant displayed a reduced level of autophosphorylation activity, and reduced activation of SEK1, JNK/SAPK, and p38/RK, whereas the T278A mutant, which exhibited close to normal autophosphorylation whereas mutation of Thr^{278} to alanine only displayed a reduced level of autophosphorylation whereas mutation of Thr^{278} to alanine only resulted in relatively minor reduction of SEK1 phosphorylation seems likely that both Thr^{277} and Ser^{281} but not Thr^{278} need to be phosphorylated for normal MLK-3 function toward SEK1.

Because MLK-3 lies downstream of HPK1 in the stress signaling pathway and is directly phosphorylated by HPK1 (21), we also asked whether HPK1 could activate MLK-3 by phosphorylating MLK-3 activation loop. We identified the Ser^{281} residue as the HPK1-phosphorylation site, indicating that both autophosphorylation and HPK1 phosphorylation could activate MLK-3 via the kinase activation loop which underscores the complex regulation of MLK-3 activation. This mode of regulation, by autophosphorylation and transphosphorylation by another kinase(s) within the activation loop has also been reported for the tyrosine kinase Lck (37).

We have shown previously that MLK-3 homodimerization is required for its autophosphorylation, critical for its activation, and can be induced by Cdc42 (27). In this report, we in fact demonstrated that autophosphorylation is essential for MLK-3 activation. Taken together, these data indicate that MLK-3 employs an activation mechanism similar to that of receptor tyrosine kinases; that is, induced dimerization leads to autophosphorylation and the subsequent activation of MLK-3. Based on the fact that all MLK family members (except MLK1 which has not been reported) display autophosphorylation activity and bear tandem leucine zippers (2–12), it is reasonable to speculate that all MLK family members utilize a dimerization-based activation mechanism which ultimately leads to autophosphorylation and activation. Within the MLK family, DLK has also been shown to also employ a dimerization-based activation mechanism (38). Moreover, it is noteworthy that the two residues corresponding to the MLK-3 potential autophosphorylation sites are conserved among other MLK family members. As shown in Fig. 8, the Thr^{277} residue is conserved in MLK1 and MLK2 (but substituted with a serine in DLK/MUK/ZPK and LZK), whereas the Ser^{281} residue is conserved in all.
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MLK family members. Hence it is tempting to hypothesize that these two residues may also be crucial for the autophosphorylation, thus activation, of other MLK family members.

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