

Autoinhibition of Mixed Lineage Kinase 3 through Its Src Homology 3 Domain*

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Mixed lineage kinase 3 (MLK3) is a serine/threonine protein kinase that functions as a mitogen-activated protein kinase kinase kinase to activate the c-Jun NH₂-terminal kinase pathway. MLK3 has also been implicated as an I κ B kinase kinase in the activation of NF- κ B. Amino-terminal to its catalytic domain, MLK3 contains a Src homology 3 (SH3) domain. SH3 domains harbor three highly conserved aromatic amino acids that are important for ligand binding. In this study, we mutated one of these corresponding residues within MLK3 to deliberately disrupt the function of its SH3 domain. This SH3-defective mutant of MLK3 exhibited increased catalytic activity compared with wild type MLK3 suggesting that the SH3 domain negatively regulates MLK3 activity. We report herein that the SH3 domain of MLK3 interacts with full-length MLK3, and we have mapped the site of interaction to a region between the zipper and the Cdc42/Rac interactive binding motif. Interestingly, the SH3-binding region contains not a proline-rich sequence but, rather, a single proline residue. Mutation of this sole proline abrogates SH3 binding and increases MLK3 catalytic activity. Taken together, these data demonstrate that MLK3 is autoinhibited through its SH3 domain. The critical proline residue in the SH3-binding site of MLK3 is conserved in the closely related family members, MLK1 and MLK2, suggesting a common autoinhibitory mechanism among these kinases. Our study has revealed the first example of SH3 domain-mediated autoinhibition of a serine/threonine kinase and provides insight into the regulation of the mixed lineage family of protein kinases.

Virtually all physiological processes are regulated by reversible protein phosphorylation. Therefore protein kinases and phosphatases should be highly regulated enzymes. Physical interactions with other signaling molecules can modulate protein kinase activity by changing subcellular location, autophosphorylation state, or conformation of a protein kinase. In the absence of intermolecular associations, many kinases are kept in an inhibited state that is maintained by intramolecular interactions.

Mixed lineage kinase 3 (MLK3)¹ also called Src homology 3

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¹ The abbreviations used are: MLK, mixed lineage kinase; SH3, Src homology 3; MAPK, mitogen-activated protein kinase; MKKK, MAPK kinase kinase; MKK, MAPK kinase; JNK, c-Jun NH₂-terminal kinase;

(SH3) domain-containing proline-rich kinase (1), is a serine/threonine kinase that functions as a mitogen-activated protein kinase (MAPK) kinase kinase (MKKK) to phosphorylate and activate the dual specific kinases, MAPK kinase 4 (MKK4) (2) and MKK7 (3) which, in turn, can phosphorylate and activate c-Jun NH₂-terminal kinase (JNK). Recent evidence suggests that I κ B kinase α (IKK α) and IKK β are also substrates of MLK3, and that MLK3 acts as an IKK kinase to activate the NF- κ B pathway in response to T cell receptor costimulation (4).

MLK3 contains several domains that are predicted to mediate protein-protein interactions including an SH3 domain, a leucine zipper, and a Cdc42/Rac interactive binding (CRIB) motif (Fig. 1). The small GTPase Cdc42, in its activated state, binds to MLK3 through the centrally located CRIB motif, and increases MLK3 catalytic activity (5–7). This activation is accompanied by a change in the phosphopeptide map pattern of *in vivo* labeled MLK3, suggesting that Cdc42 binding induces activating phosphorylation event(s) on MLK3 (7). A leucine zipper that resides NH₂-terminal to the CRIB motif is necessary for MLK3 homo-oligomerization (8). Work in our laboratory has shown that zipper-mediated oligomerization is not required for MLK3 activation by Cdc42, but instead is critical for proper interaction and phosphorylation of a downstream target, MKK4, and subsequent JNK activation (9).

The NH₂-terminal portion of MLK3 contains an SH3 domain. SH3 domains are independently folding modules of about 60 amino acids. Although different SH3 domains have distinct ligand preferences (10), the consensus binding site is composed of a short proline-rich sequence, often Pro-Xxx-Xxx-Pro preceded or followed by basic amino acids (11–14). The SH3 domain of MLK3 has been shown to interact with a proline-rich region in hematopoietic progenitor protein kinase-1 (15), but an effect on MLK3 activity has not been demonstrated.

Since MLK3 contains a large proline-rich COOH-terminal region (Fig. 1), we were interested in the possibility that the SH3 domain of MLK3 might bind to this region in an autoregulatory fashion. In the work presented here, we show that the SH3 domain of MLK3 does indeed play a critical role in autoregulation. However, unexpectedly, the SH3 domain binds to a region between the zipper and CRIB motifs that lacks a classical SH3-binding site. Point mutations, either in the SH3 domain or in the newly identified SH3-binding site of MLK3, which disrupt this interaction, result in increased MLK3 activity. The data presented here indicate that the catalytic activity of MLK3 is negatively regulated by its SH3 domain. This is the first demonstration of SH3-mediated autoinhibition of a serine/threonine kinase.

IKK α , I κ B kinase α ; IKK β , I κ B kinase β ; CRIB, Cdc42/Rac-interactive binding; HA, hemagglutinin; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; JIP, JNK interacting protein.

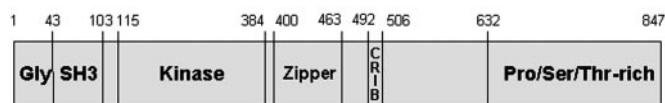


FIG. 1. **Schematic of MLK3.** The numbers in the diagram represent amino acid number. The glycine-rich region (amino acid 1–42) is denoted by *Gly*. *CRIB* stands for “Cdc42/Rac interactive binding” motif.

EXPERIMENTAL PROCEDURES

DNA Constructs and Mutagenesis—Construction of the cytomegalovirus-based expression vectors carrying the cDNAs for wild type MLK3 (pRK5-NFlag.*mlk3*), MLK3 L410P, and MLK3 Δ 430–486 has been described elsewhere (7, 9).² A series of truncation variants of MLK3 were generated by 15 cycles of amplification of pRK5-NFlag.*mlk3* using the polymerase chain reaction with *Pfx* polymerase (Invitrogen). The same 5' oligonucleotide was used for the construction of a series of variants with successive COOH-terminal deletions: 5'-GCATTAGCT-AGCACCATTGGACTACAAG-3'.

The following oligonucleotides were used as 3' primers: pRK5-NFlag.*mlk3* 1–635, 5'-CGTTAAGGATCCTCAAGAGCTGCTACCGCG-3'; pRK5-NFlag.*mlk3* 1–598, 5'-CGTTAAGGATCCTCAGGATGAGTCTCTGA-3'; pRK5-NFlag.*mlk3* 1–529, 5'-CGTTAAGGATCCTCAGCGGGAAAGGTGGG-3'; pRK5-NFlag.*mlk3* 1–485, 5'-CGTTAAGGATCCTCAGCGCGGAGCTTGCT-3'; pRK5-NFlag.*mlk3* 1–386, 5'-CGTTAAGGATCCTCATTCCTAGGACCTG-3'. The amplified *mlk3* fragments were subcloned in-frame with the *Flag* coding sequence into pRK5-NFlag.*mlk3* using *NheI* and *Bam*HI.

For the construction of the hemagglutinin (HA)-tagged MLK3 variants, the following oligonucleotides were used in the polymerase chain reaction reactions with pRK5-NFlag.*mlk3* as the template; pCGN-HA.*mlk3* 1–114, 5'-CGTTAGTCTAGAATGGAGCCCTTGAAGAG-3' and 5'-GCATTAGGATCCTCAGAAGCTGGCCACCTCGC-3'; pCGN-HA.*mlk3* 115–399, 5'-CGTTAGTCTAGACAGGAGCTGCGGCTGG-3' and 5'-GCATTAGGATCCTCACCAGCCTTCTGCATGG-3'; pCGN-HA.*mlk3* 400–591, 5'-CGTTAGTCTAGAAAGCGCGAGATCCAGGG-3' and 5'-GCATTAGGATCCTCAGTACCATGTGGCTTCG-3'; pCGN-HA.*mlk3* 592–847, 5'-CGAATGTCTAGACTGGATTTCAGATGACTC-3' and 5'-GCATTAGGATCCTCAAGGCCCGCTTCCGGC-3'. The amplified *mlk3* fragments were subcloned in-frame with the HA coding sequence into the pCGN mammalian expression vector (16) using *XbaI* and *Bam*HI.

Variants of MLK3 containing point mutations were constructed using the Quick Change site-directed mutagenesis method (Stratagene) with pRK5-NFlag.*mlk3* as the template DNA using *Pfx* polymerase (Invitrogen) and 15 cycles of amplification. The following oligonucleotides, and their reverse complements, were used as mutagenesis primers: pRK5-NFlag.*mlk3* Y52A, 5'-GGACAGCCCTGTTCGACGCCGAGCCAGTGG-3'; pRK5-NFlag.*mlk3* P469A, 5'-GTGGACCGCGAGCGAGCGCACGTGC-3'. The presence of the appropriate mutations were confirmed by DNA sequencing (MSU DNA Sequencing Facility).

A glutathione *S*-transferase (GST) expression vector (pGEM-2T-SH3) carrying the cDNA encoding the SH3 domain of MLK3 (amino acids 43–104) was constructed by polymerase chain reaction-mediated amplification of the corresponding coding sequence from pRK5-*mlk3* followed by subcloning into the pGEM-2T vector. The Y52A mutation was introduced by site-directed mutagenesis of pGEM-2T-SH3 using the same oligonucleotides that were used to construct pRK5-NFlag.*mlk3* Y52A.

Expression and Purification of GST Fusion Proteins—GST and GST fusion proteins (GST-SH3 and GST-SH3 Y52A) were expressed in *Escherichia coli* and purified using glutathione-Sepharose 4B, according to the manufacturer's protocol (Amersham Pharmacia Biotech). Eluted fractions containing the GST fusion protein, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie Blue staining, were pooled and concentrated to about 1 mg/ml using a Centrprep concentrator (Amicon).

Cell Culture, Transfections, and Lysis—Human fetal kidney 293 cells were cultured on 100-mm dishes and transfected using the calcium phosphate method as previously described (7). Cells were harvested 16 h after transfection, and lysed by the addition of 1 ml of lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM sodium fluoride, 1 mM Na₄PP₆, 100 μ M β -glycerophosphate, 1 mM Na₃VO₄, 2 mM phenylmethylsulfonyl

fluoride, and 0.15 units/ml aprotinin) as described previously (7).

Immunoprecipitations and GST Pull-down Assays—Antibodies against the proteins of interest were prebound to protein A-agarose beads for 30 min at room temperature: MLK3 antiserum (0.25 μ g/ μ l slurry) and M2 monoclonal antibody (Sigma) directed against the *Flag* epitope (0.45 μ g/ μ l slurry). For the GST pull-down experiment, the GST fusion proteins were preincubated with glutathione-Sepharose 4B resin for 30 min. Clarified lysate (400 μ l) was incubated with 20 μ l of antibody-bound Protein A-agarose or with 20 μ l of glutathione-Sepharose 4B resin prebound with 5 μ g of GST fusion protein for 90 min at 4 °C. Immunoprecipitates and GST pull-downs were washed with HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). Immunoprecipitates used for kinase assays were washed three times with HNTG buffer containing 1 M LiCl, three times with HNTG buffer, and twice with kinase assay buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM MnCl₂, 10 mM MgCl₂, 0.1 mM Na₃VO₄).

Gel Electrophoresis and Western Blot Analysis—Proteins from lysates and immunoprecipitates were resolved by SDS-PAGE according to Laemmli (17) and transferred to nitrocellulose membranes. The membranes were immunoblotted using MLK3 antiserum (1 μ g/ml), M2 *Flag* monoclonal antibody (9 μ g/ml), or HA antibody (BAbCO) (5 μ g/ml), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Invitrogen). Western blots were developed by the chemiluminescence method. Multiple exposures of the Western blots were developed, and densitometry (NIH Image) of unsaturated films was used to determine relative expression levels.

In Vitro Kinase Assays—Kinase assays were performed in 20 μ l of kinase assay buffer containing 50 μ M ATP and 5 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) (PerkinElmer Life Science), 10 μ g of mixed histones (Roche Molecular Biochemicals), and the reactions were carried out for either 15 or 30 min at room temperature as previously described (7). Following the kinase assay, proteins were separated by SDS-PAGE. Gels were rinsed in phosphate-buffered saline, dried, and the incorporation of radioactivity into the kinase or substrates was determined by phosphorimaging (Molecular Dynamics).

RESULTS

Point Mutation in the SH3 Domain Increases the In Vitro Kinase Activity of MLK3—Sequence and structural analyses of SH3 domains reveal three highly conserved aromatic amino acids that participate in ligand binding (18, 19). Replacement of any one of these conserved residues with alanine in the SH3 domain of the adaptor protein Sem-5/Grb2 abolishes binding to its proline-rich targets (18). The corresponding conserved residues in the SH3 domain of MLK3 are Tyr⁵², Trp⁸³, and Tyr⁹⁹. To deliberately disrupt the function of the SH3 domain of MLK3, site-directed mutagenesis was used to substitute the tyrosine residue at position 52 with an alanine residue. The catalytic activity of this SH3 mutant, MLK3 Y52A, was compared with that of wild type MLK3.

Cells transiently expressing MLK3 or MLK3 Y52A were lysed, the MLK3 variants were immunoprecipitated, and *in vitro* kinase assays were performed using histones as an exogenous substrate. Data from a representative experiment are shown in Fig. 2. Based on three independent experiments, MLK3 Y52A exhibits a 2-fold increase in autophosphorylation activity and a 2.5-fold increase in histone phosphorylation activity when compared with wild type MLK3. These data suggest that the catalytic activity of MLK3 is negatively regulated by its functional SH3 domain.

The SH3 Domain of MLK3 Interacts with MLK3—The finding that a disruptive mutation in its SH3 domain increases MLK3 activity, coupled with the fact that MLK3 contains nine potential SH3 binding sequences (Pro-Xxx-Xxx-Pro) in its COOH-terminal 220 amino acids, led us to investigate whether the SH3 domain may bind to MLK3 itself. The SH3 domain of MLK3 (amino acids 43–104) was expressed as a fusion protein with GST in *E. coli* and purified. The ability of MLK3 to interact with its SH3 domain in an intermolecular fashion was assessed in GST pull down experiments. As shown in Fig. 3, both wild type MLK3 and MLK3 Y52A associate with GST-SH3 but not with GST. The observation that wild type MLK3 asso-

² In our earlier published work we have referred to MLK3 as the Src homology 3 domain containing proline-rich kinase.

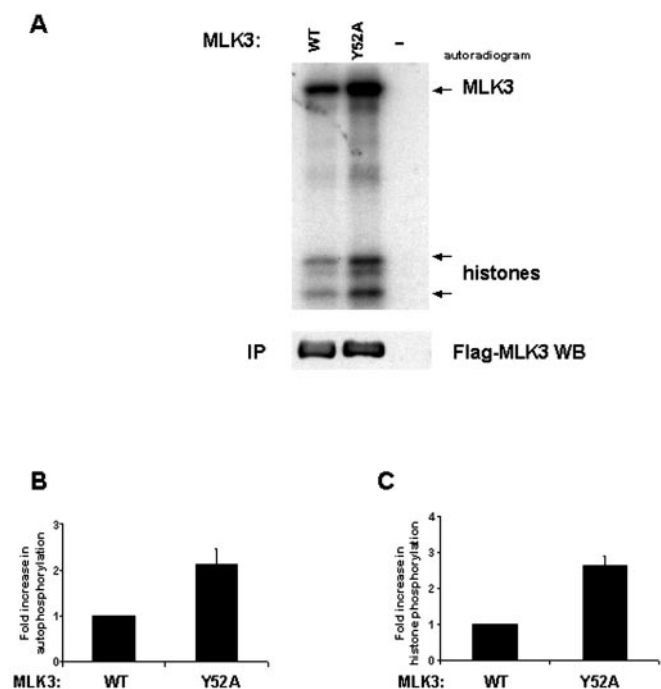


FIG. 2. Effect of a point mutation in the SH3 domain of MLK3 on catalytic activity. Cells were transfected with expression vectors containing the cDNAs for Flag-tagged wild type (WT) MLK3 or MLK3 Y52A. A minus sign indicates that a control empty vector was transfected. The MLK3 variants were isolated from cellular lysates by immunoprecipitation (IP) using the Flag antibody, and kinase activity was assessed *in vitro* as described under “Experimental Procedures.” **A**, *in vitro* kinase assay of MLK3 and MLK3 Y52A using histones as a substrate. The top panel shows an autoradiogram with bands corresponding to MLK3 autophosphorylation and histone phosphorylation indicated by arrows. A Western blot (WB) of the immunoprecipitated MLK3 variants using the Flag antibody is shown in the bottom panel. **B** and **C**, the mean \pm S.E. for fold increase in MLK3 autophosphorylation and histone phosphorylation from three independent experiments is shown. Data was quantitated by phosphorimaging and normalized to MLK3 expression levels as described under “Experimental Procedures.”

ciates with GST-SH3 to a lesser extent than does MLK3 Y52A suggests that an SH3-mediated intramolecular interaction within wild type MLK3 competes with the intermolecular binding of GST-SH3.

Identification of the SH3-binding Region within MLK3—Variants with progressive deletions from the COOH terminus of MLK3 were constructed with an NH₂-terminal Flag tag, expressed, and tested for their ability to associate with GST-SH3. MLK3-(1–635) and MLK3-(1–598) lack the Pro/Ser/Thr-rich region; MLK3-(1–529) ends 23 amino acids after the CRIB motif; MLK3-(1–485) terminates just after the zipper motif; and MLK3-(1–386) contains the NH₂ terminus through the kinase domain. All variants expressed at similar levels in 293 cells (Fig. 4A, bottom panel). To create a more appropriate negative control for the GST pull down experiment, the Tyr residue in GST-SH3 that corresponds to Tyr⁵² in MLK3 was replaced with Ala, thus giving rise to GST-SH3 Y52A. Both fusion proteins were expressed in *E. coli*, purified, and used in GST pull down assays to test their binding to the Flag-tagged MLK3 variants. Surprisingly, as shown in Fig. 4A, deletion of the Pro/Ser/Thr-rich COOH-terminal region does not prevent association with GST-SH3. In fact, of all the truncation variants tested, only MLK3-(1–386) fails to detectably bind to GST-SH3. This suggests that the SH3-binding site is within amino acids 387–485 of MLK3. However, it is also conceivable that the SH3-binding site may be present but masked in MLK3-(1–386). In any case, one can conservatively conclude that a bind-

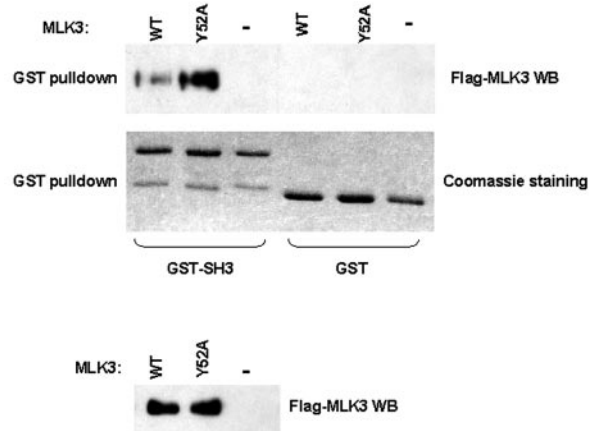


FIG. 3. Assay for association of the SH3 domain of MLK3 with full-length MLK3. Cells were transfected with expression vectors containing the cDNAs for MLK3 variants. Cellular lysates expressing the indicated MLK3 variants were incubated with glutathione-Sepharose 4B resin to which purified GST-SH3 or GST had been prebound. **Top panel**, the presence or absence of bound MLK3 variants was assessed by Western blotting with the Flag antibody. **Middle panel**, equal loading of GST-SH3 or GST on the glutathione-Sepharose 4B resin was confirmed by Coomassie staining. **Bottom panel**, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates with the Flag antibody. The data shown is representative of four independent experiments.

ing site for MLK3’s SH3 domain is present in amino acids 1–485 of MLK3. All MLK3 variants fail to associate with GST-SH3 Y52A, indicating that introduction of this point mutation successfully blocks the binding ability of the SH3 domain.

To further define the SH3-binding site within MLK3, individual or tandem domains of MLK3 were constructed as NH₂-terminal HA-tagged variants, expressed, and analyzed for their capacity to interact with the SH3 domain of MLK3. MLK3-(1–114) contains the NH₂-terminal glycine-rich region and the SH3 domain; MLK3-(115–399) contains the kinase domain; MLK3-(400–591) includes the zipper region, the CRIB motif, and an adjacent stretch of basic amino acids; and MLK3-(592–847) contains the COOH-terminal Pro/Ser/Thr-rich region. All variants expressed at similar levels although MLK3-(592–847) apparently undergoes some proteolytic degradation (Fig. 4B, bottom panel). Data from GST pull down assays show that, of these variants, only MLK3-(400–591) retains the ability to interact with GST-SH3 (Fig. 4B). As expected, none of the variants associates with GST-SH3 Y52A. Taking together all of the results from the GST pull down experiments, we conclude that the binding site for MLK3’s SH3 domain is within amino acids 400–485 of MLK3.

To confirm that the SH3-binding region lies within MLK3-(400–485), we took advantage of a previously constructed deletion mutant, MLK3-(Δ 430–486), which lacks the COOH-terminal half of the zipper and the flanking stretch of basic amino acids (7). As shown in Fig. 5, MLK3-(Δ 430–486) fails to associate with GST-SH3. These data suggest that the critical SH3 binding determinants lie within amino acids 430–485 of MLK3. However, an alternative explanation is that disruption of zipper-mediated oligomerization prevents access to the SH3-binding site. We recently reported that a leucine zipper point mutant, MLK3 L410P, behaves as a monomer (9). Our results show that GST-SH3 associates with both MLK3 L410P and wild type MLK3 to approximately the same extent (Fig. 5).

Point Mutation in the SH3-binding Site Abolishes Binding to the SH3 Domain and Increases MLK3 Activity—An SH3-binding region has been mapped to amino acids 400–485 of MLK3. The predicted leucine zipper which comprises amino acids

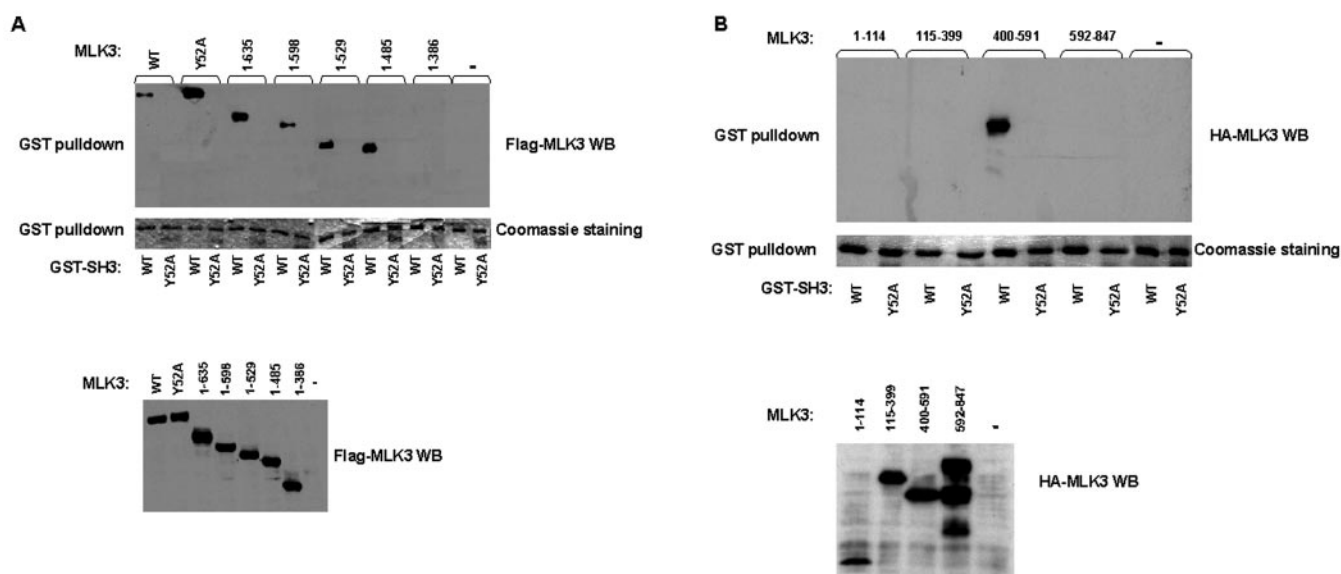


FIG. 4. **Mapping of the SH3-binding region within MLK3.** Cells were transfected with expression vectors containing the cDNAs for MLK3 truncation variants. The numbers in the figure represent amino acid numbers in MLK3. *A* and *B*, cellular lysates expressing the indicated MLK3 truncation variants were incubated with glutathione-Sepharose 4B resin to which purified GST-SH3 or GST-SH3 Y52A had been prebound. *Top panels*, the presence or absence of bound MLK3 variants was assessed by Western blotting with the Flag antibody (*A*) or the HA antibody (*B*). *Middle panels*, equal loading of GST-SH3 or GST-SH3 Y52A on the glutathione-Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panels*, the expression of MLK3 truncation variants was assessed by Western blotting of cellular lysates with the Flag antibody (*A*) or the HA antibody (*B*). The data shown is representative of at least four independent experiments.

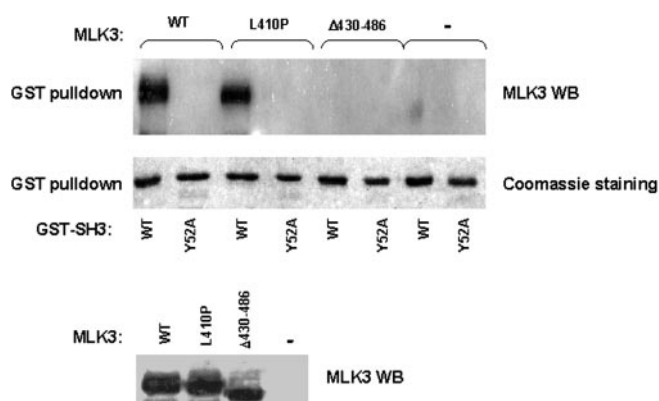


FIG. 5. **Effect of zipper mutations on the SH3 binding.** Cells were transfected with expression vectors containing the cDNAs for MLK3, MLK3 L410P, and MLK3- Δ 430–486. Cellular lysates expressing the indicated MLK3 variants were incubated with glutathione-Sepharose 4B resin to which purified GST-SH3 or GST-SH3 Y52A had been prebound. *Top panel*, the presence or absence of bound MLK3 variants was assessed by Western blotting with the MLK3 antibody. *Middle panel*, equal loading of GST-SH3 or GST-SH3 Y52A on the glutathione-Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates with MLK3 antibody. The data shown is representative of three independent experiments.

400–463 of MLK3 is devoid of proline residues, and GST-SH3 binding does not require zipper-mediated homo-oligomerization of MLK3 (Fig. 5), suggesting that the zipper domain should not contain the SH3-binding region. Amino acids 463–485 of MLK3 are therefore predicted to be crucial for the binding of the SH3 domain. As shown in Fig. 6A, no typical SH3 binding motif of Pro-Xxx-Xxx-Pro is found in this region of MLK3, although a single proline residue is found at position 469 and four contiguous arginine residues (472–475) are present. Substitution of all four arginine residues with neutral glutamine residues reduces, but does not abolish, binding of MLK3 to GST-SH3 (data not shown). The sole proline residue in this region was substituted with an alanine residue by site-directed mutagenesis and the ability of this mutant, MLK3 P469A, to

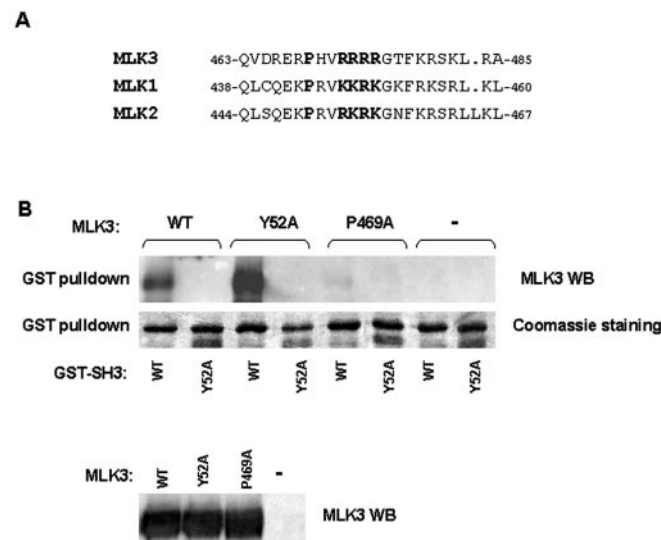


FIG. 6. **Alignment of the SH3-binding region of MLK3 with MLK1 and MLK2 and effect of MLK3 P469A on binding of SH3 domain.** *A*, alignment of the SH3-binding region of MLK3 with MLK family members, MLK1 and MLK2. Amino acid numbers are indicated to the left and right of each sequence. The sole proline residue and the four contiguous basic residues are shown in bold. *B*, cells were transfected with expression vectors containing the cDNAs for the indicated MLK3 variants. Cellular lysates expressing the MLK3 variants were incubated with glutathione-Sepharose 4B resin to which purified GST-SH3 or GST-SH3 Y52A had been prebound. *Top panel*, the presence or absence of bound MLK3 variants was assessed by Western blotting with MLK3 antibody. *Middle panel*, equal loading of GST-SH3 or GST-SH3 Y52A on the glutathione-Sepharose 4B resin was confirmed by Coomassie staining. *Bottom panel*, the expression levels of MLK3 variants were assessed by Western blotting of cellular lysates with MLK3 antibody. The data shown is representative of four independent experiments.

associate with MLK3's SH3 domain was tested in a GST pull down assay. As shown in Fig. 6B, MLK3 P469A does not detectably associate with GST-SH3, indicating that Pro⁴⁶⁹ is critical for SH3 binding.

A point mutation in the SH3 domain increases MLK3 activity (Fig. 2), presumably because the mutation disrupts an au-

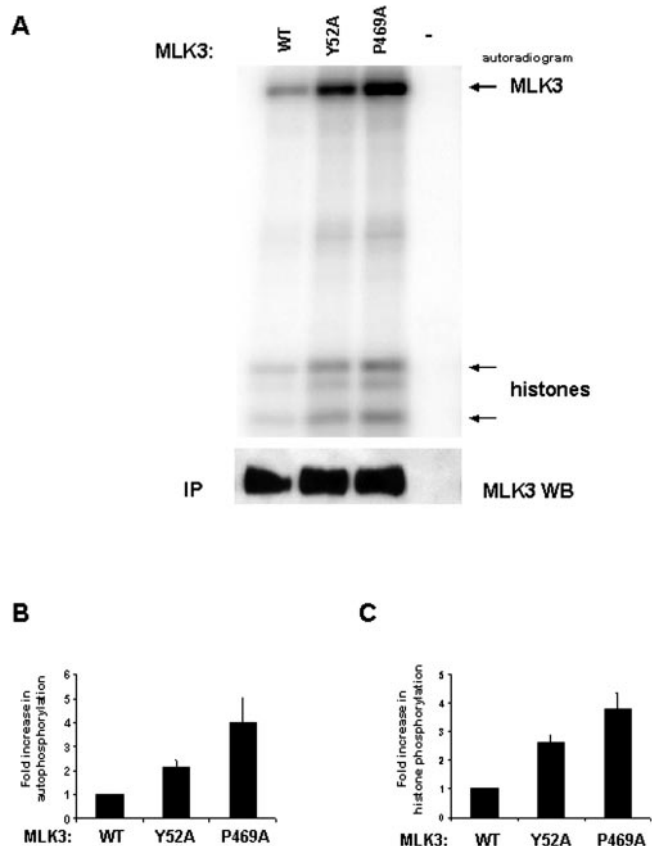


FIG. 7. Catalytic activity of MLK3 P469A. *A*, *in vitro* kinase assay of MLK3 variants using histone as a substrate. The *top panel* shows an autoradiogram with bands corresponding to MLK3 autophosphorylation and histone phosphorylation indicated by *arrows*. *A*, Western blot of the immunoprecipitated samples using a Flag antibody is shown in the *bottom panel*. *B* and *C*, the mean \pm S.E. for fold increase in MLK3 autophosphorylation and histone phosphorylation from three independent experiments is shown. Data was quantitated by phosphorimaging and normalized to MLK3 expression levels as described under "Experimental Procedures."

toinhibitory interaction. It has now been determined that Pro⁴⁶⁹ is critical for binding to MLK3's SH3 domain. A reasonable prediction, therefore, is that mutation of Pro⁴⁶⁹ should likewise disrupt an autoinhibitory interaction and increase MLK3 activity. Indeed, as measured in an *in vitro* kinase assay (Fig. 7), MLK3 P469A has about 4-fold higher autophosphorylation and histone phosphorylation activity than does wild type MLK3. These data argue that MLK3 is autoinhibited by a physical interaction between its SH3 domain and a SH3-binding site that is located between its zipper and CRIB motif.

DISCUSSION

Protein phosphorylation is a ubiquitous regulatory event in biology. Many pathological processes are associated with dysregulation of protein kinase activity. It is not surprising, therefore, that nature has evolved and coordinated multiple mechanisms to exquisitely control the activity of protein kinases. Many protein kinases are maintained in low activity or inactive states through intermolecular or intramolecular association with inhibitory molecules or domains. These inhibitory interactions may be disrupted in response to appropriate stimuli, allowing the protein kinase to adopt an active conformation.

SH3 domains were first identified as targeting domains that through binding to proline-rich sequences mediate intermolecular associations among signaling molecules (13, 14). Extensive structural information has revealed three aromatic residues

conserved in SH3 domains that participate in binding to proline-rich ligands which usually adopt left-handed polyproline type II helices (18, 19). MLK3 contains an NH₂-terminal SH3 domain. Notably, proline residues comprise 24% of the COOH-terminal 220 amino acids. Our finding that the mutation of one of the conserved aromatic residues in MLK3, Tyr⁵², to Ala renders MLK3 more active (Fig. 2), suggested to us that MLK3's SH3 domain might bind to MLK3 itself to negatively regulate its activity. Pull down experiments showed that full-length MLK3 associates with the SH3 domain of MLK3 fused to GST (Fig. 3). However, the extent of association of MLK3 Y52A with the GST-SH3 fusion protein is much greater than that of wild type MLK3 (Fig. 3), suggesting that the functional SH3 domain of wild type MLK3 competes with exogenous GST-SH3. These results argue that an intramolecular association involving the SH3 domain of wild type MLK3 negatively regulates MLK3 activity.

Despite the fact that the COOH-terminal Pro/Ser/Thr-rich region of MLK3 contains nine Pro-Xxx-Xxx-Pro sequences, deletion of this region has no effect on association of MLK3 with GST-SH3 (Fig. 4A). Instead, a series of mapping experiments has identified a sequence located between the zipper and CRIB motifs that is important for the interaction of MLK3 with its SH3 domain (Fig. 4B). Unexpectedly, this sequence is not proline-rich but, rather, contains a single proline residue at position 469. Mutation of this sole proline residue to alanine in MLK3 abolishes binding to GST-SH3. In accord with the supposition that MLK3 is autoinhibited by an SH3-mediated intramolecular interaction, replacement of Pro⁴⁶⁹ with Ala increases the catalytic activity of MLK3.

While MLK3 represents the first demonstrated example of SH3-mediated autoinhibition of a serine/threonine kinase, the well studied tyrosine kinases of the Src family offer some interesting parallels. Crystal structure and biochemical studies have revealed an autoinhibitory intramolecular association that involves both the SH2 domain and the SH3 domain of Src (20–22). A COOH-terminal phosphorylated Tyr provides a ligand for the SH2 domain, and the SH3 domain of Src binds to its so-called "SH2-kinase linker" region, a sequence located between the SH2 and kinase domains. These intramolecular interactions constrain the movement of the two lobes of the kinase domain and therefore, block the binding of ATP, keeping Src in an inactive form (reviewed in Refs. 23–25). Interestingly, the SH3 binding sequence in Src, and also in its relative Fyn, harbors only a single proline residue, analogous to what we have discovered in MLK3, even though the preferred ligand of Src's SH3 domain was found by phage display methods to be the proline-rich sequence Leu-Xxx-Xxx-Arg-Pro-Leu-Pro-Xxx-Pro (10).

One might imagine that the SH3 binding sequence in the "zipper-CRIB linker" region of MLK3 would provide a suboptimal ligand for MLK3's SH3 domain. The high effective concentration afforded in intramolecular associations in all likelihood allows this otherwise weak interaction to occur. Indeed, evolutionary selection may have given rise to a relatively low affinity intramolecular ligand for MLK3's SH3 domain so that it might be outcompeted by the presentation, in response to an appropriate physiological signal, of a high affinity proline-rich ligand on another signaling molecule. For instance, binding of a proline-rich sequence in the Nef protein of human immunodeficiency virus-1 to the SH3 domain of Hck, a Src family member expressed primarily in myeloid cells, overcomes autoinhibition, dramatically increasing the activity of Hck (26, 27).

Signaling molecules that overcome SH3-mediated autoinhibition of MLK3 have yet to be identified. One potential candidate is the MKKK kinase hematopoietic progenitor protein

kinase-1 that contains proline-rich sequences through which it interacts with the SH3 domain of MLK3 (15) and that, like MLK3, can activate both the JNK pathway and the NF- κ B pathway (28, 29). Furthermore, hematopoietic progenitor protein kinase-1 phosphorylates kinase-inactive MLK3 in an *in vitro* kinase assay (15). However, no effect of hematopoietic progenitor protein kinase-1 on MLK3 activity has been observed in our hands or reported in the literature.

The JNK pathway scaffold proteins, JNK interacting protein 1 (JIP1), JIP2 and JIP3, have been shown to bind to mixed lineage kinases including MLK3 (30–32). Co-transfection of JIP1, JIP2, or JIP3 with MLK3 in COS-7 cells enhances the activation of JNK by MLK3 (31, 32). This may suggest that binding of JIP increases MLK3 activity. It is conceivable that JIP binding might relieve SH3-mediated autoinhibition of MLK3. However, the mixed lineage kinase family member, dual leucine zipper kinase, which lacks an SH3 domain, also binds to JIP1 and JIP2 (33), and recent data from Holzman's laboratory suggests that JIP-associated dual leucine zipper kinase is catalytically inactive (34). Further studies are necessary to clarify these issues.

We previously reported that activated Cdc42 binds to MLK3 in a CRIB-dependent manner and results in a change in the *in vivo* phosphorylation pattern of MLK3 as judged by phosphopeptide mapping (7). The close proximity of the newly identified SH3 binding sequence of MLK3 to its CRIB motif may suggest interplay between Cdc42-mediated activation and SH3-mediated autoinhibition of MLK3. The precise mechanistic relationship is likely to be complex and is currently under investigation in our laboratory.

The mixed lineage family of serine/threonine kinases are so-named for the sequence similarity in their catalytic domains to both tyrosine and serine/threonine kinases. The major function ascribed to this group of protein kinases is activation of the JNK pathway, specifically by acting as MKKKs to phosphorylate and activate MKK4 or MKK7. MLK1 (35), MLK2 (36, 37), and MLK3 share the same general domain arrangement, which includes an NH₂-terminal SH3 domain and centrally located zipper and CRIB motifs. However, the sequences in their COOH termini diverge. The more distantly related mixed lineage kinases include leucine zipper-bearing kinase (38), dual leucine zipper kinase/leucine zipper protein kinase/MUK (39–41), leucine-zipper and sterile- α motif kinase (42), and MLK-like mitogen-activated protein triple kinase (43). These kinases lack both SH3 domains and CRIB motifs. In this report we have identified a single proline residue in MLK3 that is required for binding and autoinhibition through MLK3's SH3 domain. Interestingly, this proline residue is conserved in the closely related family members MLK1 and MLK2 (Fig. 6A), but not in the more distantly related mixed lineage kinases. Thus this newly discovered mechanism of SH3-mediated autoinhibition of MLK3 is predicted to apply to the regulation of MLK1 and MLK2 as well.

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