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Phosphoregulation of Mixed-Lineage Kinase 1 Activity by Multiple Phosphorylation in the Activation Loop†

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ABSTRACT: Mixed-lineage kinase 1 (MLK1) is a mitogen-activated protein kinase kinase kinase capable of activating the c-Jun NH_2-terminal kinase (JNK) pathway. Full-length MLK1 has 1104 amino acids and a domain structure identical to MLK2 and MLK3. Immunoblot and mass spectrometry show that MLK1 is threonine (and possibly serine) phosphorylated in or near the activation loop. A kinase-dead mutant is not, consistent with autophosphorylation. Mutation to alanine of any of the four serine or threonine residues in the activation loop reduces both the activity of the recombinant kinase domain and JNK pathway activation driven by full-length MLK1 expressed in mammalian cells. Furthermore, the gel mobility of the mutant MLK1s is closer to that of the kinase-dead than wild type, consistent with reduced phosphorylation. Thr312 is the key residue: MLK1[T312A] retains only basal activity (about 1−2% of wild type), and its gel mobility is indistinguishable from kinase-dead. Thr312 does not suffice, however; phosphorylation of multiple sites is necessary for full activation of MLK1. An activation mechanism consistent with these data involves phosphorylation of multiple sites in the activation loop, with phosphorylation of Thr312 required for full phosphorylation. This mechanism is broadly similar to that previously reported for MLK3 [Leung, I. W., and Lassam, N. (2001) *J. Biol. Chem. 276*, 1961−1967], but the key residue differs.

The mixed-lineage kinases (MLKs)[1] are a family of serine−threonine protein kinases at the mitogen-activated protein kinase kinase kinase level that can activate the JNK pathway (*1*). Overexpression of MLKs results in apoptotic cell death in PC12 cells and primary sympathetic neurons. Conversely, expression of kinase-dead MLKs blocks apoptosis induced by trophic factor withdrawal (*2*, *3*) and by treatment with the MPTP metabolite MPP^+ (*4*). The small molecule indolocarbazole CEP-1347 (KT7515) inhibits MLKs at the same concentrations at which it promotes neuronal survival under trophic factor withdrawal and at the same concentrations at which it inhibits cellular activation of the JNK pathway (*4*, *5*). Together, these data implicate MLKs in apoptotic cell death and imply that MLK activity must be regulated stringently.

Several studies have elucidated mechanisms regulating the activity of one family member, MLK3. Tandem leucine zippers COOH terminal to the kinase domain mediate dimerization of MLK3 (*6*, *7*). Interaction with activated cdc42 enhances dimerization (*8*). Dimerization of MLK3 in turn enhances autophosphorylation. Phosphorylation at two key sites in the activation loop of MLK3, T277 and S281, results in MLK3 activation. In addition to autophosphorylation, the S281 site can also be phosphorylated by hematopoietic progenitor kinase 1 (*9*). Mass spectrometry has identified other phosphorylation sites in MLK3 coexpressed with activated cdc42 (*10*), but their relationship to MLK3 activation remains to be determined.

As a prelude to purification of a uniformly phosphorylated form of the closely related MLK1 (MAP3K9), for structural determination by X-ray crystallography, we have studied the role of phosphorylation in the activation of MLK1. We present here biochemical and biophysical evidence that MLK1 is activated by autophosphorylation in (or near) the activation loop. The activation loops of the MLK family are highly homologous, and so one might predict that the same residues would be key to their activation. Functional data presented here, however, demonstrate that the key residue for activation of MLK1, Thr312, differs from the key residue for activation of MLK3. The data presented here also demonstrate that full activation of MLK1 requires phosphorylation of multiple sites.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents. Anti-Xpress antibody was from Invitrogen. Anti-phospho-c-Jun(Ser73) and anti-phospho-

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[1] Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence; ECF, enhanced chemifluorescence; ESI-MS, electrospray ionization−mass spectrometry; GST, glutathione *S*-transferase; JNK, c-Jun NH_2-terminal kinase; KD, kinase domain; LC-MS, liquid chromatography−mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization−time of flight mass spectrometry; MBP, myelin basic protein; MLK, mixed-lineage kinase; TBS, Tris-buffered saline.

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MKK4(Thr261) antibodies were from Cell Signaling Technology. Mammalian expression vector pcDNA4/HisMax C, Lipofectamine Plus, Bis-Tris NuPAGE gels and associated buffers, and Colloidal Coomassie stain were from Invitrogen. The EndoFree plasmid maxi kit was from Qiagen. The QuikChange site-directed mutagenesis kit was from Stratagene. ECL and ECF reagents were from Amersham Biosciences. POROS oligoR3 sorbent was from Perseptive Biosystems. Trypsin (sequencing grade) and calf intestinal alkaline phosphatase were from Roche Applied Science. Endoproteinase LysC was from Wako Biochemicals. ZipTips were from Millipore. FluoroNUNC Maxisorp microtiter plates were from Nalge Nunc International. All other chemicals were of reagent grade or better. TBS was 25 mM Tris, pH 7.5, plus 150 mM NaCl. Stock solutions of sodium orthovanadate were activated according to ref *11*.

Cloning of MLK1. A partial sequence of MLK1, encompassing the kinase domain and the leucine zipper, has been published (*12*); a plasmid containing this sequence was kindly provided by Donna S. Dorow. This plasmid was used to obtain a partial MLK1 nucleotide sequence. A genomic DNA file including this sequence had been deposited in GenBank (Accession Number AC004816). A putative coding region for full-length MLK1 was deduced from this file, based on homology with MLK2 (*13*) and consensus splice sites. Primers based on a region of predicted sequence 3′ to the published sequence were used to screen a pool of human cDNAs (Edge Biosystems). Of five positive clones, four contained the MLK1 sequence (with a few corrections to predicted splice sites) and together extended to the TAG stop codon. None included the putative first exon, which was cloned and assembled using a combination of 5′-RACE and PCR. The complete coding sequence consists of 3315 base pairs (split into 12 exons in the genomic sequence) and codes for a protein of 1104 amino acids with a predicted molecular mass of 122 kDa.[2] The sequence has been scanned against the GenBank database, and the only significant similarities to human sequences are to incomplete MLK1 sequences (GenBank Accession Numbers XM027237 and AF251442, both of which begin 3′ to the ATG start codon proposed here). The sequence also has high homology to two *Mus musculus* MLK1 sequences (GenBank Accession Numbers XM147685 and NM177395). Both of these include the 5′ untranslated sequence and assign the ATG start codon in agreement with the assignment proposed here.

Full-length MLK1 was subcloned into pcDNA4/HisMax C for mammalian expression. This construct contained two NH_2-terminal epitope tags, namely, a hexahistidine peptide and the Xpress peptide. The tagged MLK1 protein contained 1138 amino acids and had a predicted molecular mass of 126 kDa. Mutations were introduced using the QuikChange kit and were verified by nucleotide sequencing.

The kinase domains $MLK1_{KD}$ and $MLK3_{KD}$ were expressed as GST fusion proteins in Sf21 cells via baculovirus-mediated infection and purified by glutathione affinity as described in ref *5*. The amino acid sequences of both GST-kinase domain fusion proteins are presented in supplemental Figure 2. Mutations were introduced and verified as above. For in vitro kinase assay substrates, inactive MKK4b[K113A] and the NH_2 terminus of the JNK substrate c-Jun (residues 1−79) were each subcloned into pGEX-KT (*14*), expressed in *Escherichia coli* as GST fusion proteins, and purified by standard glutathione affinity methods (*15*).

Gel Electrophoresis and Immunoblotting. Proteins were separated by SDS−polyacrylamide gel electrophoresis on Bis-Tris gels. For direct visualization, gels were stained with Colloidal Coomassie. For immunoblotting, proteins were electrophoresed to a nitrocellulose or PVDF membrane. The membrane was blocked with 5% (w/v) BSA in TBS, probed with the indicated primary antibody, washed with 0.2% (v/v) Triton X-100 in TBS, and probed with horseradish peroxidase-labeled secondary antibody for ECL or alkaline phosphatase-labeled secondary antibody for ECF. ECL blots were visualized on film. ECF blots were visualized and quantitated on a Storm 840 phosphorimager (Molecular Dynamics). Both development time and photomultiplier voltage were adjusted to ensure the signal was in the linear range.

Kinase Activity. Activity of $MLK1_{KD}$ and $MLK3_{KD}$ was determined by phosphorylation of MBP with [γ-^{32}P]ATP in MultiScreen format as described in ref *5*. Activity of $MLK1_{KD}$ was also determined by phosphorylation of MKK4 in an ELISA assay modeled after the TrkA assay described in ref *16*. A 96-well microtiter plate (FluoroNUNC Maxisorp) was coated overnight at 4 °C in a humidified chamber with 10 μg/mL GST-MKK4 diluted in TBS and then washed three times with wash buffer (0.05% Tween-20 in TBS). All subsequent incubations used 0.1 mL/well for 1 h at 37 °C in a humidified chamber unless otherwise indicated. The plate was blocked with block buffer (3% BSA and 0.2% Tween-20 in TBS), washed three times with wash buffer, and then washed three times with TBS. $MLK1_{KD}$ was prepared as a 10-fold concentrated stock in 20 mM HEPES containing 0.1% BSA and diluted into reaction buffer (20 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM activated sodium orthovanadate, and 30 μM ATP) to initiate the kinase reaction. As a negative control, EDTA was added to selected wells to a final concentration of 0.1 M prior to reaction. After 30 min at 37 °C, addition of EDTA to 0.1 M terminated the kinase reaction. The plate was washed three times with wash buffer. The phosphorylated product was detected by incubation with anti-phospho-MKK4, washing, incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase, washing, and reaction with the fluorogenic alkaline phosphatase substrate 4-methylumbelliferyl phosphate (0.02 mg/mL in 1.0 M diethanolamine, pH 9.6, 5 mM $MgCl_2$) for 45 min at 37 °C. The reaction was stopped with 0.5 M dibasic sodium phosphate and the product measured in a microplate reader (Cytofluor, 360 nm excitation, 460 nm emission).

Mass Spectrometric Determination of Phosphopeptides. Purified $MLK1_{KD}$ was separated by SDS−PAGE. The protein band was excised, washed with 50% acetonitrile/10 mM ammonium bicarbonate, and digested overnight with LysC. The digest was extracted with 60% acetonitrile/10 mM ammonium bicarbonate as previously described (*17*). To verify digestion and sequence coverage, an aliquot of the extracted peptides was analyzed by LC-MS on a Model API-

[2] The nucleotide sequence for human MLK1 (MAP3K9) has been deposited in the GenBank database under GenBank Accession Number AY327900. The amino acid sequence can be accessed through the NCBI Protein Database under NCBI Accession Number AAQ23054. The sequences are also included with this paper as supplemental Figure 1.

3000 triple quadrupole (PE-Sciex) equipped with a Nano-Electrospray Source II (Protana). The detected peptides covered 40% of $MLK1_{KD}$ and 50% of the kinase-dead mutant $MLK1_{KD}$[K171A].

The presence of phosphopeptides in the digest was assessed using the precursor ion scanning method. The digest was fractionated on a nanocolumn of POROS oligoR3 sorbent as previously described (*18*). The digest was applied, washed in 0.5% formic acid, and eluted into spraying capillaries with, in succession, 20% methanol, 50% methanol, and 50% methanol plus 5% ammonium hydroxide. ESI-MS was performed on the Model API-3000 as above. For precursor ion scans, the instrument scanned in the negative ion mode for precursors of −79 Da (PO_3H). The collision energy and collision gas were optimized to produce the desired fragment ion efficiently.

The presence of phosphopeptides in the digest was also assessed following alkaline phosphatase treatment. An aliquot of the digest was dried, reconstituted in 10 mM ammonium bicarbonate, pH 8, and treated with 0.1 unit of calf intestinal alkaline phosphatase at 37 °C for 2 h. The treated digest and an untreated aliquot were desalted in parallel using ZipTips and analyzed by MALDI-TOF MS. A tryptic digest of bovine β-casein was analyzed as a positive control. MALDI-TOF MS analysis was performed on a Model ReflexIII mass spectrometer (Bruker) equipped with a 337 nm nitrogen laser. The matrix was α-cyano-4-hydroxycinnamic acid.

Overexpression and Activity of Full-Length MLK1. Plasmids expressed the cDNA of interest under control of the CMV promoter. They were purified with the EndoFree plasmid maxi kit, and the DNA concentration was determined by absorbance at 260 nm. CHO cells were maintained in Dulbecco's modified Eagle's medium plus 10% (v/v) heat-inactivated fetal bovine serum. For transfection experiments they were plated in six-well plates and transfected when between 40% and 60% confluent. CHO cells were cotransfected with expression plasmids coding for MLK1 (wild type or mutant) and HA-JNK, using Lipofectamine Plus reagent as per the manufacturer's directions, at 1 μg of DNA per well. In experiments where a given DNA was varied, total DNA was held constant at 1 μg per well by addition of empty vector pcDNA3.1. Two days after transfection the cells were washed twice with ice-cold TBS and then lysed with 0.1 mL per well of lysis buffer (Tris-HCl, 20 mM, pH 7.8; NaCl, 50 mM; Triton X-100, 1% (v/v); phenylmethanesulfonyl fluoride, 0.2 mM; E-64, 10 μM; leupeptin, 10 μM; pepstatin A, 10 μM; NaF, 20 mM; β-glycerophosphate, 25 mM; sodium orthovanadate, 2 mM). Lysates were clarified by centrifugation at 16000*g* for 10 min. Protein concentration in the lysate was determined by BCA assay using BSA as standard.

JNK Activity ELISA. JNK activity in mammalian cell lysates was determined by an ELISA assay that paralleled the MLK1 assay described above. The microtiter plate was coated with 20 μg/mL GST-c-Jun and then blocked. Concentrated assay buffer was added to each well, followed by several dilutions of lysate for each sample. Addition of ATP to 50 μM initiated the kinase reaction, which continued at 37 °C for 15 min. The final assay mixture (0.1 mL per well) contained 20 mM HEPES, pH 7.4, 0.02% BSA, 20 mM $MgCl_2$, 2 mM DTT, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM activated sodium orthovanadate, and 50 μM ATP. Addition of EDTA to 83 mM terminated the kinase reaction. The phosphorylated product was detected by incubation with anti-phospho-c-Jun(Ser73) antibody, incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase, and reaction with 4-methylumbelliferyl phosphate. For each experiment, a preliminary assay was performed with several dilutions of positive control lysate (diluted in 0.1 mg/mL BSA) to determine the range in which assay response was proportional to lysate protein. The assay was then performed for all samples with the amount of lysate protein at the top of that range (in these experiments, generally 40 ng of protein per well).

RESULTS

Cloning of Full-Length MLK1. Full-length MLK1 was cloned as described in Experimental Procedures. The sequence codes for a protein of 1104 amino acids with a predicted molecular mass of 122 kDa and a domain structure nearly identical to MLK2 and MLK3. Features in common, listed from NH_2 to COOH terminus, include a Src homology 3 (SH3) domain, a canonical protein kinase domain, a leucine zipper, a basic domain, a cdc42/rac-interactive binding motif (CRIB motif), and a large Pro- and Ser-rich domain whose sequence diverges from other MLK family members (Figure 1A).

Active MLK1 Is Threonine Phosphorylated. A common mechanism for activation of protein kinases is phosphorylation of residues in the activation loop (*19*, *20*). Phosphorylation of activation loop residues has been shown to activate the related kinase MLK3 (*9*). To test the role of phosphorylation in the activation of MLK1, the kinase domain of MLK1 was cloned into baculovirus as a GST fusion protein, expressed in Sf21 cells, and purified by glutathione affinity. In vitro, the wild-type kinase domain phosphorylated both the general protein kinase substrate MBP and the physiological MLK1 substrate MKK4 (Figure 1C,D). Mutation of the essential Lys171 to Ala abolished kinase activity. Immunoblotting demonstrated that the wild-type kinase domain was threonine phosphorylated but not tyrosine phosphorylated (Figure 1B). (No data are shown for serine phosphorylation because, in our hands, all commercial phosphoserine antibodies cross-reacted with phosphothreonine.) The inactive K171A mutant, however, was not phosphorylated. Taken together, these data are consistent with MLK1 activation by autophosphorylation on Thr (and possibly Ser) residues.

Active MLK1 Is Phosphorylated in the Activation Loop. GST-$MLK1_{KD}$ was digested with endoproteinase LysC, and the digest was analyzed by mass spectrometry. Phosphate precursor ion scanning detected phosphopeptides at 1910.6 and 1991.0 Da (Figure 2). These correspond to singly and doubly phosphorylated forms of MLK1(292−306) (predicted molecular masses 1910.0 and 1990.0 Da). This peptide includes two of the phosphorylatable residues in the activation loop, Thr304 and Thr305 (as well as one phosphorylatable residue upstream, Thr293). In a second approach, the digest was analyzed by MALDI MS before and after treatment with alkaline phosphatase. The rationale for this approach is that phosphopeptides do not ionize well under MALDI. Alkaline phosphatase treatment can thus reveal a

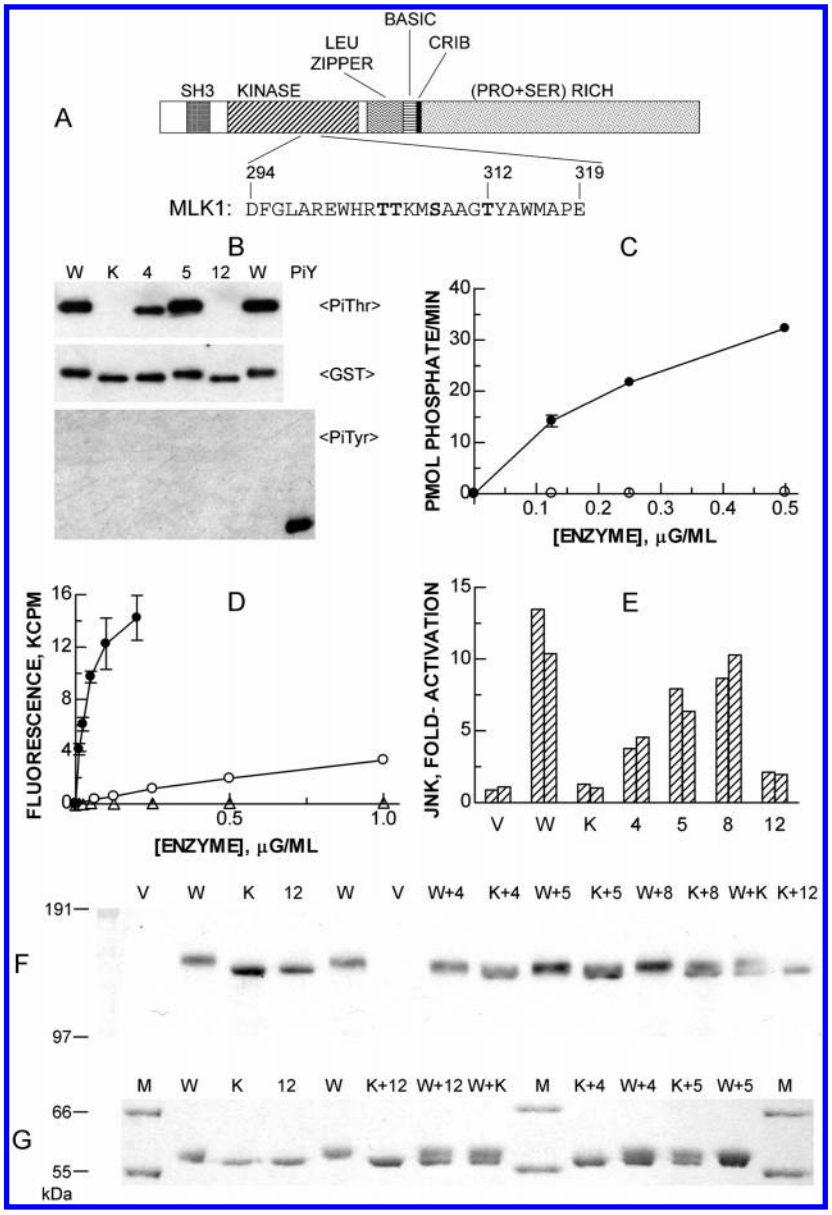

FIGURE 1: (A) Schematic of protein domains in the full-length sequence of MLK1. The activation loop sequence of MLK1 is given below the schematic, with the phosphorylatable Ser and Thr residues in boldface type. (B) Phosphorylation of $MLK1_{KD}$s. $MLK1_{KD}$s were expressed and purified as GST fusion proteins. The indicated proteins were separated by electrophoresis through 4−12% Bis-Tris gels, immunoblotted to PVDF, and probed with the indicated antibodies. Immune complexes were visualized by ECL. Key: W, wild type; K, K171A mutant; 4, T304A; 5, T305A; 12, T312A; PiY, positive control for tyrosine phosphorylation (*16*). The top of each panel aligns with the 64 kDa molecular mass standard; the bottom aligns with either 51 kDa (⟨PiThr⟩ , ⟨GST⟩) or 39 kDa (⟨PiTyr⟩). (C) Kinase activity of $MLK1_{KD}$s determined by ^{32}P phosphorylation of MBP. Filled circles (●) denote wild type; open circles (○) denote T312A. (D) Kinase activity of $MLK1_{KD}$s determined by ELISA-based detection of MKK4 phosphorylation. Filled circles (●) denote wild type; open circles (○) denote T312A; open triangles (△) denote K171A. (E) JNK pathway activation by MLK1 and its activation loop mutants. The indicated constructs were introduced into CHO cells by cotransfection with HA-JNK. Key: V, empty vector control; W, wild type; 8, S308A mutant; other abbreviations as in panel B. JNK activity is expressed as fold activation over the empty vector control. All conditions were transfected in duplicate; individual bars represent individual cultures. The result depicted is representative of two independent experiments. (F) SDS−PAGE mobilities of full-length MLK1 activation loop mutants. Full-length MLK1 constructs were expressed in CHO cells and detected by immunoblotting. Cell lysates were separated on a 4−12% Bis-Tris gel in MOPS buffer. To improve resolution, the gel was run for 1 h (when the dye front reached the bottom of the gel), the running buffer was replaced, and the gel was run 1 h more. The proteins were then transferred to nitrocellulose, probed with anti-Xpress antibody, and visualized by ECL. (G) GST-$MLK1_{KD}$s were separated on a 4−12% Bis-Tris gel in MOPS buffer and stained with Colloidal Coomassie. Key: M, molecular mass standards; other designations as in panel E. Sums denote mixtures of the indicated lysates (e.g., W + 12 denotes wild type mixed with T312A).

signal due to the corresponding dephosphorylated peptide (*21*). Alkaline phosphatase treatment of the GST-$MLK1_{KD}$ digest increased the signal of only a single peptide relative to the other peptides in the digest (data not shown). The increased signal stood at a *m*/*z* of 2404.9 Da; this *m*/*z* corresponds to MLK1(307−328) (predicted molecular mass 2404.8 Da), which includes the other two phosphorylatable residues in the activation loop, Ser308 and Thr312 (as well as two phosphorylatable residues downstream, Ser324 and Ser327). No increase at this *m*/*z* was observed upon alkaline phosphatase treatment of the kinase-dead mutant GST-MLK1[K171A]. Mass spectrometry is thus consistent with phosphorylation at two or more different sites in or near the activation loop of active MLK1.

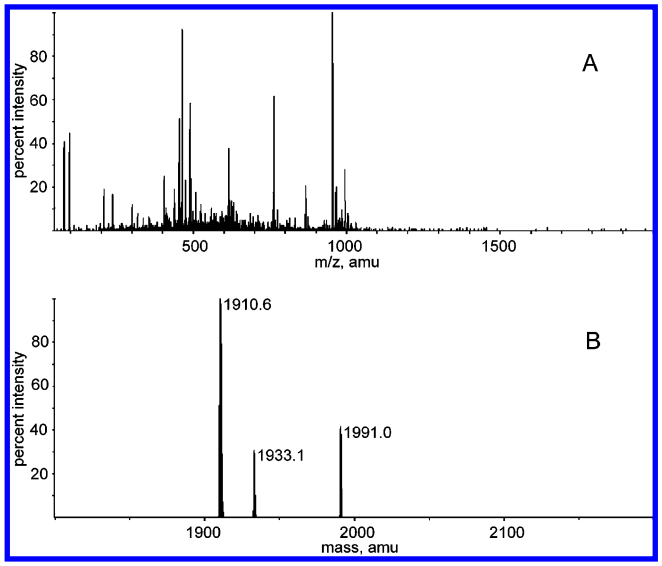

FIGURE 2: Mass spectrometric identification of MLK1 phosphorylation sites by phosphate precursor ion scan. (A) Raw data spectrum (multiply charged species) for precursors of −79 Da from a LysC digest of $MLK1_{KD}$ fractionated on a nanocolumn and eluted with 50% methanol. (B) Deconvoluted spectrum (M_r species). Peptides were observed at 1910.6 and 1991.0 Da, corresponding to singly and doubly phosphorylated forms of MLK1(292−306) (predicted molecular masses 1910.0 and 1990.0 Da). The peptide at 1933.1 was the sodium adduct of 1910.6.

Role of Phosphorylation in the Activation Loop. To test the consequences of activation loop phosphorylation for MLK1 activity, each of the four Ser or Thr residues in the activation loop was mutated to Ala in both GST-kinase domain constructs (expressed and purified from Sf21 cells) and in full-length constructs (expressed in CHO cells). The kinase domains were purified by glutathione affinity, their phosphorylation was assayed by immunoblot, and their activity was assayed in vitro. Mutation of Thr312 to Ala had the greatest effect on both phosphorylation and activity. Threonine phosphorylation of the T312A mutant was undetectable, while a parallel anti-GST blot showed that the protein load was the same (Figure 1B). Threonine phosphorylation of the T304A mutant was reduced compared to wild type, while T305A was nearly the same as wild type. The activity of the T312A mutant was not detectable by ^{32}P phosphorylation of MBP and was only 2% the activity of wild type by the more sensitive ELISA-based assay for phosphorylation of MKK4 (Figure 1C,D; Table 1). The rank order of mutant activities is wild type > T305A > T304A > T312A > K171A. (The S308A mutant was omitted from the kinase domain experiments because Sf21 cells expressed this form at substantially lower levels than the others.)

High levels of expression should drive dimerization of MLKs and, consequently, their activation in the absence of a physiological stimulus. Consistent with this expectation, mammalian cells transfected with MLK constructs express active MLKs (*5*, *6*, *8*). We therefore assessed mutant activities in full-length MLK1 by transfecting CHO cells and assaying JNK pathway activation. Immunoblot revealed that all four activation loop mutants expressed at comparable levels to wild type (data not shown). Once again, mutation of Thr312 to Ala had the greatest effect on activity, reducing JNK activation near the vector control or the kinase-dead K171A mutant (Figure 1E). The rank order of JNK activation by MLK1 constructs is wild-type > S308A ≅ T305A > T304A > T312A > K171A.

Table 1: Activity of MLK1 Activation Loop Mutants[a]

mutation	kinase domain in vitro (%)	full length in cells (%)
K171A	ND	2−10
T304A	16	20−29
T305A	37	56−59
S308A	7[b]	59−78
T312A	2	10−14

[a] Mutant activity is expressed as percent wild-type activity. ND, not detectable. The activity of kinase domains in vitro was determined by MultiScreen assay with MBP as substrate, with the exception of T312A, whose activity was determined by the more sensitive ELISA with MKK4 as substrate. Activity ratios are based on the linear portion of the activity−enzyme curve. Activity of full-length constructs was determined by JNK activation in CHO cells. The range of activities in two independent experiments is given. These activity ratios are based on transfections at a single amount of DNA. Under the transfection conditions used in these experiments, all four activation loop mutants expressed at comparable levels to wild type (data not shown). [b] The low activity of the S308A kinase domain is difficult to interpret as this form expressed in insect cells at much lower levels than the other mutants. No such difference in expression level was noted for activation loop mutants of full-length MLK1 expressed in CHO cells (including the S308A mutant).

All full-length MLK1s ran on SDS−PAGE near their predicted molecular mass (126 kDa), but their mobilities were shifted slightly (Figure 1F). Phosphorylation reduces mobility on SDS−PAGE for many proteins (*22*), including the closely related MLK3 (*5*, *23*). Significantly, if the observed mobility differences among MLK1 mutants are due to the degree of phosphorylation, they are consistent with phosphorylation leading to enzyme activation. Kinase-dead MLK1[K171A] ran significantly faster than wild type, and T312A ran almost as fast as K171A. This can be most clearly seen in the mixing experiment, where wild type and K171A (W + K) were clearly resolved but K171A and T312A (K + 12) appeared as a single band. The other activation loop mutants were not cleanly resolved from either wild type or K171A, but T305A and S308A were better separated from K171A than they were from wild type (that is, the band in mixtures with K171A looked thicker), consistent with their activity being closer to wild type. The same pattern was seen for kinase domains (Figure 1G), where the resolution can be better because the proteins are smaller. The T312A kinase domain was cleanly resolved from wild type but comigrated with K171A. (Consistent with this observation, we were unable to detect threonine phosphorylation of either K171A or T312A kinase domains by immunoblot; see Figure 1B.) T304A and T305A were intermediate in mobility to both wild type and K171A, with the thickness of the mixture "bands" suggesting that T304A was closer to K171A and T305A was closer to wild type. In summary, the mobilities of both full-length MLK1s and kinase domains rank in the order wild-type < T304A, T305A, and S308A < T312A and K171A, consistent with the rank order of their activity.

Basal Activity of MLK1[T312A]. Because mutation of Thr312 had greater effect than mutation elsewhere in the activation loop, the consequence of this mutation was examined in greater detail. Limited tryptic digestion suggested that the overall folding and stability of the mutant were not grossly different from the wild type (Figure 3).

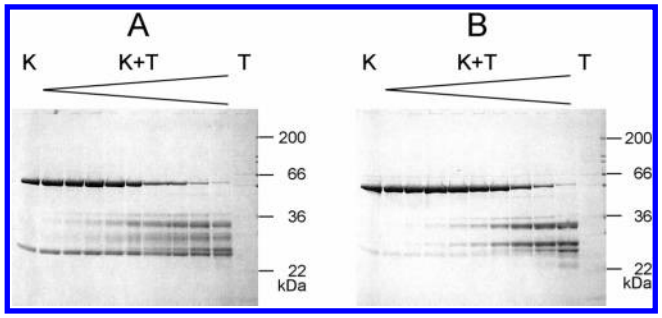

FIGURE 3: Limited tryptic digestion of (A) wild type and (B) T312A mutant forms of GST-MLK1$_{KD}$. Key: K, kinase domain alone; K + T, kinase domain plus trypsin (trypsin concentration increasing in 2-fold steps from 2.5 to 640 units/mL); T, trypsin alone (640 units/mL). For each reaction, 2 μg of kinase domain was digested by the indicated amount of trypsin in a total volume of 20 μL for 20 min at 37 °C. The gel was stained with Colloidal Coomassie.

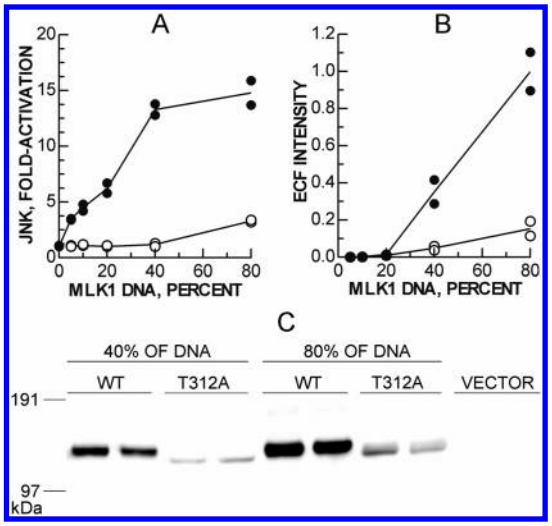

FIGURE 4: Dose−response for cellular JNK pathway activation by MLK1 and MLK1[T312A]. CHO cells were cotransfected with HA-JNK (20% of total DNA) and varying amounts of MLK1 (wild type or T312A); total DNA per well was kept constant with vector DNA. Note that the amount of HA-JNK DNA differs from the experiment in Figure 1E; the conditions in this experiment permitted variation of MLK1 expression levels. (A) JNK activity determined by ELISA. (B) Expression level determined by immunoblotting visualized with enhanced chemifluorescence (ECF) and normalized to the highest level obtained in this experiment. Filled circles (●) denote wild type; open circles (○) denote T312A. (C) Comparison of the SDS−PAGE mobilities of MLK1 and MLK1[T312A]. The immunoblots were probed with anti-Xpress antibody and visualized by ECF.

Trypsin generated a similar, though not identical, pattern of proteolytic fragments from both forms of MLK1, over a similar range of trypsin concentrations.

Consistent with the residual activity of the T312A mutant kinase domain, overexpression of MLK1[T312A] weakly activated the JNK pathway (Figure 4). Transfection with T312A DNA as 80% of the total activated JNK 3-fold over the vector, the same as transfection with wild-type MLK1 DNA as only 5% of the total. Under these conditions, T312A protein expressed at an ~100-fold higher level than wild-type protein. MLK1[T312A] therefore activated the JNK pathway with about 1% the efficacy of wild-type MLK1. This is very near the reduction in specific activity measured for the kinase domain (Table 1).

Wild-type MLK1 and MLK1[T312A] expressed in CHO cells both ran between 97 and 190 kDa on Bis-Tris gels,

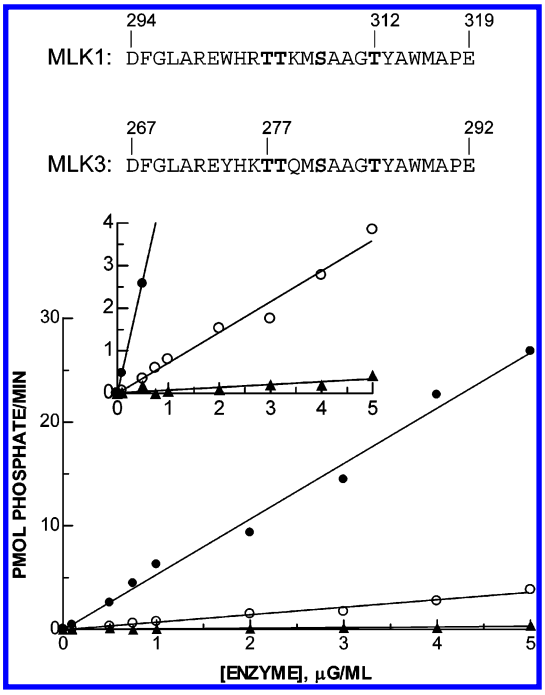

FIGURE 5: Kinase activity of GST-MLK3$_{KD}$s determined by ^{32}P phosphorylation of MBP. Filled circles (●) denote wild type; open circles (○) denote T285A; filled triangles (▲) denote T277A. The inset displays the same data on an expanded scale. The activation loop sequences of MLK1 and MLK3 are given above the graph, with the phosphorylatable Ser and Thr residues in boldface type.

consistent with their predicted molecular mass of 126 kDa (Figure 4C). The T312A mutant ran with greater mobility than wild type, but at the highest levels of expression it began to shift toward wild-type mobility. The different mobilities are consistent with phosphorylation of the wild-type protein that is absent from the mutant. Furthermore, the shift in mutant protein mobility at higher expression levels suggests there are multiple phosphorylation sites in MLK1. First, Thr312 had been mutated to a nonphosphorylatable amino acid, yet the mobility of the mutant protein still shifted. Second, the magnitude of the shift for wild-type protein was greater than for the mutant.

Comparison with MLK3. The activation loops of MLK1 and MLK3 have nearly identical sequences (Figure 5). Leung and Lassam mutated some of the phosphorylatable residues in the activation loop of MLK3 and reported that Thr277 (homologous to Thr304 of MLK1) had the most profound effect on activity (*9*). They did not report mutation of Thr285 (homologous to Thr312 of MLK1). Because mutation of Thr312 had a greater effect on the activity of MLK1 than mutation of Thr304, we introduced each of the corresponding mutations into MLK3$_{KD}$ and measured the in vitro activity of purified kinase domains (Figure 5). The data confirmed the importance of Thr277 in the activation of MLK3; the activity of the T277A mutant was only 1% the activity of the wild type. Mutation of Thr285 had less effect; the T285A mutant retained 14% of the activity of the wild type.

DISCUSSION

We have shown here that the kinase domain of MLK1 was Thr phosphorylated but that a kinase-dead mutant was not. We have shown by mass spectrometry that at least some of these phosphorylation sites were in or near the activation

loop of MLK1. Mutation of the Ser and Thr residues in the activation loop of MLK1 to nonphosphorylatable Ala reduced the activity of MLK1 by three independent measures: in vitro phosphorylation of MBP or MKK4, activation of the JNK pathway in mammalian cells, and autophosphorylation. Mutation of Thr312 had the greatest effect, reducing activity to only 1−2% of wild type. Limited tryptic digestion was inconsistent with gross unfolding of the T312A mutant protein. Together, these data are consistent with a model in which MLK1 is activated by phosphorylation in its activation loop. This phosphorylation can be autophosphorylation, as the phosphorylation was absent on the kinase-dead mutant. Phosphorylation of multiple sites is necessary for full activity.

In the most straightforward model for these data, phosphorylation of Thr312 is the key step in activation of MLK1. The evidence for phosphorylation at this site, however, is indirect. Alkaline phosphatase treatment of a tryptic digest increased the intensity of a peptide that included Thr312, implying it had been phosphorylated, but MALDI MS is not quantitative. Even though the increase in intensity was not observed in the kinase-dead mutant, these data are not as strong as phosphate precursor ion scanning. Mutation of Thr312 to Ala had a greater effect on activity than mutation of the other Thr or Ser residues in the activation loop, but an alternate explanation is that this mutation causes a conformational change that restricts the enzyme to basal activity (although limited tryptic digestion was inconsistent with gross unfolding). Yet another explanation is that unphosphorylated Thr312 plays an important role in the active conformation of MLK1. Thr312 is a key site for activation of MLK1, and to suppose that it must be phosphorylated would make parallel the activation mechanisms of MLK1 and MLK3 (*9*). The available data are consistent with this model but do not rule out others.

The experiments reported here address primarily the role of activation loop phosphorylation in MLK1 activation. In particular, the kinase domains used to measure in vitro kinase activity did not include the leucine zipper, and their activity was therefore independent of leucine zipper mediated dimerization. On the other hand, the kinase domains were expressed as GST fusion proteins, and GST can mediate dimerization of its fusion partners (*24*−*26*). Similarly, overexpression of full-length MLK1 in CHO cells should drive its dimerization in the absence of a physiological stimulus. The roles played by dimerization and by interaction with other regulatory proteins (such as rac/cdc42) in the activation of MLK1 remain to be determined.

Phosphorylation in the activation loop can affect both active site conformation and protein substrate recognition (*9*). Note, however, that the three measures of MLK1 activity reported here depend on different protein substrates. In vitro activity reflects phosphorylation of MBP or of MKK4. Cellular JNK activation reflects phosphorylation of MKK4/MKK7. Mobility shift and threonine phosphorylation reflect autophosphorylation. That all three measures rank the phosphorylation sites in the same order makes it more likely that this order reflects their importance to active site conformation and intrinsic enzyme activity.

MLK1 and MLK3 have a broadly similar mechanism of activation: phosphorylation of multiple residues in the activation loop is required for full activity, but there is a key residue whose mutation to a nonphosphorylatable alanine has a greater impact on enzyme activity. The identity of that key residue, however, differs between the two enzymes. The key residue in MLK3, Thr277, is not homologous to the key residue in MLK1, Thr312, but to Thr304, which is of secondary importance in the activation of MLK1. It may seem surprising that the key residues for activation of MLK1 and of MLK3 are not homologous when the activation loops differ by only 3 residues out of 26, and two of the three substitutions are conservative (Figure 5). The nearly identical activation loops interact, however, with different residues elsewhere in the kinase domain. A three-dimensional structure of both kinase domains would be required to explain the difference in their activation mechanism.

Although mutation of Thr312 has the most profound effect among the phosphorylatable residues in the activation loop, the T312A mutant retains a basal activity on the order of 1% the activity of the wild-type enzyme. We suggest that an unphosphorylated wild-type MLK1 may also have a small but nonzero level of kinase activity. Basal activity seems necessary to a kinase capable of activation by autophosphorylation.

Although mutation of Thr312 has the most profound effect, several lines of evidence indicate that MLK1 activation involves phosphorylation at additional sites. Mass spectrometry identified phosphorylation in two nonoverlapping peptides that cover the activation loop; phosphate precursor ion scanning identified a doubly phosphorylated form of one of these peptides. At high levels of expression in mammalian cells, the mobility of MLK1[T312A] on SDS−PAGE shifted even though Thr312 had been mutated to a nonphosphorylatable residue. Finally, the mutants T304A and T305A (and possibly S308A) had measurably and reproducibly diminished activity. These observations suggest that phosphorylation of multiple sites is necessary for full activation of MLK1. Activation of MLK3 also involves multiple phosphorylations (*9*). Why are multiple phosphorylations required for full activation of MLKs? Multiple phosphorylation has been analyzed theoretically as a mechanism for introducing cooperativity into the transition between active and inactive forms of a kinase, turning the transition into a switch (*27*). An example is the cyclin-dependent kinase inhibitor Sic1, which requires six phosphorylations to drive its ubiquitination and degradation, which in turn allows cell cycle progression from the G1 to the S phase. The requirement for multiple phosphorylations contributes to the ultrasensitive, switch-like nature of the G1/S transition (*28*). This phenomenon may be relevant to activation of the JNK pathway, which activates as a switch upon stimulation of *Xenopus laevis* oocytes (*29*). Because MLKs, when activated, can initiate apoptotic pathways via the JNK pathway, switch-like kinetics might be especially important to prevent inappropriate activation.

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SUPPORTING INFORMATION AVAILABLE

Nucleotide sequence of cDNA coding for mixed-lineage kinase 1 and the deduced protein sequence (supplemental Figure 1) and amino acid sequences of the GST-MLK_{KD}s used in this work (supplemental Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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