Dynamic Positive Feedback Phosphorylation of Mixed Lineage Kinase 3 by JNK Reversibly Regulates Its Distribution to Triton-soluble Domains

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MLK3 (mixed lineage kinase 3) is a widely expressed, mammalian serine/threonine protein kinase that activates multiple MAPK pathways. Previously our laboratory used in vivo labeling/mass spectrometry to identify phosphorylation sites of activated MLK3. Seven of 11 identified sites correspond to the consensus motif for phosphorylation by proline-directed kinases. Based on these results, we hypothesized that JNK, or another proline-directed kinase, phosphorylates MLK3 as part of a feedback loop. Herein we provide evidence that MLK3 can be phosphorylated by JNK in vitro and in vivo. Blockade of JNK results in dephosphorylation of MLK3. The hypophosphorylated form of MLK3 is inactive and redistributes to a Triton-insoluble fraction. Recovery from JNK inhibition restores MLK3 solubility and activity, indicating that the redistribution process is reversible. This work describes a novel mode of regulation of MLK3, by which JNK-mediated feedback phosphorylation of MLK3 regulates its activation and deactivation states by cycling between Triton-soluble and Triton-insoluble forms.

Protein kinases and their signaling cascades are dynamically regulated through protein-protein interactions, subcellular targeting, and phosphorylation. By differential integration of these regulatory mechanisms, a protein kinase and its signaling pathway can lead to distinct cellular responses. In addition, many protein kinases are subjected to feedback regulation, and biological signaling pathways are modulated by positive and negative regulatory feedback loops.

MLK3 (mixed lineage kinase 3) is a widely expressed mammalian serine/threonine kinase that functions as a mitogen-activated protein kinase kinase kinase (MAPKKK) and can activate multiple MAPK pathways. MLK3-induced JNK activation, through dual phosphorylation of MKK4 and/or JNKK2/MKK7, is implicated in neuronal apoptosis in response to trophic factor withdrawal (3–6). Targeted gene disruption of mlk3 decreases tumor necrosis factor-α-induced JNK activation, consistent with findings in human cell lines (8, 9). In addition, Slipper, the MLK3 homolog in Drosophila, is required for JNK-dependent dorsal closure in the fly embryo (10). MLK3 can also activate the p38 pathway (11), perhaps in a JNK-interacting protein-2-dependent fashion (12–14), and recently a scaffolding role for MLK3 in B-Raf-mediated ERK activation has emerged (15, 16).

Our laboratory has focused on understanding the mechanisms that regulate MLK3 activity and signaling. In addition to its catalytic domain, MLK3 contains several other regions important for its regulation, including an N-terminal Src homology 3 domain and a centrally located zipper followed by a COOH-terminal region of MLK3 is rich in Ser, Thr, and Pro residues, with the final 220 amino acids of MLK3 being composed of 12, 13, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acids. Activated forms of the small GTPases, Cdc42 and Rac, can bind to MLK3, cause retardation of MLK3 electrophoretic mobility, increase MLK3 volume, and 24%, respectively, of these amino acid...
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labeling coupled with mass spectrometry (25). Strikingly, seven of the identified sites, serines 524, 705, 727, 740, 758, 770, and 793, are followed immediately by a proline, thus conforming to the consensus for phosphorylation by proline-directed kinases.

The identification of sites of proline-directed phosphorylation of MLK3, together with the ability of MLK3 to activate multiple MAPK pathways, led us to hypothesize that MLK3 might be regulated through feedback phosphorylation by MAPKs. Herein we present evidence for JNK-mediated phosphorylation of MLK3 in cells. JNK also phosphorylates MLK3 in Triton-soluble domains. Enforcement of such a positive feedback loop in which the activated MAPKKK, MLK3, is phosphorylated by the downstream MAPK, JNK, leading to preferential distribution of MLK3 to a Triton-soluble fraction, whereas blockade of JNK with clarified cellular lysates and immunoprecipitates were washed as previously described (19). For the Triton X-100 biochemical fractionation, the cells were lysed as described above, and the Triton-insoluble pellets were washed in lysis buffer and then resuspended in 2× SDS loading buffer prior to SDS-PAGE.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The following reagents were purchased from Calbiochem: SP600125, SB203580, U0126, roscovitine, and kenpaullone. Calyculin A was obtained from BioMol. The mixed lineage kinase inhibitor CEP-11004 was generously provided by Cephalon, Inc. AP21967 was generously provided by Ariad Pharmaceuticals. Mouse monoclonal antibodies against phospho-c-Jun (KM-1) and green fluorescent protein as well as antibodies against Hsp90 and Cdc37 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Ca). The phospho-MLK3 antibody and the phospho-JNK antibody were from Cell Signaling Technology. The monoclonal antibody against cytochrome oxidase subunit IV was from Stressgen. Other antibodies used were the MLK3 rabbit polyclonal antibody (19), actin and FLAG mouse monoclonal antibody (Sigma), hemagglutinin (HA) mouse monoclonal antibody (Babco), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad).

Construction of Mammalian Expression Vectors and Site-directed Mutagenesis—Construction of the expression vectors pRK5-MLK3, pRK5-MLK3 K144R, pCGN-HA-MLK3, and pRK5-N-FLAG-Cdc421212 has been described elsewhere (21, 26). MLK3 K144M was generated by the QuikChange site-directed mutagenesis method (Stratagene) using the oligonucleotide 5′-CTGGTGCGCTGTGATGGCCGCTGCCC-3′ and its reverse complement.

FLAG-MKK7 K165A and glutathione S-transferase-MKK4 K129R were generated using pCDNA3-FLAG-MKK7 and pEBG-glutathione S-transferase-MKK4 as templates and the following oligonucleotides and their reverse complements: 5′-CATTTGCTGTGGCAGAATTCGGTCAACT-3′ for MKK7 and 5′-CACATTTGCGAGTTGAGAATTCGGTCAACT-3′ for MKK4. Other mammalian expression vectors include pSRa3HA-JNK2-JNK1 and pCMS-EGFP.

Cell Culture and Transfections—Human embryonic kidney (HEK) 293 cells and HeLa cells were cultured as described previously (19, 20). Transfections were performed using the calcium phosphate technique or using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. MCF-7/iFLAG-MLK3 cells inducibly expressing FLAG-MLK3 have been described elsewhere (9). FLAG-MLK3 expression was induced by the addition of 50 nM AP21967 for 20 h.

Cell Lysis and Immunoprecipitation—Cells were lysed in 1% Triton lysis buffer, containing 50 mm HEPES (pH 7.5), 150 mm NaCl, 1.5 mm MgCl2, 2 mm EGTA, 1% Triton X-100, 10% glycerol, 10 mm NaF, 1 mm Na3VO4, 100 μm β-glycerophosphate, 1 mm Na2VO4, 2 mm phenylmethylsulfonyl fluoride, and complemented with a mixture of protease inhibitors (Sigma). Following sonication for 40 s in a sonication bath to enhance disruption of organelles, lysates were clarified by centrifugation at 14,000 rpm at 4 °C for 15 min. For immunoprecipitations, antibodies against the proteins of interest were prebound to Protein A-agarose beads at room temperature for 30 min and incubated with clarified cellular lysates for 90 min at 4 °C. The immunoprecipitates were washed as previously described (19). For the Triton X-100 biochemical fractionation, the cells were lysed as described above, and the Triton-insoluble pellets were washed in lysis buffer and then resuspended in 2× SDS loading buffer prior to SDS-PAGE.

Gel Electrophoresis and Western Blot Analysis—Cellular lysates and immunoprecipitates were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes that were immunoblotted using appropriate antibodies. Western blots were developed by the chemiluminescence method as described previously (19).

Pro-Q Diamond and Silver Staining—HEK 293 cells were transfected with vectors encoding MLK3 or MLK3 K144M as described. MLK3 was immunoprecipitated as described above and resolved by SDS-PAGE. The gel was stained with Pro-Q Diamond stain (Invitrogen) following the manufacturer’s instructions. The image was acquired using a Molecular Imager FX Pro Plus scanner and PDQuest version 7.4 (Bio-Rad). The gel was then silver-stained using SilverQuest (Invitrogen) following the manufacturer’s instructions. Quantitations were done by densitometry (Image); available on the World Wide Web at rsb.info.nih.gov/ij/).

In Vivo Labeling and Phosphopeptide Mapping—12 h after transfection, HEK 293 cells were washed twice with phosphate-free medium (Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum (Sigma)) and incubated at 37 °C for 1 h. The cells were then pretreated with the different MAPK pathway inhibitors (40 μm SP600125, 6 μm SB203580, or 10 μm U0126) or vehicle Me2SO for 1 h before the addition of 1 mCi/ml [32P]orthophosphate (PerkinElmer Life Sciences) and further incubated for 4 h at 37 °C. Cells were washed twice with ice-cold PBS and then lysed as described above. MLK3 was immunoprecipitated as described above and resolved by SDS-PAGE. Incorporation of radiolabel into MLK3 was quantified by phosphorimaging and normalized to the MLK3 expression levels, determined by den-
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sitometry (ImageJ). Radiolabeled bands were excised from the gel and digested with trypsin (Roche Applied Science), and phosphopeptide maps were developed as described previously (25).

In Vitro Phosphorylation of MLK3—HEK 293 cells were transfected with a vector encoding MLK3 K144M. 20 h after transfection, the cells were washed and lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors. MLK3 K144M was immunopurified from clarified cell lysates. Kinase reactions were carried out in 50 μl of kinase buffer (50 mM Tris-Cl, pH 7.5, 0.1 mM EGTA, 10 mM MgCl2, 0.1% β-mercaptoethanol) containing 10 μM ATP, 10 μCi of [γ-32P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences), and 0.5 μg (57 milliunits) of recombinant, activated JNK1α1 (Upstate Biotechnology, Inc.) at 37 °C for 30 min. Proteins were fractionated by SDS-PAGE, and the incorporation of radioactivity into MLK3 was measured by phosphorimaging.

RESULTS

JNK Is Required for in Vivo Phosphorylation of MLK3—Using mass spectrometry coupled with comparative phosphotryptic peptide mapping, we previously identified 11 in vivo phosphorylation sites on MLK3 (25). In our strategy, HEK 293 cells expressing MLK3 with its activator Cdc42V12 for 16 h were subsequently labeled with [32P]orthophosphate for an additional 4 h. After affinity purification and trypsin digestion of MLK3, the resultant radiolabeled phosphotryptic peptides were resolved by chromatography, and their sequences were determined using a combination of matrix-assisted laser desorption ionization mass spectrometry and liquid chromatography-tandem mass spectrometry. Thus, only those sites that are relatively labile (dephosphorylated) and incorporate substantial radiolabel (rephosphorylated) during the 4-h labeling period could potentially be identified.

MLK3 contains 27 potential proline-directed kinase sites, most of which are found in the COOH-terminal region, which is rich in proline, serine, and threonine (26). Examination of the identified phosphorylation sites revealed that seven of the 11 sites conform to the consensus sequence for phosphorylation by proline-directed kinases, and six of the seven sites reside in the COOH-terminal region. This suggests that MLK3 is phosphorylated by proline-directed kinases. Additionally, we have found that, whereas wild type MLK3 incorporates substantial radiolabel under our experimental conditions, a kinase-defective MLK3 variant incorporates very little radiolabel.3

Total phosphate incorporation into MLK3 was determined using the Pro-Q diamond dye. This fluorescent stain detects phosphate groups attached to tyrosine, threonine, or serine residues in a protein (27, 28). MLK3 or a kinase-inactive version MLK3 K144M was expressed in HEK 293 cells. Immunopurified MLK3 variants were resolved by SDS-PAGE. After staining the gel with Pro-Q Diamond, densitometry was used to quantify the relative incorporation of Pro-Q Diamond stain into the MLK3 variants (Fig. 1A). After correcting for expression levels, the extent of Pro-Q Diamond staining was determined to be 40% less for kinase-defective MLK3 than for wild type MLK3. The increased phosphorylation of wild type MLK3 over that of inactive MLK3 is consistent with MLK3 autophosphorylation and/or feedback phosphorylation emanating from active MLK3. Given that MLK3 is implicated in the activation of multiple MAPK pathways, it is reasonable to hypothesize that, upon MLK3-induced activation, MAPKs might phosphorylate MLK3.

To decipher which MAPK(s) might contribute to proline-directed phosphorylation of MLK3, the impact of MAPK pathway inhibitors on the incorporation of radiolabeled phosphate into MLK3 was determined using the same conditions we used in our original mass spectrometry studies. Concentrations of SP600125, U0126, and SB203580 required for inhibition of JNK, ERK (through inhibition of its upstream activator MEK), and p38, respectively, were experimentally determined in HEK 293 cells coexpressing MLK3 and activated Cdc42 (data not shown). Cells were pretreated with MAPK pathway inhibitors prior to and during in vivo labeling and, after affinty purification and SDS-PAGE, the incorporation of radiolabel into MLK3 was quantitated by phosphorimaging. Based on three independent experiments, incorporation of radiolabel into MLK3 was reduced by 70% in the presence of SP600125 (Fig. 1B). In contrast, inhibition of the p38 and ERK pathways does not reduce in vivo phosphorylation of MLK3 (Fig. 1B). In fact, treatment with SB203580 enhances the phosphorylation of MLK3 by about 2-fold. This may reflect the established antagonism between p38 and JNK signaling (29).

Phosphotryptic peptide maps of MLK3 isolated from cells treated with each of the MAPK pathway inhibitors separately, as well as all three together, were generated. The radiolabeled MLK3 was excised from the gel and trypsin-digested, identical cellular equivalents of the phosphotryptic peptides were resolved by thin-layer electrophoresis/TLC, and radioactive phosphopeptides were detected by phosphorimaging. Phosphotryptic peptide maps from the different samples were processed and imaged in parallel so that the intensities of spots between maps could be directly compared. Treatment of cells with SP600125, but not with other MAPK pathway inhibitors, yielded phosphopeptide maps that showed a decrease in intensity of essentially all of the spots corresponding to identified phosphorylation sites (Fig. 1C). Given that more than 80% of the total intensity of phosphotryptic peptides is accounted for by phosphopeptides containing proline-directed kinase sites, these findings are not altogether unexpected. Thus, the activity of JNK, but not of other MAPKs, is apparently responsible for the dynamic COOH-terminal proline-directed phosphorylation of MLK3.

Manipulation of JNK Activity Alters the Phosphorylation of MLK3—Coexpression with activated Cdc42 retards the electrophoretic mobility of MLK3 due to phosphorylation at sites other than in the activation segment (20). The reduced in vivo phosphorylation of Cdc42-activated MLK3 observed upon treatment of cells coexpressing activated Cdc42 and MLK3 with SP600125 is correlated with a more rapidly migrating, hypophosphorylated form of MLK3 (Fig. 1). Because SP600125 may target other protein kinases in addition to JNK (30), we sought alternative means to manipulate cellular JNK activity so

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3 P. O. Vacratsis and K. A. Gallo, unpublished data.
as to provide further support for the idea that JNK phosphorylates MLK3 in vivo.

MLK3 activates the JNK pathway through phosphorylation of MKK4 (2) and MKK7/JNKK2 (31). Catalytically inactive versions of MKK4 (MKK4 K129R) and MKK7/JNKK2 (MKK7 K165A) can function as dominant negative proteins to block JNK activity in cells. The blockade of JNK activation is most efficient when the inactive versions of MKK4 and MKK7 are coexpressed (32). Therefore, increasing amounts of expression vectors for MKK4 K129R and MKK7 K165A were simultaneously transfected into HEK 293 cells, together with vectors encoding MLK3 and activated Cdc42. Upon introduction of increasing amounts of dominant negative MKK4 and MKK7, efficient inhibition of the Cdc42-MLK3-induced JNK activity in cells was achieved, as judged by the decrease in phospho-c-Jun levels (Fig. 2A, second panel). Under these conditions of efficient JNK inhibition, the electrophoretic mobility of MLK3 was restored to that observed for MLK3 in the absence of Cdc42. These results are similar to the effects of SP600125 on MLK3, suggesting that the effects of SP600125 are, indeed, mediated through inhibition of JNK and that the retarded mobility of MLK3 observed upon coexpression with Cdc42 is mediated, at least in part, through JNK phosphorylation.

FIGURE 1. Effect of MAPK pathway inhibitors on in vivo phosphorylation of MLK3. A, ectopically expressed MLK3 and MLK3 K144M were immunopurified from HEK 293 cells and resolved by SDS-PAGE. Pro-Q Diamond staining was used to detect total phosphorylation levels (left). Total amounts of proteins were detected by silver staining of the same gel (right). An asterisk denotes the position of the MLK3 band. Data from three independent experiments were normalized to total protein levels and quantitated as described under “Experimental Procedures” (bottom). B, HEK 293 cells transiently coexpressing MLK3 and Flag-Cdc42V12 were preincubated in phosphate-free medium containing either SP600125, SB203580, U0126, the combination of all three inhibitors, or carrier (MeSO (DMSO)) as described under “Experimental Procedures.” After in vivo labeling with 32P-inorganic phosphate for 4 h in the presence of the MAPK pathway inhibitors, MLK3 was immunoprecipitated from cleared lysates and resolved by SDS-PAGE. Top, autoradiogram of immunoprecipitated MLK3. Middle, Western blot of a fraction of the immunoprecipitated MLK3 using an MLK3 antibody. Bottom, Western blot of Flag-Cdc42V12 in cellular lysates using a Flag antibody. Incorporation of radioactivity into MLK3 was quantified by phosphorimaging and normalized to MLK3 expression levels as described under “Experimental Procedures.” The means ± S.E. of three independent experiments are shown. C, phosphotryptic peptide maps of MLK3 from cells metabolically labeled in the presence of MAPK inhibitors. The bands corresponding to radiolabeled MLK3 from each sample were excised from the gel and subjected to trypsin digestion. Portions of the recovered peptides corresponding to equal cellular equivalents were analyzed by thin-layer electrophoresis (TLE) in the first dimension and TLC in the second dimension. Identical exposure times of the phosphopeptide maps were analyzed by phosphorimaging. wt, wild type.
constitutively active JNK when expressed in cells (36). To determine whether selective activation of JNK promotes MLK3 phosphorylation, HeLa cells were transfected with expression vectors for MLK3 and the fusion protein JNKK2-JNK1. As shown in Fig. 2B, the introduction of activated JNK in cells results in the appearance of slower migrating forms of MLK3, indicative of phosphorylation of MLK3. Since the majority of the identified phosphorylation sites of MLK3 conform to the consensus for proline-directed kinases, the simplest explanation for our data is that JNK directly phosphorylates MLK3 in cells. To determine whether recombinant, activated JNK can directly phosphorylate MLK3 in vitro, it was necessary to eliminate the background of MLK3 autophosphorylation, and therefore a kinase-defective version of MLK3 (K144M) was expressed, immunopurified from HEK 293 cells, and used as a substrate for recombinant, active JNK in an in vitro kinase assay. As shown in Fig. 2C, JNK phosphorylates MLK3 K144M in vitro, and this phosphorylation results in a decreased electrophoretic mobility of MLK3 (Fig. 2C, bottom). These data support the hypothesis that MLK3 is a direct substrate of JNK.

Feedback Phosphorylation Regulates MLK3 Protein Levels in Cellular Lysates—One intriguing observation in the experiments described above was an apparent correlation between the cellular levels of activated JNK and the levels of MLK3 protein present in cellular lysates. As shown in Fig. 2A, when JNK activity is diminished, less MLK3 is detected. This effect is specific to MLK3, since the levels of FLAG-Cdc42 in cellular lysates, which is expressed using an identical vector, are unchanged in response to JNK inhibition. Likewise, the amount of MLK3 in cellular lysates (Fig. 2B) increases when the corresponding cells express the constitutively active JNK fusion protein, whereas the levels of green fluorescent protein used as a transfection control remain constant. These observations suggest the existence of a positive feedback loop involving MLK3-mediated JNK activation and JNK-mediated phosphorylation of MLK3, leading to increased hyperphosphorylated, active MLK3 in cellular lysates and sustaining activation of the JNK pathway.

The JNK-phosphorylated MLK3 is activated, as judged by activation loop phosphorylation. Activation loop phosphorylation due at least in part to autophosphorylation (20), COOH-terminal phosphorylation by JNK, or both may be required for the increased levels of MLK3 in cellular lysates. As shown previously (20), Cdc42 promotes activation loop phosphorylation of wild type MLK3, increases MLK3 protein levels in cellular lysates, and potentiates JNK activation (Fig. 3A). However, catalytically inactive MLK3 K144R is refractory to regulation by Cdc42 (Fig. 3A), indicating that the enhanced levels of MLK3 in cellular lysates caused by coexpression with the activated GTPase require activation loop phosphorylation and/or JNK activation. To dissociate activation loop phosphorylation, which requires MLK3 kinase activity, from JNK-mediated phosphorylation of MLK3, the constitutively active JNKK2-JNK1 fusion protein was employed. As shown in Fig. 3B, expression of the constitutively active JNKK2-JNK1 fusion protein increases the protein levels in cellular lysates and retards the mobility of both wild type and kinase-defective MLK3, indi-
cating that feedback phosphorylation by JNK, rather than activation segment phosphorylation per se, is the critical determinant in dictating JNK-mediated enhanced levels of MLK3 in cellular lysates.

**Inhibition of JNK Reduces the Levels of Activated MLK3 in Cellular Lysates**—To study the regulation of MLK3 by JNK phosphorylation in a more reproducible, homogeneous system, a stable clone of a human breast cancer cell line, termed MCF-7/iFLAG-MLK3, was used (9). In this cell line, transcription of FLAG-MLK3 is controlled by the small molecule dimerizer AP21967. The induced FLAG-MLK3 potently activates the JNK pathway (9). Consistent with the high activity of MLK3 in this system, the electrophoretic mobility of MLK3 is retarded to a degree comparable with that observed when activated Cdc42 is transiently coexpressed with MLK3 (Fig. 4A).

To test the effects of JNK inhibition on MLK3, MCF-7/iFLAG-MLK3 cells were induced for 20 h with AP21967, washed to remove the inducer, and then treated with different concentrations of SP600125 for 2 h. Fig. 4B shows that inducibly expressed Flag-MLK3 is active, as judged by Western blotting with an antibody specific for activation loop phosphorylation of MLK3. The levels of FLAG-MLK3 in cellular lysates are dramatically decreased upon treatment with the JNK inhibitor,
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in a concentration-dependent manner. Interestingly, the activation loop phosphorylated form seems to disappear more quickly than the net FLAG-MLK3, which may indicate that the active form of MLK3 is most sensitive to regulation by JNK.

Recently, a pharmacological inhibitor of the mixed lineage kinase family, CEP-11004, has been shown in animal models to attenuate JNK-induced apoptotic death of dopaminergic neurons of the substantia nigra (37). Therefore, CEP-11004 was used in our inducible expression system to corroborate the effects on MLK3 protein levels observed when using the JNK inhibitor SP600125. MCF-7 cells inducibly expressing FLAG-MLK3 were incubated with 400 nM CEP-11004, and as shown in Fig. 4C, the levels of phosphorylated c-Jun are decreased dramatically, indicating that JNK activity is potently inhibited. In addition, CEP-11004 reduces the levels of activated and total MLK3 protein in cellular lysates, in a manner similar to that of the JNK inhibitor.

The effect of SP600125 appears to be specific for MLK3, since it had no impact on the levels of a FLAG-tagged estrogen receptor inducibly expressed in MCF-7 cells using the identical expression system and vectors (data not shown). Northern blots using mRNA derived from the MCF-7 cells inducibly expressing FLAG-MLK3 showed that SP600125 did not alter the mRNA level of FLAG-MLK3 (data not shown). These data argue for posttranslational modulation of activated MLK3 by JNK.

Inhibition of Other Proline-directed Kinase Pathways Has No Impact on MLK3 Levels—In addition to the MAPKs, cyclin-dependent kinases and glycogen synthase kinase 3 are proline-directed kinases. To test whether other MAPKs might directly or indirectly regulate protein levels of MLK3, the p38 and ERK pathways were blocked using pharmacological inhibitors. As shown in Fig. 5A, neither the p38 inhibitor, SB203580, nor the MEK inhibitor, U0126, altered the levels of FLAG-MLK3 protein, in agreement with the results from the in vivo labeling experiment. In addition, reactivity with the MLK3 activation loop phosphorylation-specific antibody was maintained, indicating that active MLK3 was unaffected. Likewise, inhibition of the cyclin-dependent kinases with roscovitine (38) or of glycogen synthase kinase 3 with kenpaullone (39) failed to impact levels of total MLK3 or active MLK3 found in cellular lysates (Fig. 5B). Together these data support the idea that inhibition of JNK, but not of other proline-directed kinases, reduces the level of active MLK3.

Dephosphorylation of MLK3 Reduces Its Levels in Cellular Lysates—Our data provide evidence for positive feedback phosphorylation of MLK3 by JNK. We surmise that the JNK phosphorylation sites are relatively labile, given that they incorporate substantial radioactivity during a 4-h labeling period. This raises the idea that JNK and an opposing phosphatase target the same COOH-terminal phosphorylation sites and that MLK3 levels are regulated by the balance between the two. JNK inhibition would be expected to shift the balance toward dephosphorylation and reduction of MLK3 in cellular lysates. If COOH-terminal dephosphorylation signals to down-regulate active MLK3 levels, inhibition of the responsible phosphatase should protect MLK3 levels from JNK inhibition. To test this hypothesis, the FLAG-MLK3-expressing cells were treated with calyculin A, a broad-spectrum serine/threonine phosphatase inhibitor (40, 41), together with the JNK inhibitor. As predicted, calyculin A blocked the effect of SP600125 on MLK3 protein levels and MLK3 activity (Fig. 6), consistent with the model that dephosphorylation of JNK phosphorylation sites somehow down-regulates the levels of MLK3 found in cellular lysates.

JNK Inhibition Redistributes MLK3 to a Triton-insoluble Fraction—One way by which inhibition of JNK might reduce the cellular levels of MLK3 is through ubiquitination and proteasome-mediated degradation of dephosphorylated MLK3. Although MLK3 can be ubiquitinated when ectopically expressed with ubiquitin in HEK 293 cells, increased ubiquitination of MLK3 upon JNK inhibition, even in the presence of proteasome inhibitors, was not observed (data not shown).

We therefore wondered whether JNK-mediated phosphorylation of MLK3 might impact the distribution, rather than the protein stability, of MLK3. To test this idea, the Triton X-100-insoluble pellets obtained after cellular lysis were examined for both the presence and the activation state of MLK3. Briefly, cells inducibly expressing FLAG-MLK3 were incubated with SP600125 alone or with SP600125 in the presence of calyculin
A. Cells were disrupted in the standard 1% Triton X-100-containing buffer, the cellular lysates were removed after centrifugation and, after rinsing in lysis buffer, the Triton-insoluble pellets were resolubilized in SDS-containing buffer. Equal cellular equivalents from the Triton X-100-soluble fractions and the SDS-resolubilized Triton X-100 pellets were examined by Western blotting using identical exposure times and conditions. As shown in Fig. 7, JNK inhibition results in a dramatic shift of MLK3 from a Triton-soluble to a Triton-insoluble fraction, and this redistribution is prevented by the addition of calyculin A. Thus, whereas the total cellular amount of MLK3 is largely unaffected by the manipulation of the phosphorylation state of MLK3, the distribution of MLK3 is profoundly impacted by its phosphorylation status. The MLK3 that redistributes to the Triton-insoluble fraction upon JNK inhibition is largely inactive, as judged by its trace reactivity with the phospho-MLK3 antibody, consistent with a JNK-mediated positive feedback loop.

FIGURE 6. Impact of phosphatase inhibition on MLK3 levels on cellular lysates. FLAG-MLK3 expression was induced using AP21967 in MCF-7/FLAG-MLK3 cells for 20 h. Cells were then incubated with 15 μM SP600125 for 1 h in the presence or absence of 50 nM calyculin A. The cells were then lysed, and the distribution of MLK3 was analyzed by Western blotting using identical exposure times and conditions. As shown in Fig. 7, JNK inhibition results in a dramatic shift of MLK3 from a Triton-soluble to a Triton-insoluble fraction, and this redistribution is prevented by the addition of calyculin A. Thus, whereas the total cellular amount of MLK3 is largely unaffected by the manipulation of the phosphorylation state of MLK3, the distribution of MLK3 is profoundly impacted by its phosphorylation status. The MLK3 that redistributes to the Triton-insoluble fraction upon JNK inhibition is largely inactive, as judged by its trace reactivity with the phospho-MLK3 antibody, consistent with a JNK-mediated positive feedback loop.

The Triton-soluble fractions contain phospho-c-Jun, cytochrome oxidase IV, and ERp72, but these proteins are largely absent from the Triton-insoluble fractions, indicating that the nucleus, mitochondria, and endoplasmic reticulum membranes are efficiently solubilized during the cellular lysis procedure. In a recent study from our laboratory, Hsp90 and its co-chaperone Cdc37 were identified as cochaperones of MLK3 (9). As shown in Fig. 7, the Triton-insoluble fractions are largely devoid of Hsp90 and Cdc37, which may suggest that the dephosphorylation-dependent redistribution of MLK3 to detergent-insoluble domains requires disruption of the MLK3-Hsp90/Cdc37 complex.

Distribution of MLK3 between Triton-soluble and Triton-insoluble Fractions Is Reversible—Our findings that the Triton-insoluble domains contain inactive MLK3 and largely lack the MLK3 chaperones Hsp90/Cdc37 might indicate that the redistributed, insoluble, hypophosphorylated MLK3 is irreversibly aggregated or inactivated. To test whether the ability of hypophosphorylated, inactive MLK3 to signal to JNK could be restored by altering its phosphorylation status, FLAG-MLK3 expression was induced, and the cells were treated with 15 μM SP600125 for 1 h. The JNK inhibitor was then removed by placing the cells in fresh medium either with or without calyculin A for different periods of time. The cells were then lysed and the distribution of MLK3 was analyzed by Western blotting. As shown in Fig. 8, upon removal of the JNK inhibitor, a significant portion of MLK3 redistributed to the soluble fraction and regained its activation status, as judged by activation loop phosphorylation. The portion of MLK3 that remained insoluble also recovered its activation status. Removal of the JNK inhibitor, followed by phosphatase inhibition with calyculin A resulted in quantitative reactivation of MLK3 and redistribution to the soluble fraction. These data indicate that the phosphorylation/dephosphorylation cycle of MLK3 modulates the distribution of MLK3 in a reversible manner.

FIGURE 7. Redistribution of MLK3 to detergent-insoluble fractions. FLAG-MLK3 expression was induced using AP21967 in MCF-7/FLAG-MLK3 cells for 20 h. Cells were then treated with 15 μM SP600125 (SP) for 1 h, in the presence or absence of 50 nM calyculin A. The cells were then lysed, and identical cellular equivalents from the Triton-soluble and Triton-insoluble fractions were analyzed by Western blotting (WB), using appropriate antibodies and identical exposure times.

DISCUSSION
Many signal transduction pathways are modulated by feedback loops. In this work, we provide evidence for a JNK-mediated feedback phosphorylation of MLK3, which results in enhanced MLK3/JNK signaling. When phosphorylation-dependent positive feedback is blocked through JNK or MLK3 inhibition, MLK3 is rapidly inactivated and redistributed to a Triton-insoluble fraction. This JNK phosphorylation-depend-
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Results in active, solubilized MLK3, providing evidence for reversibility of this process, at least in short term treatments.

Feedback phosphorylation of MAPKKks by their downstream MAPKs is not without precedent. For instance, in yeast, pheromone induces the three-component MAPK pathway of Ste11 (MAPKKK)-Ste7 (MAPK kinase)-Fus3/Kss1 (kinase suppressor of SST2 1) (MAPK). Fus3/Kss1-mediated feedback phosphorylation of active Ste11 targets this MAPKKK for proteasome-mediated degradation (43). In the Raf-MEK-ERK pathway, both B-Raf and Raf-1 are negatively regulated by ERK phosphorylation (44–46). Recently, ERK-mediated feedback phosphorylation was shown to inactivate and desensitize Raf-1 rather than target Raf-1 for degradation. Hyperphosphorylated Raf is recycled through dephosphorylation by protein phosphatase 2A after Pin1-dependent isomerization (47). In addition, ERK-mediated feedback phosphorylation of B-Raf results in the dissociation of mitogen-induced B-Raf/Raf-1 heterodimers to terminate signaling (48).

In contrast to these examples of negative feedback phosphorylation of MAPKKks by their downstream MAPKs, in the MLK3-MKK4-7-JNK pathway, our evidence indicates that JNK-mediated phosphorylation of MLK3 constitutes a positive feedback loop. In Xenopus oocyte maturation, progesterone treatment leads to the accumulation of the MAPKKK Mos (49–51). This positive feedback of the Mos-MEK-ERK pathway is manifested primarily through stabilization of mos mRNA and accompanying increased translation of Mos. JNK has been reported to phosphorylate other MAPKKks. For instance, JNK binds and phosphorylates the MAPKKK, MEKK1, in the MEKK1-MKK4-JNK (52) pathway. Interestingly, MEKK1 functions not only as a MAPKKK and scaffold but also as an E3 ubiquitin ligase and undergoes ubiquitination. However, ubiquitinated MEKK1 is not rapidly degraded but instead is rendered catalytically inactive (53). MLK2 can also be phosphorylated by JNK, but precise phosphorylation sites and whether they impact MLK2 cellular levels were not reported (54).

Our finding that JNK phosphorylation regulates the solubility of a MAPKKK is novel. While this work was under review, it was reported that cellular stresses like camptothecin and sorbitol can induce MLK3 activation and stabilization. Ablation of JNK expression by small interfering RNA or with dominant negative JNK1 blocked camptothecin-induced increase in MLK3 protein levels, supporting a model for a positive feedback regulation of MLK3 stability by JNK (55). However, the subcellular distribution of the stabilized MLK3 was not analyzed. In our inducible system, JNK inhibition does not reduce the total amount of MLK3 but rather changes its subcellular distribution.

We recently published that Hsp90/Cdc37 act as cochaperones for MLK3. The Triton-insoluble fraction containing inactive MLK3 is largely devoid of Hsp90 and lacks detectable Cdc37, suggesting that Hsp90/Cdc37 dissociation from MLK3 might be involved in its redistribution to Triton-insoluble fractions. Dissociation of Hsp90 from the IKK complex was recently shown to result in its transient, reversible Triton-insolubility (56), whereas long term stress signals result in degradation of IKK, but a role for IKK phosphorylation in this process has not been established. It is conceivable that long term inhib-
bition of JNK may ultimately result in degradation of MLK3. Studies are under way to address the fate of the insoluble MLK3 under long term JNK inhibition and to determine the role of Hsp90/Cdc37 in regulating this process.

Phosphorylation has been shown to regulate a cycle of cytoskeletal association, dissociation, and turnover of the cytoskeletal adapter protein ankyrin (57). It is quite possible that the Triton-insoluble MLK3 represents localizations (61, 62). In particular, signaling molecules that localize to caveola-enriched rafts (59), rafts (63–67). It is possible that the Triton-insoluble inactive MLK3 represents localization of MLK3 to caveola-enriched rafts. Studies are under way to identify the specific membrane microdomain and/or cytoskeletal elements with which inactive, hypophosphorylated MLK3 associates.

Acknowledgment—We thank Stancy Liou for critical reading of the manuscript. We gratefully acknowledge the estate of Lela M. Soulby for partial funding of the ACS grant to K. A. G.

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