Mixed lineage kinase 3 (MLK3) functions as a mitogen-activated protein kinase kinase kinase to activate multiple mitogen-activated protein kinase pathways. Our current studies demonstrate that lack of MLK3 blocks signaling of activated Cdc42 to c-Jun N-terminal kinase, giving strong support for the idea that Cdc42 is a physiological activator of MLK3. We show herein that Cdc42, in a prenylation-independent manner, targets MLK3 from a perinuclear region to membranes, including the plasma membrane. Cdc42-induced membrane targeting of MLK3 is independent of MLK3 catalytic activity but depends upon an intact Cdc42/Rac-interactive binding motif, consistent with MLK3 membrane translocation being mediated through direct binding of Cdc42. Phosphorylation of the activation loop of MLK3 requires MLK3 catalytic activity and is induced by Cdc42 in a prenylation-independent manner, arguing that Cdc42 binding is sufficient for activation loop autophosphorylation of MLK3. However, membrane targeting is necessary for full activation of MLK3 and maximal signaling to JNK. We previously reported that MLK3 is autoinhibited through an interaction between its N-terminal SH3 domain and a proline-containing sequence found between the leucine zipper and the CRIB motif of MLK3. Thus we propose a model in which GTP-bound Cdc42/Rac binds MLK3 and disrupts SH3-mediated autoinhibition leading to dimerization and activation loop autophosphorylation. Targeting of this partially active MLK3 to membranes likely results in additional phosphorylation events that fully activate MLK3 and its ability to maximally signal through the JNK pathway.

The activation of protein kinases and the specification of their signaling pathways is a highly orchestrated process that is accomplished through dynamic and reversible events including phosphorylation, molecular interactions with proteins or other effector molecules, and subcellular targeting. Altered subcellular localization may impact protein kinase activation, signaling, or both. For instance, whether a protein kinase encounters an activating protein may depend upon its subcellular localization. Alternatively, many protein kinases have multiple in vivo substrates and signaling pathways, and spatiotemporal localization of protein kinases provides a mechanism by which substrate and signaling specificity can be achieved.

Mixed lineage kinase 3 (MLK3) was first characterized as a mitogen-activated protein kinase kinase kinase (MAP3K) that activates the c-Jun N-terminal kinase (JNK) pathway through the dual phosphorylation of mitogen-activated protein kinase kinases 4/7 (MKK4/7) (1, 2). The *Drosophila* MLK, called Slipper, is critical for the JNK-dependent process of dorsal closure in the fly embryo (3). MLK3-induced JNK activation is implicated in apoptosis of neuronal cells in response to trophic factor withdrawal (4–7). MLK3 also activates JNK in Jurkat T lymphocytes (8) and MCF-7 breast cancer cells (9) in response to tumor necrosis factor α treatment. MLK3 activates the p38 pathway through phosphorylation of MKK3/6 (10), although this activity may be dependent upon the scaffold JIP2 (11, 12). Transforming growth factor β-induced apoptosis of hepatocytes is reportedly dependent on MLK3-induced activation of p38 (13). MLK3 also contributes to extracellular signal-regulated kinase (ERK) activation (14), and gene silencing of MLK3 using siRNA blocks B-Raf-mediated ERK activation and proliferation (15, 16).

Deciphering how MLK3 is regulated is critical to our ultimate understanding of how MLK3 integrates different MAPK signaling pathways. Autoregulatory interactions are key to controlling MLK3 activity and signaling. The catalytic domain of MLK3 is flanked by an N-terminal SH3 domain and a centrally located zipper and Cdc42/Rac-interactive binding (CRIB) motif. Zipper-mediated homo-oligomerization is required for full activity of MLK3, proper substrate phosphorylation, and activation of the JNK pathway (17, 18). Work from our lab indicates that MLK3 is autoinhibited through an interaction between its SH3 domain and a proline-containing sequence within MLK3 (19).

Phosphorylation also contributes to the regulation of MLK3. Site-directed mutagenesis data indicate that activation loop phosphorylation of Thr277 and Ser281 is critical for MLK3 activity (20). In addition MLK3 is reported to be negatively regulated by Akt phosphorylation (21). Finally, in vivo labeling coupled with mass spectrometry has revealed multiple sites of phosphorylation of MLK3, most of which are clustered at the C terminus (22), whose functions are currently under study.

Cdc42 and Rac are Rho family GTPases that regulate diverse cellular processes including actin cytoskeleton remodeling, vesicular transport, endocytosis, cell cycle progression, cellular transformation, motility, and cell polarity (23–27). Like all members of the Ras superfamily of GTPases, Cdc42 and Rac are able to associate with cellular membranes by virtue of posttranslational prenylation of the Cys of the C-terminal CAAX motif (28, 29). A second signal for membrane localization, found in the so-called hypervariable region immediately upstream of the CAAX motif, typically contains either palmitoylation sites (30) or a series of basic residues (31) (see Fig. 2).

Activated forms of the small GTPases Cdc42 and Rac interact with MLK3 in a CRIB motif-dependent manner to increase the autophosphorylation.
Cdc42-induced Phosphorylation and Membrane Targeting of MLK3

phorylation of MLK3 and substrate phosphorylation activity (32–34) and to potentiate MLK3-induced activation of JNK (10, 33, 34). However, little is known about how GTPases activate MLK3.

Herein we provide evidence that Cdc42 induces JNK signaling through endogenous MLK3. Furthermore we show that activated Cdc42 translocates MLK3 to membranes and induces activation loop phosphorylation of MLK3. The data presented support a mechanism whereby Cdc42 binding is sufficient for activation loop autoprophorylation of MLK3, but prenylation-dependent, Cdc42-induced membrane targeting of MLK3, which does not in itself require MLK3 activity, is required for full activation of MLK3 and signaling to JNK. This work provides important insight into the molecular mechanism by which Cdc42 activates MLK3 and its signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The phospho-MLK3 (Thr277/Ser283), phospho-SEK1/MLK4 (Thr281), phospho-MKK7 (Ser322/Thr323), phospho-SAPK/JNK (Thr183/Tyr185), phospho-c-Jun (KM-1) mouse monoclonal antibody was from Santa Cruz Biotechnology, Inc. The FLAG M2 monoclonal antibody and actin mouse monoclonal antibody were purchased from Cell Signaling Technology, Inc. The phospho-c-Jun (KM-1) mouse monoclonal antibody was from Cell Signaling Technology, Inc. The phospho-SEK1/MLK3 (Thr277/Tyr278), phospho-SAPK/JNK (Thr202/Tyr204) monoclonal antibodies were purchased from Cell Signaling Technology, Inc. The phospho-c-Jun (KM-1) mouse monoclonal antibody was from Santa Cruz Biotechnology, Inc. The phospho-c-Jun (KM-1) mouse monoclonal antibody was from Cell Signaling Technology, Inc. The phospho-SEK1/MLK3 (Thr277/Tyr278), phospho-SAPK/JNK (Thr202/Tyr204) monoclonal antibodies were purchased from Cell Signaling Technology, Inc. The phospho-c-Jun (KM-1) mouse monoclonal antibody was from Santa Cruz Biotechnology, Inc. The phospho-c-Jun (KM-1) mouse monoclonal antibody was from Cell Signaling Technology, Inc.

**siRNA**—The human mlk3 siRNA sequence is derived from the sequence 5’-GGGCAGTGACGTGCTAGTTT-3’ as described previously (15). The negative control siRNA sequence is derived from the sequence 5’-GGGCAGGCAGTGCAGACCT-3’. HeLa cells were plated in 6-well plates and transfected with 50 nmol of siRNA oligonucleotide/well using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 20 h, transfections using FLAG-Cdc42V12 expression vector were performed; and after another 20 h, the cells were lysed.

**Expression Vectors and Site-directed Mutagenesis**—The construction of the cytochalasin-based expression vectors containing the cDNA for the wild type MLK3 (pRK5-mlk3) has been described elsewhere (35). The expression plasmid construct encoding the N-terminal FLAG (34) and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad).

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Cdc42-induced Phosphorylation and Membrane Targeting of MLK3

presented in red) and LP 650 nm (TO-PRO-3 iodide, represented in blue), respectively, as emission filters (Fig. 4).

Immune Complex Kinase Assays—For measurement of MLK3 activity in the plasma-enriched fractions, an immune complex kinase assay of the solubilized P16.9 fractions was performed. The relative amounts of MLK3 in the P16.9 fractions from cells expressing MLK3 alone or MLK3 plus Cdc42V12 were determined by densitometry (NIH Image) and adjusted to ensure equal amounts of immunoprecipitated MLK3 from the P16.9 fractions. Solubilized portions of the P16.9 fractions were incubated with 20 μl of protein A-agarose beads prebound with MLK3 antibody for 90 min at 4 °C. The MLK3 kinase assay was carried out as described previously (18), except that 10 μg of purified GST-MKK4 was used as substrate. The extent of GST-MKK4 phosphorylation was determined by Western blotting with an anti-phospho-MKK4 antibody.

For measurement of MLK3 activity in total cellular lysates, the MLK3 precipitates were washed twice with lysis buffer and twice with kinase buffer. 8 μg of recombinant, catalytically inactive GST-MKK7 K165A (9) was used as substrate, and the reaction was carried out for 20 min at room temperature. The extent of GST-MKK7 phosphorylation was determined by Western blotting with a phospho-MKK7 antibody.

RESULTS

Cdc42-induced JNK Activation Requires MLK3—Previous studies have demonstrated that activated Cdc42 binds MLK3 and increases its catalytic activity (33, 34). However, these studies have relied upon ectopically expressed MLK3. To determine whether Cdc42 regulates JNK signaling through endogenous MLK3, Cdc42-induced JNK activation was measured in HeLa cells in which MLK3 expression was abolished using RNA interference (15). As shown in Fig. 1, Cdc42-induced JNK activation is reduced at least 5-fold upon transfection with human-specific siRNA but not the control siRNA, indicating that MLK3 is a major physiological target of Cdc42 in signaling to JNK.

Membrane Targeting-defective Variants of Activated Cdc42 Are Able to Interact with MLK3—The C-terminal CAAX motif of Cdc42 serves as a target for geranylgeranylation, thus endowing Cdc42 with the ability to associate with membranes. Therefore, it is tempting to speculate that prenylated, activated Cdc42 localizes MLK3 to cellular membranes. To study the potential of Cdc42-induced membrane targeting of MLK3, variants of Cdc42 that fail to undergo prenylation and/or lack the basic secondary membrane targeting motifs were constructed as diagrammed in Fig. 2A. Specifically, the prenylation site Cys188 in the CAAX motif of Cdc42V12 was mutated to Ser (Fig. 2A). In yeast, this analogous change...
Cdc42-induced Phosphorylation and Membrane Targeting of MLK3

Activated Cdc42 Targets MLK3 to a Plasma Membrane-enriched Fraction—To determine whether Cdc42 impacts the subcellular distribution of MLK3, biochemical fractionation experiments were performed using HEK 293 cells transiently expressing MLK3 and variants of constitutively active Cdc42V12. The distribution of MLK3 between a soluble (S16.9) and a plasma membrane-enriched pellet (P16.9) fraction was assessed by Western blotting. Data from a representative experiment are shown in Fig. 3A. Based on five independent experiments, MLK3 is distributed approximately equally between the S16.9 and P16.9 fractions when expressed alone. In contrast, upon coexpression with activated Cdc42V12, MLK3 is found predominantly in the P16.9 fraction. Taken together, these data support the hypothesis that functional prenylation is required for Cdc42-mediated targeting and activation of MLK3 at the plasma membrane.

Activated Cdc42 and MLK3 Colocalize at the Plasma Membrane—To examine the subcellular localization of Cdc42V12 and MLK3 in cells, confocal microscopy experiments were performed using HeLa cells transfected with vectors encoding MLK3 and Cdc42V12 variants. MLK3 alone displays prominent perinuclear staining along with punctate, vesicular patterns and limited diffuse cytosolic staining (Fig. 4, top panel). These data are in general agreement with reports that MLK3 localizes to the Golgi (40) and centrosome regions (41). Cdc42V12 has been previously shown to localize to the plasma membrane (42). When MLK3 is coexpressed with Cdc42V12, they clearly colocalize at the plasma membrane (Fig. 4, center panel). In contrast the prenylation-defective, activated Cdc42V12 consistently displays cytosolic staining. In the presence of prenylation-defective Cdc42V12, MLK3 fails to localize to the plasma membrane and largely maintains perinuclear staining (Fig. 4, bottom panel). Taken altogether, these data further support the idea that activated Cdc42V12 targets MLK3 to the plasma membrane.

Requirements of MLK3 Membrane Targeting by Activated Cdc42—To determine the requirements for Cdc42-induced membrane targeting of MLK3, variants of MLK3 were expressed alone and with activated Cdc42 in HEK 293 cells, and the subcellular distribution of MLK3 was examined by Western blotting of equal cellular equivalents in SDS-PAGE. As shown in Fig. 5A, both wild type MLK3 and the catalytically inactive variant, MLK3 K144R, are efficiently targeted to the plasma membrane-enriched fraction when expressed with Cdc42V12, indicating that MLK3 activity is not required for Cdc42-induced targeting.

MLK3 translocation may depend on direct physical association with Cdc42 or, alternatively, may be an indirect effect of activated Cdc42 signaling. To discern between these possibilities, we made use of the variant MLK3 I492A,S493A, in which two of the conserved residues in the CRIB motif have been changed to Ala residues. This variant, which has been shown to be defective in Cdc42V12 binding (34), fails to undergo Cdc42-mediated translocation as shown in Fig. 5A. These data strongly support a mechanism whereby direct interaction with activated Cdc42 is responsible for membrane targeting of MLK3.
FIGURE 4. Subcellular localization of MLK3. HeLa cells, transiently expressing MLK3 with or without FLAG-tagged Cdc42V12 variants, were fixed, and MLK3 was stained using a rabbit MLK3 antibody and a secondary antibody conjugated with Alexa Fluor 546. FLAG-tagged Cdc42V12 variants were detected by mouse monoclonal FLAG antibody and a secondary antibody conjugated with Alexa Fluor 488. The nuclei were stained with TO-PRO-3 iodide. Using confocal laser scanning microscopy, more than 50 cells were examined for each transfection. Representative images are shown with FLAG-Cdc42, MLK3, and the nuclei represented in green, red, and blue, respectively. Bar, 10 μm.

FIGURE 5. Requirements of MLK3 membrane targeting by activated Cdc42 and activation loop phosphorylation of MLK3. A, requirements of MLK3 membrane targeting by activated Cdc42. HEK 293 cells were transiently transfected with expression vectors containing cDNAs as indicated. Subcellular fractionation experiments were performed as described in the legend to Fig. 3. The samples for all of the fractions were loaded as equal cellular equivalents. The distribution of MLK3 and FLAG-Cdc42 were shown by Western blotting (WB) using the MLK3 or FLAG antibody respectively.

B, Cdc42 induces activation loop (auto)phosphorylation of MLK3. HeLa cells were transiently transfected with expression vectors containing cDNAs for MLK3 variants with or without FLAG-Cdc42V12, as indicated. Equal amounts of total protein from cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies. The data shown are representative of three independent experiments. WT, wild type.
Cdc42 Induces Activation Loop (Auto)-Phosphorylation of MLK3—Within the catalytic domain of protein kinases resides the so-called activation loop, whose phosphorylation often alters its conformation rendering the kinase (usually) catalytically active (43–46). Site-directed mutagenesis data support phosphorylation of the activation loop within the kinase domain of MLK3 as critical for MLK3 activity (20). Activation loop phosphorylation of MLK3 was assessed by Western blotting of cellular lysates with a phosphospecific antibody directed against activation loop-phosphorylated MLK3 (Thr(Ph)277/Ser(Ph)281). As shown in Fig. 5B, wild type MLK3 lacks activation loop phosphorylation, which is markedly enhanced upon coexpression with activated Cdc42. The kinase-defective variant of MLK3 is effectively targeted by Cdc42 to the membrane fraction (Fig. 5A) but fails to undergo activation loop phosphorylation (Fig. 5B), indicating that activation loop phosphorylation is due, at least in part, to autophosphorylation.

If direct association with Cdc42 is required for MLK3 translocation, as our data above indicate, and if translocation is needed for MLK3 activation, it is reasonable to predict that the CRIB mutant of MLK3 would not be activated by Cdc42. In accord with this hypothesis, MLK3 I492A,S493A exhibits very minimal activation loop phosphorylation whether expressed with or without activated Cdc42 (Fig. 5B). As shown in Fig. 5B, activation loop phosphorylation, to a first approximation, mirrors MLK3-induced JNK activation, as judged by Western blotting of phospho-JNK in cellular lysates.

Cdc42 Induces Activation Loop Phosphorylation Independent of Membrane Targeting—Catalytic activity and GTPase binding are required for Cdc42-induced activation loop phosphorylation of MLK3. As expected, the activation loop phosphorylated MLK3 resides in the membrane fraction (Fig. 6A). One reasonable hypothesis is that Cdc42-induced membrane targeting is critical for MLK3 activation loop phosphorylation. However, we find that both the prenylation-defective and the prenylation-competent versions of Cdc42 induce a comparable increase in activation loop phosphorylation of MLK3, indicating that the Cdc42-mediated increase in activation loop phosphorylation of MLK3 does not depend upon membrane targeting (Fig. 6B). Taken altogether, these data imply that Cdc42 activates MLK3, at least in part, by inducing activation loop (auto)phosphorylation. Furthermore, this function of Cdc42 is independent of its ability to associate with cellular membranes and likely ensues from the physical association of the kinase with the small GTPase.

Membrane Targeting Contributes to Full Activation of MLK3—Both prenylation-competent and prenylation-defective variants of Cdc42V12 promote activation loop phosphorylation of MLK3 to the same extent. However, activation loop phosphorylation may not be sufficient for full activation of MLK3. To determine whether membrane targeting contributes to the activation of MLK3 by Cdc42, in vitro MLK3 immune complex catalytic activity assays were performed from HeLa cells expressing MLK3 and Cdc42V12 variants. Recombinant, catalytically inactive GST-MKK7 was used as a substrate, and the extent of phosphorylation of the activation segment of MKK7 was measured using a phospho-specific antibody directed against MKK7. Control experiments were performed to confirm that a linear relationship exists between the amount of phospho-GST-MKK7 loaded and the resulting signal quantitated using ImageJ software (data not shown).
As demonstrated previously, coexpression of MLK3 with activated Cdc42V12 enhances the in vitro catalytic activity of MLK3 (Fig. 7). We have consistently observed that coexpression with Cdc42 increases the protein levels of MLK3, and thus it is necessary to correct the MLK3 activity for the amount of protein present. Based on three independent experiments using HeLa cells and normalizing for the amount of immunoprecipitated MLK3, MLK3 catalytic activity is increased 5.5-fold upon coexpression with prenylation-competent Cdc42V12 over that of MLK3 alone, whereas the prenylation-defective Cdc42 V12C188S, K/R4Q induces only a 2.5-fold activation of MLK3. These data indicate that membrane targeting is required for the full activation of MLK3 induced by Cdc42.

Prior work from our lab identified Ser555 and Ser556 of MLK3 as sites that incorporate radiolabeled phosphate in vivo upon coexpression of MLK3 with activated Cdc42 (22), leading us to hypothesize that these might be membrane targeting-dependent, activating phosphorylation sites. However, an MLK3 variant in which these phosphorylation sites have been substituted with alanine was fully activated by Cdc42-induced membrane targeting, suggesting that other phosphorylation events/posttranslational modifications are responsible for the enhanced activation of MLK3 in response to membrane targeting.

Enhanced MLK3 Signaling to JNK Requires Cdc42-induced Membrane Targeting—Because full activation of MLK3 requires Cdc42-induced membrane targeting, one might expect membrane targeting to affect MLK3 signaling. One of the best described functions of MLK3 is as a MAPKKK that activates the JNK pathway (2). In addition, activated Cdc42 potentiates MLK3-induced JNK activation (34). To determine the impact of membrane targeting of MLK3 by Cdc42 on JNK activation, MLK3-induced JNK activation in cells was assessed using either the phospho-specific antibodies directed against activated JNK (Thr(P)183/Tyr(P)185) and/or c-Jun (Ser(P)63). MLK3 alone induces basal phosphorylation of JNK and c-Jun that is markedly enhanced upon coexpression with prenylation-competent Cdc42V12 in HeLa cells (Fig. 8A). In contrast, despite its full ability to induce activation loop phosphorylation of MLK3, prenylation-defective Cdc42 V12C188S, K/R4Q induces only weakly potentiated MLK3 activation of JNK and c-Jun in cells.

Additional experiments show that prenylated Cdc42 alone, or MLK3 alone, only weakly activate JNK, whereas expression of the two together results in synergistic JNK activation (Fig. 8B, fourth panel), as judged by phospho-JNK Western blotting. Furthermore, only when MLK3 has been activated by prenylated Cdc42 are there significant levels of activated MKK7 in cells, as measured by immunoblotting of cellular lysates with a phospho-MKK7 antibody (Fig. 8B, third panel). From these data we conclude that functional targeting to the plasma membrane contrib-

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FIGURE 7. MLK3 activity induced by Cdc42V12 variants. HeLa cells were transfected with or without Cdc42V12 variants as indicated. A, in vitro immune complex kinase assay of MLK3 using recombinant GST-MKK7 K165A as a substrate. The top panel shows phosphorylation of GST-MKK7 K165A as determined by Western blotting (WB) using a phospho-specific antibody against MKK7; the second panel shows a Western blot of the immunoprecipitated (IP) MLK3 from the in vitro kinase assays; the third, fourth, and fifth panels are Western blots indicating the levels in cellular lysates of MLK3, FLAG-Cdc42V12 variants, and, as a loading control, actin. B, quantitation of MLK3 activity assay. Phosphorylation of GST-MKK7 was quantitated by densitometry and normalized to levels of immunoprecipitated MLK3 as described under “Experimental Procedures.” The means ± S.E. for fold increase in phosphorylation of GST-MKK7 K165A from three independent experiments are shown.

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Y. Du and K. Schachter, data not shown.
utes to MLK3-induced activation of JNK by Cdc42, most likely reflecting the increased catalytic activity of membrane-targeted MLK3.

**DISCUSSION**

In this study we investigated the mechanism by which the small GTPase Cdc42 activates MLK3. Depletion of cellular MLK3 using RNA interference prevents Cdc42-induced JNK activation, implicating MLK3 as physiological target of Cdc42. Previous work from our lab showed that GTP-bound Cdc42 associates with and activates MLK3 in a CRIB motif-dependent manner (34). To assess the contribution of Cdc42-induced membrane targeting to MLK3 activation and signaling, a membrane-targeting defective variant of Cdc42V12 was engineered. This membrane targeting-defective counterpart has no detectable membrane association yet retains the ability to associate with MLK3 in coimmunoprecipitation assays. In these studies, biochemical fractionation experiments, along with confocal microscopy, indicate that prenylation-competent but not prenylation-defective activated Cdc42 targets MLK3 from a perinuclear region to heavy membranes, including the plasma membrane. These results are consistent with the idea that Cdc42 and MLK3 signal at the plasma membrane.

Recently a biosensor approach revealed that active Cdc42 is present not only at the cell periphery but also within the cell body near the cell periphery and at the trans-Golgi apparatus (47). Given recent data regarding Ras signaling on endosomes as well as on the plasma membrane (48–51) and considering the ability of both Cdc42 (47, 52) and MLK3 to localize to endomembrane structures (40), it would not be at all surprising if under certain conditions Cdc42 activates MLK3 on these cellular membranes.

Phosphorylation within the so-called “activation loop” promotes the active conformation of many protein kinases. Substitution of phosho-

![FIGURE 8. Effect of Cdc42V12 variants on MLK3-induced JNK signaling.](image)
mimetic Asp residues for Thr277 and Ser281 within the activation loop results in active MLK3 (20). Interestingly, using a phospho-specific antibody, we found that GTP-bound Cdc42 potently induces activation loop phosphorylation of wild type MLK3. However, Cdc42-induced membrane targeting is not required for activation loop phosphorylation because both prenylation-competent and prenylation-defective variants of Cdc42 promote activation loop phosphorylation of MLK3 to the same extent. Furthermore, we show that Cdc42-induced activation loop phosphorylation requires MLK3 activity. These data imply that physical association with activated Cdc42 causes activation loop (auto)-phosphorylation of MLK3.

It is possible that Cdc42 activates MLK3 indirectly through one of the MAPKKK kinases, such as the p21-activated kinase (PAK), which is known to be activated by Cdc42 and Rac (53–56). However, our finding that a GTPase binding-defective version of MLK3 cannot be activated by Cdc42 strongly argues for Cdc42 as a direct effector of MLK3.

The N-terminal region of PAK harbors a CRIB motif-containing p21-binding domain that overlaps with an autoinhibitory domain (53–55). The binding of the active, small GTPase disrupts this autoinhibitory conformation, resulting in autophosphorylation of the activation loop and activation of PAK (53, 54). Our lab has previously reported that MLK3 is autoinhibited through association of its N-terminal SH3 domain with a sequence containing Pro669, which, based at least on primary sequence, is situated in close proximity between the zipper domain (amino acids 400–462) and the CRIB motif (amino acids 498–514) (19). Thus although they utilize different structural domains/elements, PAK and MLK3 appear to share a common theme of GTPase-mediated disruption of autoinhibition. In the case of PAK, crystal structures reveal a trans autoinhibited dimeric form (56), and experimental evidence indicates that activated Cdc42 dissociates the autoinhibited PAK dimer leading to cis-autophosphorylation. In contrast, zipper-mediated dimerization/oligomerization is critical for MLK3 substrate phosphorylation and signaling (17, 18). Although the precise oligomerization state of neither the autoinhibited nor the activated form of MLK3 has been experimentally determined, it has been suggested, based upon communoprecipitation of differentially tagged versions of MLK3, that Cdc42 induces dimerization/oligomerization of MLK3 (17, 57).

From the results discussed above, it might be concluded that physical association with Cdc42 is sufficient for MLK3 activation. However, in vitro kinase assays for MLK3 activity revealed that maximal activation of MLK3 requires Cdc42-induced membrane targeting. The inference is that activation loop (auto)phosphorylation is required, but insufficient, for full activation of MLK3, and is consistent with the idea that membrane targeting-dependent phosphorylation events are necessary for the full activation of MLK3 and its signaling to JNK. It is also conceivable that enhanced accessibility of cellular substrates to MLK3 might contribute to its signaling to JNK.

The process of Ras-induced Rap activation shares some similar features with Cdc42-induced MLK3 activation. GTP-bound Ras binds and translocates c-Raf to the plasma membrane (36, 58–61) and endomembranes (48–51). Full activation of c-Raf requires activation loop phosphorylation (62) as well as membrane-dependent phosphorylation, reportedly by PAK (63, 64) and Sdc (65–67). However, although prenylation-defective, activated Cdc42 induces activation loop phosphorylation and partial activation of MLK3, prenylation-defective variants of Ras fail to activate c-Raf (39, 66). A prenylation-defective variant of Ras has been shown to bind Raf (39), but binding-induced activation loop phosphorylation has not been examined.

The protein kinase(s) responsible for the membrane targeting-dependent phosphorylation of MLK3 are as yet unidentified, but two MAPKKKs, germinal center kinase (68) and hematopoietic progenitor protein kinase 1 (69), which have been implicated in MLK3 activation, emerge as candidate MLK3 kinases. Although hematopoietic progenitor protein kinase 1 is restricted to cells of hematopoietic lineage, both MLK3 and germinal center kinase are widely expressed in cell and tissue types (35, 70). Interestingly it has been shown that hematopoietic progenitor protein kinase 1 can be recruited to lipid rafts (71), and germinal center kinase is found at Golgi and plasma membranes (72).

One of the major functions ascribed to MLK3 is as a MAPKKK for activation of the JNK pathway. Our finding that the prenylation-defective, activated Cdc42 variant fails to fully potentiate MLK3-induced JNK activation supports the idea that plasma membrane localization of MLK3 is an important facet of the mechanism by which Cdc42 activates MLK3-induced JNK signaling. We propose that activated Cdc42 associates with autoinhibited MLK3 through its CRIB motif, disrupting SH3-mediated autoinhibition and inducing zipper-mediated dimerization of MLK3 and subsequent (auto)phosphorylation within the activation loop of MLK3. This localization-independent, allosteric activation loop phosphorylation accounts for about half of the full activation of MLK3. As a consequence of Cdc42-induced membrane targeting, MLK3 undergoes additional phosphorylation events that are required for its full activation.

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