

Hsp90/p50^{cdc37} Is Required for Mixed-lineage Kinase (MLK) 3 Signaling*

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Hua Zhang[‡], Wei Wu[§], Yan Du[¶], Sarah J. Santos[‡], Susan E. Conrad[‡], Jack T. Watson[§],
Nicholas Grammatikakis^{**}, and Kathleen A. Gallo^{‡¶}

From the [‡]Cell and Molecular Biology Program, Departments of [¶]Physiology, ^{||}Biochemistry and Molecular Biology, and [§]Chemistry, Michigan State University, East Lansing, Michigan 48824 and ^{**}Boston University School of Medicine, Boston, Massachusetts 02118

Mixed-lineage kinase 3 (MLK3) is a mitogen-activated protein kinase (MAPK) kinase that activates MAPK pathways, including the c-Jun NH₂-terminal kinase (JNK) and p38 pathways. MLK3 and its family members have been implicated in JNK-mediated apoptosis. A survey of human cell lines revealed high levels of MLK3 in breast cancer cells. To learn more about MLK3 regulation and its signaling pathways in breast cancer cells, we engineered the estrogen-responsive human breast cancer cell line, MCF-7, to stably, inducibly express FLAG epitope-tagged MLK3. FLAG-MLK3 complexes were isolated by affinity purification, and associated proteins were identified by in-gel trypsin digestion followed by liquid chromatography/tandem mass spectrometry. Among the proteins identified were heat shock protein 90 α , β (Hsp90) and its kinase-specific co-chaperone p50^{cdc37}. We show that endogenous MLK3 complexes with Hsp90 and p50^{cdc37}. Further experiments demonstrate that MLK3 associates with Hsp90/p50^{cdc37} through its catalytic domain in an activity-independent manner. Upon treatment of MCF-7 cells with geldanamycin, an ansamycin antibiotic that inhibits Hsp90 function, MLK3 levels decrease dramatically. Furthermore, tumor necrosis factor α -induced activation of MLK3 and JNK in MCF-7 cells is blocked by geldanamycin treatment. Our finding that geldanamycin treatment does not affect the cellular levels of the downstream signaling components, MAPK kinase 4, MAPK kinase 7, and JNK, suggests that Hsp90/p50^{cdc37} regulates JNK signaling at the MAPK kinase level. Previously identified Hsp90/p50^{cdc37} clients include oncoprotein kinases and protein kinases that promote cellular proliferation and survival. Our findings reveal that Hsp90/p50^{cdc37} also regulates protein kinases involved in apoptotic signaling.

Signal transduction processes invariably involve protein-protein interactions. The accumulation of knowledge regarding cell signaling over the past several years has led to an appreciation for the idea that supramolecular signaling complexes

exist within cells and that the cellular environment is highly organized. Knowledge of the components of signaling complexes is important in understanding signaling processes. Methods such as the yeast two-hybrid system are often useful for characterizing binary interactions, but are of limited utility in defining multicomponent complexes. Recently, affinity purification of protein complexes and identification of their components using mass spectrometry has become feasible.

The mixed-lineage kinases (MLKs)¹ are a family of seven mammalian serine/threonine kinases that function as mitogen-activated protein kinase (MAPK) kinase kinases (MAPKKKs) to activate the c-Jun N-terminal kinase (JNK) pathway (1). In some experimental settings, the MLKs may also activate the extracellular signal-regulated kinase (ERK), p38 MAPK, and NF- κ B pathways (1–4). The MLKs have garnered attention as important mediators of apoptosis, particularly in neuronal cells (5–7).

MLK3 activates the JNK pathway through phosphorylation and activation of the dual specific kinases, MAPK kinase 4 (MKK4) (8) and MKK7 (9). Overexpressed MLK3 also modestly activates the p38 MAPK pathway (10–12). JNKs and p38 MAPKs are activated by diverse extracellular stimuli, including cytokines, osmotic stress, UV light, and heat shock (13). However, it has been demonstrated that more than a dozen MAPKKKs activate the JNK and p38 pathways. The signals that specifically activate endogenous MLK3 are not well characterized. Recently, it has been shown that treatment of Jurkat T lymphocytes with tumor necrosis factor- α (TNF α) activates endogenous MLK3, as judged by the ability of MLK3 to phosphorylate recombinant MKK4 *in vitro* (14).

In addition to its catalytic domain, MLK3 contains an amino-terminal Src-homology 3 domain, a centrally located zipper region, and a Cdc42/Rac interactive binding (CRIB) motif, which mediate protein-protein interactions to regulate MLK3 activity and/or signaling. The small Rho family GTPases, Cdc42 and Rac, in their activated states, bind to MLK3 in a CRIB motif-dependent manner and increase MLK3 catalytic activity (15). Previous work in our lab has shown that MLK3 is autoinhibited through an interaction between its amino-terminal Src-homology 3 domain and a sequence located between its zipper and CRIB motifs (16).

Affinity purification coupled with mass spectrometry (MS)

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^{‡¶} To whom correspondence should be addressed: Dept. of Physiology, 4180 Biomedical and Physical Sciences Bldg, Michigan State University, East Lansing, MI 48824. Tel.: 517-355-6475 (ext. 1159); Fax: 517-355-5125; E-mail: gallok@msu.edu.

¹ The abbreviations used are: MLKs, mixed-lineage kinases; MAPK, mitogen-activated protein kinase; MAPKKKs, MAPK kinase kinases; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; MKK4, MAPK kinase 4; TNF α , tumor necrosis factor- α ; CRIB, Cdc42/Rac-interactive binding; MS, mass spectrometry; GST, glutathione S-transferase; HA, hemagglutinin; LC/MS/MS, liquid chromatography tandem MS; CID, collision-induced dissociation; HEK, human embryonic kidney; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

has surfaced as a popular tool to fish out protein complexes. Several groups have successfully applied a one-step purification procedure using an anti-FLAG antibody-bound resin to capture FLAG-tagged protein complexes (17, 18). To better understand the regulation and signaling of MLK3, we employed the FLAG affinity purification coupled with mass spectrometry to identify proteins that interact with MLK3. We report the identification of the molecular chaperones Hsp90/p50^{cdc37} as MLK3 binding partners. The work described herein demonstrates the functional importance of the complex formation with Hsp90/p50^{cdc37} on the stability and signaling of MLK3.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Geldanamycin and other chemicals were purchased from Sigma. The phospho-c-Jun (KM-1) mouse monoclonal antibody, MKK4 rabbit polyclonal antibody, MKK7 goat polyclonal antibody, JNK rabbit polyclonal antibody, Hsp90 rabbit polyclonal antibody, p50^{cdc37} mouse monoclonal antibody, and glutathione S-transferase (GST) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Other antibodies used were the MLK3 rabbit polyclonal antibody (15), actin mouse monoclonal antibody (Sigma), hemagglutinin (HA) mouse monoclonal antibody (BAbCO), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad).

Plasmid Constructs and Mutagenesis—Construction of mammalian expression vectors for wild-type MLK3 (pRK5-*NFLAG.mlk3*), MLK3 K144A, the MLK3 truncation variant (pCGN-*HA.mlk3* 115–399), and pGST-p50^{cdc37} have been described elsewhere (16, 19). Two other HA-tagged MLK3 variants were constructed by PCR using the following oligonucleotides with pRK5-*NFLAG.mlk3* as the template: pCGN-*HA.mlk3* 1–399, 5'-CGTTAGTCTAGAATGGAGCCCTTGAAGAG-3' and 5'-GCATTAGGATCCTCACCAGCCTTCTGCATGG-3'; pCGN-*HA.mlk3* 400–847, 5'-CGTTAGTCTAGAAGCGCGAGATCCAGGG-3' and 5'-GCATTAGGATCCTCAAGCCCCGCTTCCGGC-3'.

The amplified *mlk3* fragments were subcloned in-frame with the HA coding sequence into the pCGN mammalian expression vector using XbaI and BamHI.

The bacterial expression vector of GST-MKK7, kindly provided by Dr. Roger Davis (University of Massachusetts Medical School), was used as a template to create the kinase-defective mutant GST-MKK7 K165A using the QuikChange site-directed mutagenesis method (Stratagene).

Generation of an MCF-7 Cell Line Inducibly Expressing MLK3—A cell line inducibly expressing MLK3 was generated using the ARGENT regulated transcription system (Ariad Pharmaceuticals, Inc.). A BamHI-XhoI fragment, containing the G418 resistance (*neo*) gene, was subcloned from pIRESneo2 vector (Clontech) into the pC₄N₂-R_HS3H-ZF3 plasmid vector (Ariad Pharmaceuticals, Inc.). The pC₄N₂-R_HS3H-ZF3 *neo* plasmid was transfected into MCF-7 cells, and clones were selected in Dulbecco's modified Eagle's medium with 5% fetal bovine serum (Invitrogen) and 400 µg/ml G418 (Sigma). Individual clones were maintained in the same medium containing 40 µg/ml G418.

The target gene vector pLH-Z₁₂-I-PL-*FLAG-mlk3* was constructed by PCR-mediated amplification of the corresponding coding sequence from pRK5-*FLAG-mlk3* followed by subcloning into the target gene vector at the HindIII and ClaI sites. The pLH-Z₁₂-I-PL-*FLAG-mlk3* plasmid was transfected into a stable MCF-7 clone derived from the selection described above. Clones were selected in the same medium containing 40 µg/ml G418 and 50 µg/ml hygromycin for about 2 weeks. Individual clones were tested for inducible expression of FLAG-MLK3 after growth in the presence of 50 nM AP21967 for 20 h. Individual clones inducibly expressing FLAG-MLK3 were maintained in normal medium containing 40 µg/ml G418 and 10 µg/ml hygromycin. The clone MCF-7/iFLAG-MLK3 was used for further studies.

Isolation of FLAG-MLK3 Complexes—MCF-7/iFLAG-MLK3 cells (1×10^8) were cultured in the presence of 100 nM AP21967 for 20 h to induce FLAG-MLK3 expression. An equal number of cells were cultured in the absence of AP21967 as a negative control. Cells were disrupted in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM sodium fluoride, 1 mM Na₄PP₃, 100 µM β-glycerophosphate, 1 mM Na₃VO₄) containing a mixture of protease inhibitors (Sigma). Clarified cellular lysates were incubated with 400 µl of anti-FLAG M2 agarose gel (Sigma) for 90 min at 4 °C. The resin was collected by centrifugation and was washed with Tris-buffered saline three times. FLAG-MLK3 complexes were eluted from the resin by incubation for 60 min at 4 °C with 300 ng/µl 3× FLAG peptide (Sigma) in Tris-buffered saline and acetone precipitated. The precipitated pro-

teins were redissolved in water and loading buffer and resolved by gel electrophoresis on 4–12% gradient gels (NuPage, Invitrogen).

In-gel Trypsin Digestion—Visible protein bands were excised from the Coomassie-stained gel and chopped into 1-mm³ pieces. Proteins contained in gel pieces were reduced with 10 mM dithiothreitol and then alkylated with 55 mM iodoacetamide. The gel pieces were dehydrated with 100% acetonitrile and dried using a Speed-Vac. After digestion with sequencing-grade trypsin (13 ng/µl; Promega) at 37 °C overnight, tryptic peptides were extracted with 60% acetonitrile/1% trifluoroacetic acid and were completely dried. The dried sample was reconstituted by the addition of 8 µl of 1% formic acid.

Nano-electrospray Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)—LC/MS/MS was performed on a Finnigan LCQ-Dea mass spectrometer (Thermo Finnigan, San Jose, CA). A portion of the tryptic peptides (typically 6 µl out of a 12-µl sample) was loaded onto a peptide capillary trap (Michrom); after washing the trap with 1% aqueous formic acid, the trap was connected to a C18 PicoFrit column (NewObjectives, 75-µm inner diameter × 5 cm), and peptides were eluted with a linear gradient of acetonitrile (5–60%) in 1% formic acid. The Top3 mode was used to obtain the MS/MS data during collision-induced dissociation (CID). The SEQUEST software version 2 (TurboSequest; Thermo Finnigan) performed *in silico* tryptic digestion of proteins in the NCBI Protein Database, calculated their possible CID fragments, and compared the latter with the experimentally determined CID spectra of MLK3-associated proteins for purposes of identification.

Cell Lines and Transfections—MCF-7 cells were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (Invitrogen). MCF-7 cells were pretreated for 24 h with 5 µM geldanamycin or with Me₂SO. Cells were then treated with TNFα for the indicated periods of time, harvested, and lysed as described previously (16). A portion of the cells was pelleted and boiled in SDS-loading buffer and used for Western blotting of the phospho-c-Jun level.

Human embryonic kidney (HEK) 293 cells were maintained in Ham's F-12/low glucose Dulbecco's modified Eagle's medium (1:1) (Invitrogen) supplemented with 8% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin. HEK 293 cells were transfected using the calcium phosphate method as described previously (15). Cells were harvested 16 h after transfection and lysed as described previously (16).

GST Pulldown Assays—Mammalian constructs (pGST or pGST-p50^{cdc37}) encoding GST or GST-p50^{cdc37}, respectively, were cotransfected with expression vectors encoding MLK3 truncation variants in HEK 293 cells. Cells were lysed and 400 µl of the clarified lysate were incubated with 20 µl of glutathione-Sepharose 4B resin (Amersham Biosciences) for 90 min at 4 °C. The pelleted resin was washed twice with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), and bound proteins were resolved by SDS-PAGE.

Immunoprecipitations—Clarified lysates were incubated with antibody-bound protein A-agarose for 90 min at 4 °C. Immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MnCl₂, 10 mM MgCl₂, 0.1 mM Na₃VO₄).

In Vitro Kinase Assays—Immunoprecipitated MLK3 was incubated in 20 µl of kinase assay buffer containing 10 µM ATP, 5 µCi [γ -³²P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences) and 6 µg of recombinant GST-MKK7 K165A for 30 min at room temperature. After the kinase assay, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the incorporation of radioactivity into the GST-MKK7 was quantitated by phosphorimaging (Molecular Dynamics). The membrane was Western blotted with MLK3 antibody and GST antibody sequentially to determine the MLK3 and GST-MKK7 levels.

SDS-PAGE and Western Blot Analysis—Lysates and GST pull-downs of proteins were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and Western blotted using appropriate antibodies. Western blots were developed by the chemiluminescence method as described previously (15).

RESULTS

Inducible Expression of MLK3 in MCF-7 cells—Because Western blotting of cellular lysates revealed that MLK3 is expressed at high levels in breast cancer cell lines (data not shown), MCF-7 cells were chosen as a source for MLK3-interacting proteins. To establish a reliable system for affinity purification of MLK3 complexes, MCF-7 cells were engineered to inducibly express FLAG-tagged MLK3 using the ARGENT sys-

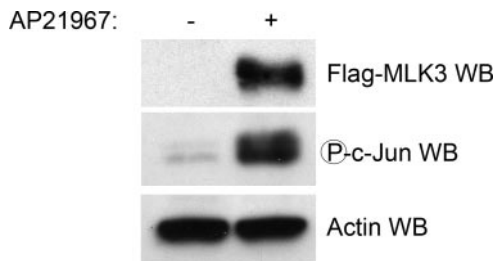


FIG. 1. Inducible expression of FLAG-tagged MLK3. A selected clone from the MCF-7 cells engineered to inducibly express FLAG-MLK3 was cultured either in the presence or absence of AP21967 (100 nM) for 20 h. Cells were lysed, and cellular lysates were subjected to Western blotting (WB) analysis. *Top panel*, the presence of FLAG-MLK3 in the total cellular lysates was assessed by Western blotting using the FLAG antibody. *Middle panel*, the level of phosphorylated c-Jun in the total cellular lysates was assessed by Western blotting using an antibody directed against phospho-c-Jun. *Bottom panel*, equal loading of the cellular lysates was confirmed by Western blotting using an antibody directed against actin.

tem (Ariad Pharmaceuticals, Inc.) wherein transcription of a specified gene is controlled by the small molecule dimerizer AP21967 (20). We refer to this inducible cell line as MCF-7/iFLAG-MLK3. As shown in Fig. 1, FLAG-MLK3 was inducibly expressed upon the addition of AP21967. Because MLK3 activates the JNK pathway, the phosphorylation of the transcription factor c-Jun as detected by a phospho-c-Jun-specific antibody was used as a measure of the FLAG-MLK3 activity *in vivo*. The phosphorylation of c-Jun on Ser⁶³ dramatically increased upon induced expression of FLAG-MLK3 (Fig. 1), indicating that the inducibly expressed FLAG-MLK3 is catalytically active and activates the JNK pathway.

Purification of MLK3 Complexes—Inducibly expressed FLAG-MLK3 was immunoprecipitated with anti-FLAG M2 affinity gel as described under “Experimental Procedures.” FLAG-MLK3 complexes were eluted by the addition of a peptide consisting of three tandem repeats of the FLAG epitope (3× FLAG peptide) and resolved by SDS-PAGE. Proteins were stained with Coomassie Blue. Uninduced MCF-7/iFLAG-MLK3 cells were used as a negative control. As shown in Fig. 2A, a prominent band representing FLAG-MLK3 is present only in the induced sample. In addition, there are multiple bands present in the induced sample but absent in the uninduced sample, suggesting that these bands represent MLK3-interacting proteins.

Identification of p50^{cdc37} and Hsp90 in MLK3 Complexes—Two prominent Coomassie-stained bands of ~90 (p90) and 50 kDa (p50) (Fig. 2A) present only in the sample with induced MLK3 expression were subjected to further analysis. These bands were excised from the gel and digested with trypsin. The resulting tryptic peptides were subjected to LC/MS/MS. SEQUEST was used to match MS/MS with proteins in the NCBI Protein Database. Based upon these analyses, the p90 band contained Hsp90 α and Hsp90 β , and the p50 band contained the Hsp90 co-chaperone p50^{cdc37}. Representative MS/MS data from Hsp90 α and p50^{cdc37} are shown in Fig. 2, B and C, respectively.

To verify that Hsp90 and p50^{cdc37} are present in FLAG-MLK3 complexes, they were isolated as described previously and probed by Western blotting using antibodies directed against Hsp90 and p50^{cdc37}. As expected, both Hsp90 and p50^{cdc37} are detected in FLAG-MLK3 complexes but not in control samples derived from uninduced MCF-7/iFLAG-MLK3 cells (Fig. 2D).

Hsp90 is an evolutionarily conserved heat shock protein that functions as a molecular chaperone to prevent the aggregation and to maintain the homeostasis of client proteins. The client proteins of eukaryotic Hsp90 include steroid hormone receptors

and certain protein kinases (reviewed in refs. 21, 22). The co-chaperone p50^{cdc37} is required for the interaction between Hsp90 and its client protein kinases.

Endogenous MLK3 Is Associated with Hsp90 and p50^{cdc37}—Although Hsp90 and p50^{cdc37} are clearly components of FLAG-MLK3 complexes, it is conceivable that the association of FLAG-MLK3 with Hsp90/p50^{cdc37} may be an artifact resulting from the high expression levels of FLAG-MLK3 in the induced MCF-7/iFLAG-MLK3 cells. However, immunoprecipitates of endogenous MLK3 from MCF-7 cellular lysates contain endogenous Hsp90 and p50^{cdc37} (Fig. 3). Only a small proportion of the total cellular pool of Hsp90 and p50^{cdc37} is associated with MLK3 (data not shown). This observation is not surprising as Hsp90 and p50^{cdc37} are relatively abundant proteins that have multiple client proteins.

Hsp90/p50^{cdc37} Regulate the Level of MLK3 in MCF-7 Cells—Certain ansamycin antibiotics, such as geldanamycin, occupy the ATP/ADP binding pocket of Hsp90 to inhibit its chaperone activities (23, 24) and, as such, often decrease the cellular levels of Hsp90 client proteins. To test whether the stability of MLK3 depends upon the chaperone activity of Hsp90, the impact of geldanamycin treatment on levels of endogenous MLK3 in MCF-7 cells was examined. Indeed treatment of MCF-7 cells for 24 h with increasing concentrations of geldanamycin decreased the levels of MLK3, as judged by Western blotting (Fig. 4A). A concentration of 1 μ M geldanamycin is sufficient to trigger the loss of MLK3 in MCF-7 cells. In addition, geldanamycin treatment reduces the amount of cellular MLK3 in a time-dependent manner (Fig. 4B). On average, the levels of MLK3 were reduced by more than 80% at 24 h. These results indicate that Hsp90/p50^{cdc37} regulates the level of endogenous MLK3 in MCF-7 cells and support the supposition that MLK3 is a *bona fide* client of Hsp90/p50^{cdc37}. Others have noted that Hsp90 inhibitors, including 17-allylamino-17-demethoxygeldanamycin (17-AAG) and herbimycin A, induce cell-cycle arrest of MCF-7 cells (25, 26). To determine whether geldanamycin inhibits MCF-7 cell growth, equal numbers of cells were plated and then treated with 5 μ M geldanamycin or Me₂SO for 24 h. Based on experiments performed twice in triplicate, there were 51% fewer cells after treatment with 5 μ M geldanamycin as compared with controls cells, in accord with previously published reports.

Hsp90/p50^{cdc37} Associates with the Kinase Domain of MLK3—The ability of Hsp90 to interact with and stabilize its client protein kinases depends upon its cochaperone p50^{cdc37}. To determine which domains of MLK3 are responsible for interaction with Hsp90/p50^{cdc37}, the ability of NH₂-terminal HA-tagged truncated variants of MLK3 to interact with coexpressed GST-p50^{cdc37} and endogenous Hsp90 was examined using a GST-pulldown assay. The presence of endogenous Hsp90 and the MLK3 variants in the GST-p50^{cdc37} complexes was assessed by Western blotting. Data from GST-pulldown assays show that GST-p50^{cdc37}, but not GST, associates with endogenous Hsp90 (Fig. 5A). Only MLK3 1–399, which includes the glycine-rich region, Src-homology 3 domain, kinase domain, and the isolated kinase domain, MLK3 115–399, are present in complexes of GST-p50^{cdc37}/endogenous Hsp90 (Fig. 5A). In contrast, MLK3 400–847 (which includes the zipper region, CRIB motif, and the proline-rich carboxyl-terminal region) fails to interact with GST-p50^{cdc37}/endogenous Hsp90 (Fig. 5A). As expected, none of the MLK3 variants associates with GST. Based on these experiments, we conclude that MLK3 interacts with Hsp90/p50^{cdc37} through its kinase domain. This conclusion is consistent with the findings that LKB1 (18), I κ B kinase (27), MOK (28), and Raf (29) associate with Hsp90/p50^{cdc37} through their kinase domains.

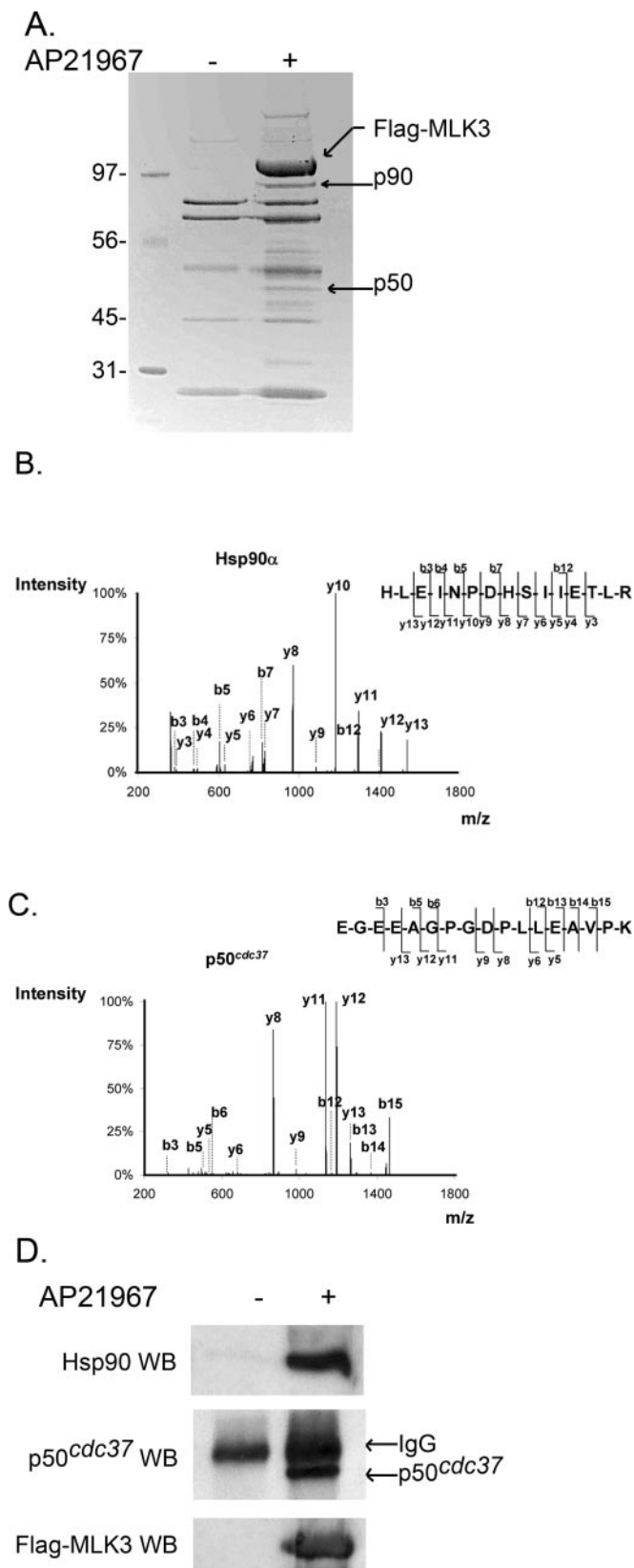


FIG. 2. Affinity purification of FLAG-MLK3 complexes and identification of Hsp90/p50^{cdc37} by LC/MS/MS. A, MCF-7 cells engineered to inducibly express FLAG-MLK3 were cultured either in the presence or absence of AP21967 for 20 h. Cleared cellular lysates were immunoprecipitated with anti-FLAG M2 agarose. Immunoprecipitates were washed, and FLAG-MLK3 complexes were eluted with 3 \times FLAG peptide. After acetone precipitation, FLAG-MLK3 and associated proteins were resolved by SDS-PAGE on a 4–12% acrylamide gradient gel and stained with Coomassie Blue. Molecular mass markers are

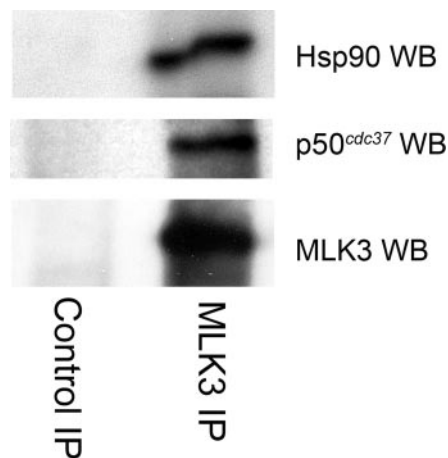


FIG. 3. Endogenous MLK3 is associated with Hsp90 and p50^{cdc37}. MLK3 was immunoprecipitated from MCF-7 cellular lysates using an MLK3 antibody prebound to protein A agarose beads. A control immunoprecipitation was performed in parallel using a GST antibody. The presence of Hsp90, p50^{cdc37}, and MLK3 was assessed by Western blotting using the indicated antibodies.

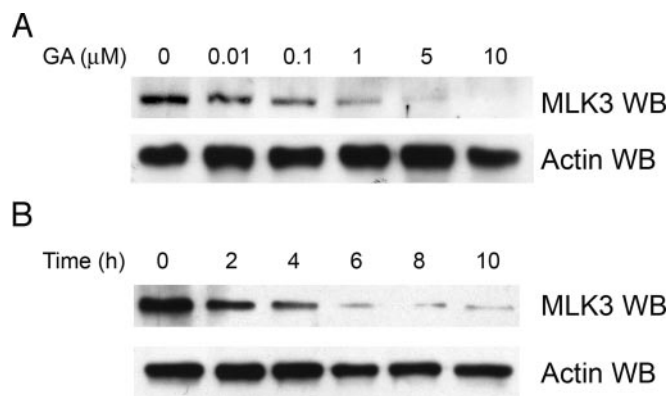


FIG. 4. Geldanamycin decreases the endogenous level of MLK3. MCF-7 cells were treated with various concentrations of geldanamycin (GA) for 24 h (A) or with 5 μ M of geldanamycin for the indicated times (B). The endogenous level of MLK3 was assessed by Western blotting of cellular lysates using the MLK3 antibody. Western blotting for endogenous actin was used to confirm that equal cell equivalents were loaded.

The Association of MLK3 with Hsp90/p50^{cdc37} Is Independent of Its Activity—Because Hsp90/p50^{cdc37} bind the catalytic domain of MLK3, it is conceivable that the activation state of MLK3 may affect this interaction. We tested the ability of three forms of MLK3 that reflect different activation states to associate with Hsp90/p50^{cdc37}: wild-type MLK3, Cdc42-activated wild-type MLK3, and the kinase-defective MLK3 K144A (15). After transient expression of MLK3 variants with or without activated Cdc42, MLK3 variants were immunoprecipitated, and the presence of endogenous Hsp90 and endogenous p50^{cdc37} was assessed by Western blotting using appropriate antibodies. As shown in Fig. 6, equal amounts of Hsp90/

indicated on the left in kDa. B, MS/MS of one peptide from p90, [M+H]²⁺ = 894.67. The data shown are representative of MS/MS from 12 Hsp90 α peptides and 9 Hsp90 β peptides. C, MS/MS of one peptide from p50, [M+H]²⁺ = 855.27. The observed y and b fragment ions corresponding to the indicated peptide sequence are shown. D, after purification of MLK3 complexes from MCF7/iFLAG-MLK3 cells, MLK3-associated proteins were separated by SDS-PAGE. The presence of Hsp90, p50^{cdc37}, and FLAG-MLK3 was assessed by Western blotting with antibodies directed against Hsp90, p50^{cdc37}, and the FLAG epitope, respectively. The p50^{cdc37} and the anti-FLAG M2 antibody (IgG) heavy chain are indicated by arrows in the middle panel.

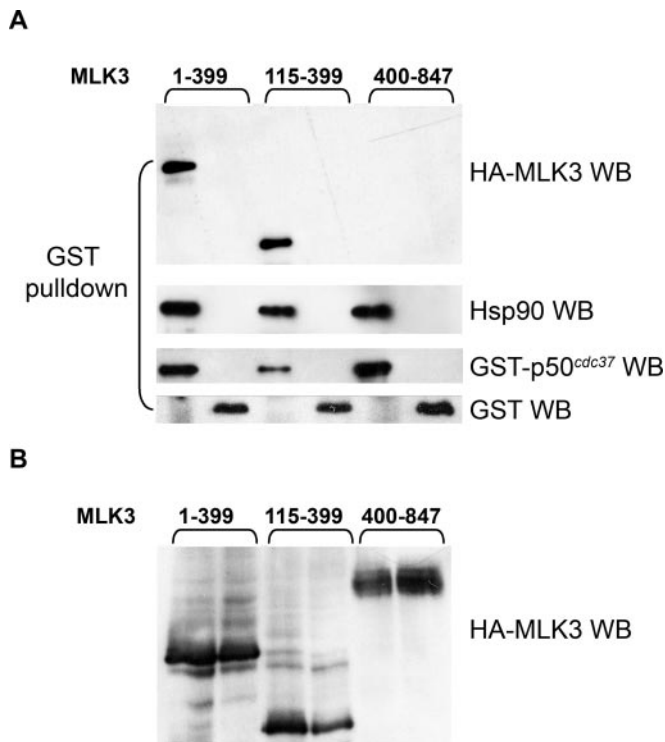


FIG. 5. Hsp90/p50^{cdc37} associate with the catalytic domain of MLK3. HEK 293 cells were transfected with expression vectors containing the cDNA for MLK3 truncation variants and GST-p50^{cdc37} (or GST). The numbers in the figure represent amino acid numbers in MLK3. Cellular lysates expressing the indicated MLK3 truncation variants and GST-p50^{cdc37} (or GST) were incubated with glutathione-Sepharose 4B resin. *A*, upper two panels, the presence or absence of bound MLK3 variants and Hsp90 was assessed by Western blotting using the HA antibody and Hsp90 antibody, respectively; lower two panels, amounts of GST-p50^{cdc37} or GST bound to the glutathione-Sepharose 4B resin were assessed by Western blotting with GST antibody. *B*, the expression levels of MLK3 variants in cellular lysates probed with the HA antibody. The data shown are representative of three independent experiments.

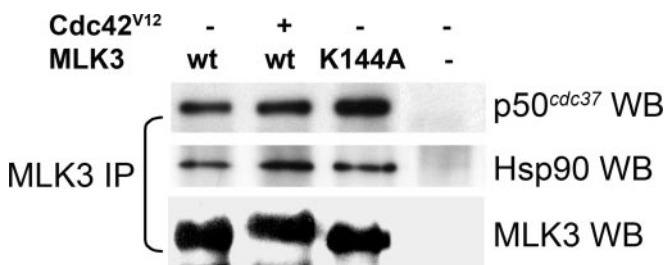


FIG. 6. Association of MLK3 variants with Hsp90/p50^{cdc37}. HEK 293 cells were transfected with expression vectors for wild-type (*wt*) MLK3, with or without Cdc42^{V12}, or for kinase-defective MLK3 (K144A). MLK3 was immunoprecipitated from cellular lysates using MLK3 antibody. The presence or absence of p50^{cdc37}, Hsp90, and MLK3 was assessed by Western blotting using the indicated antibodies.

p50^{cdc37} associate with the MLK3 variants under these conditions. These data support the idea that MLK3 activity does not influence its interaction with Hsp90/p50^{cdc37}.

Hsp90/p50^{cdc37} Regulate TNF α -induced Activation and Signaling of MLK3 in MCF-7 Cells—TNF α treatment has been shown to activate endogenous MLK3 in Jurkat T cells (14). We examined whether TNF α increases MLK3 activity in MCF-7 cells. After 30 min of treatment with TNF α , endogenous MLK3 was immunoprecipitated, and its activity was assessed by an *in vitro* kinase assay using recombinant, catalytically inactive GST-MKK7 as a substrate. As shown in Fig. 7, the basal activity of endogenous MLK3 is low. Upon TNF α treatment,

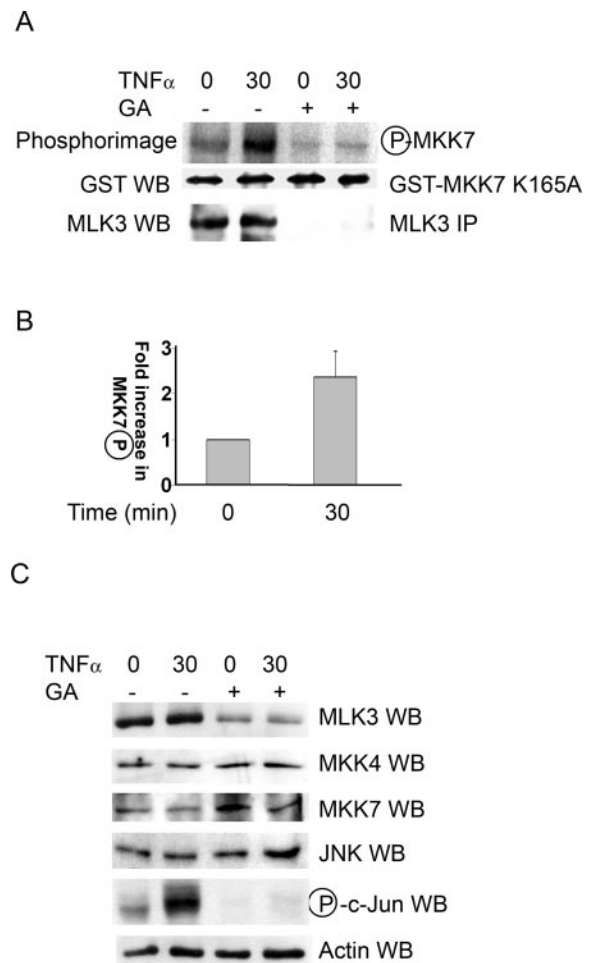


FIG. 7. Geldanamycin abolishes TNF α -mediated activation of MLK3 and JNK. MCF-7 cells were pretreated with geldanamycin or the vehicle Me₂SO for 24 h, then treated with TNF α for the indicated time and lysed. *A*, *in vitro* kinase assay of MLK3 using GST-MKK7 K165A as a substrate. MLK3 was isolated from cellular lysates by immunoprecipitation using an MLK3 antibody, and kinase activity was assessed *in vitro* using recombinant GST-MKK7 K165A as a substrate. *Top panel*, phosphorimage with bands corresponding to MKK7 phosphorylation; *bottom panel*, Western blot of the immunoprecipitated MLK3; *middle panel*, equal amounts of GST-MKK7 substrate in the kinase assay samples were confirmed by Western blotting using an antibody against GST. *B*, the mean \pm S.E. for fold increase in GST-MKK7 K165A phosphorylation from three independent experiments. *C*, equal amounts of protein from cellular lysates were resolved by SDS-PAGE and Western blotted using the indicated antibodies.

the catalytic activity of MLK3 increases about 2.5-fold (Fig. 7, *A* and *B*). The activation of endogenous MLK3 coincides with JNK activation in cells, as measured by the phosphorylation of c-Jun (Fig. 7*C*). These data are consistent with TNF α -induced, MLK3-mediated JNK activation.

If TNF α -induced JNK activation involves MLK3, we would expect that geldanamycin treatment should block JNK activation in this setting. MCF-7 cells were incubated with or without geldanamycin for 24 h and then treated with TNF α for 30 min. As shown in Fig. 7, geldanamycin pretreatment abolished the TNF α -induced activation of MLK3. Indeed, under these conditions, TNF α fails to activate JNK. In addition, geldanamycin treatment lowers the basal level of phosphorylated c-Jun in MCF-7 cells. It is possible that geldanamycin directly affects the levels of JNK or its proximal upstream kinases, MKK4 and MKK7. However, Western blotting revealed that the geldanamycin has no impact on the levels of MKK4, MKK7, or JNK in MCF-7 cells (Fig. 7*C*). Taken together, these results support

the idea that Hsp90/p50^{cdc37} regulate TNF α -induced JNK activation at the MAPKKK (MLK3) level.

DISCUSSION

To better understand MLK3 signaling, especially in the context of breast cancer cells, we sought to identify MLK3-interacting proteins in the estrogen-responsive breast cancer cell line, MCF-7. Our strategy was to inducibly express FLAG-tagged MLK3, isolate its complexes, and identify associated proteins using mass spectrometry. The induced FLAG-MLK3 is constitutively active, as judged by a corresponding increase in the cellular level of phosphorylated c-Jun. The uninduced MCF-7 cells served as a useful control for proteins that non-specifically bind the affinity resin. In this way, several Coomassie-stained protein bands present only in complexes of induced MLK3 expression were identified. Bands of 90 and 50 kDa, which were of similar staining intensity, were found to be Hsp90 and p50^{cdc37}, respectively.

Several protein kinases have been reported as clients of Hsp90 and p50^{cdc37}. In the cases of LKB1 (18), I κ B kinase (27), Raf (29), and MOK (28), where domain interactions have been mapped, Hsp90/p50^{cdc37} associates with the kinase domains of these protein kinases. Accordingly we found that the catalytic domain of MLK3 is sufficient for its association with recombinant p50^{cdc37}. Given their highly homologous catalytic domains, it seems likely that other MLKs may be clients of Hsp90/p50^{cdc37}.

Our finding that a kinase-defective variant of MLK3 and a Cdc42-activated form of MLK3 associate with Hsp90/p50^{cdc37} to a similar extent suggests that complex formation with Hsp90/p50^{cdc37} is independent of MLK3 kinase activity. This is analogous to the finding that Raf-1, another MAPKKK that activates the ERK rather than JNK pathway, is in complex with Hsp90/p50^{cdc37} in both its inactive cytosolic form as well as in its membrane-associated, activated form (30).

Most protein kinases that interact with Hsp90/p50^{cdc37} are stabilized through this association. Occupation of the ATP/ADP binding site of Hsp90 by geldanamycin still permits its interaction with p50^{cdc37} but inhibits binding to client protein kinases. Our findings that endogenous MLK3 interacts with Hsp90/p50^{cdc37} and that MLK3 levels are sensitive to geldanamycin suggest that MLK3 is stabilized through its interaction with Hsp90/p50^{cdc37}, although it is conceivable that the geldanamycin-induced reduction in MLK3 levels might occur indirectly through the down-regulation of another geldanamycin-sensitive Hsp90 client protein. For instance, geldanamycin also inhibits the endoplasmic reticulum-localized heat shock protein GRP94 (31); however, we failed to detect GRP94 as an MLK3-associated protein in our mass spectrometry experiments.

Recently, it has been reported that TNF α activates endogenous MLK3, leading to JNK activation in Jurkat T lymphocyte cells (14). In our study, we have shown that TNF α activates endogenous MLK3 and JNK with similar kinetics in MCF-7 breast cancer cells. Pretreatment of the MCF-7 cells with geldanamycin abolishes TNF α -induced MLK3 activation, in parallel with the loss of MLK3. Yet under these conditions, the cellular levels of MKK4, MKK7, and JNK remain constant. Indeed, it has been reported previously that the conventional MAPKs, ERK, p38, and JNK, fail to interact with Hsp90/p50^{cdc37} (28). Our findings are consistent with the idea that the geldanamycin-induced blockade of JNK signaling is accomplished through destabilization of MLK3. However, it is conceivable that other TNF α -activated MAPKKKs, such as apoptosis signal-regulating kinase 1 (32, 33) or mitogen-activated protein kinase kinase kinase 1 (34), might also be targeted by geldanamycin. We also cannot rule out the existence of other geldanamycin-sensitive protein kinases that function up-

stream of MAPKKKs to activate JNK. For example, there are reports, albeit controversial, that receptor interacting protein, a geldanamycin-sensitive protein kinase that primarily functions in TNF α -induced NF- κ B activation (35), may also effect TNF α -induced JNK activation (36, 37).

Our finding that geldanamycin impinges on JNK signaling at the MAPKKK (MLK3) level, but not at the MAPKK (MKK4/MKK7) or at the MAPK (JNK) levels, is reminiscent of what has been learned about Hsp90/p50^{cdc37} regulation of the ERK/MAPK pathway. Hsp90/p50^{cdc37} controls the stability of both active, membrane-associated and inactive, cytosolic Raf-1 (38) but not of MEK or ERK (39). Furthermore, phorbol 12-myristate 13-acetate-induced activation of ERK is abolished by geldanamycin treatment, apparently through destabilization of Raf-1 (39). The cumulative evidence may indicate a more general role of Hsp90/p50^{cdc37} in stabilizing MAPKKKs.

The defined repertoire of Hsp90 clients has included primarily steroid hormone receptors, oncoprotein kinases, and protein kinases associated with cellular proliferation or survival, including the estrogen receptor (40), Raf (19, 29, 30, 38, 39, 41), CDK4 (42), Akt (43, 44), ErbB2 (45), and Bcr-Abl (46). Yet recent findings challenge this paradigm. Hsp90/p50^{cdc37} has been demonstrated to interact with and stabilize LKB1, a serine/threonine kinase which functions as a tumor suppressor gene (18). MLK3 and its family members have been implicated in programmed cell death in neurons and neuronal-like cells through their ability to activate the JNK cascade (5, 6). In addition, mutations in the *mlk4* gene have been identified in human colorectal cancer (47). In one instance, the mutated *mlk4* gene encodes a kinase with a truncated catalytic domain, suggesting that *mlk4* might function as a tumor suppressor gene (43) rather than as a promoter of proliferation.

Geldanamycin and other ansamycins have been shown to inhibit the growth of many tumor cell lines, including MCF-7 cells; and the geldanamycin analogue, 17-AAG, has entered clinical trials for treating various types of cancer (48). Because of the multiple clients ascribed to Hsp90, the net effect of geldanamycin on cell proliferation is likely multifactorial and, as such, it is impossible to know whether depletion of MLK3 is at all responsible for geldanamycin-induced growth arrest of MCF-7 cells. Although MLKs have been implicated in primarily apoptotic signaling, the function(s) of MLK3 in breast cancer cells are not understood. It will be important to more thoroughly probe the signaling events and cellular responses mediated through and impacted by MLK3 in cancer cells.

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