

## Zipper-mediated Oligomerization of the Mixed Lineage Kinase SPRK/MLK-3 Is Not Required for Its Activation by the GTPase cdc 42 but Is Necessary for Its Activation of the JNK Pathway

MONOMERIC SPRK L410P DOES NOT CATALYZE THE ACTIVATING PHOSPHORYLATION OF Thr<sup>258</sup> OF MURINE MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4\*

Received for publication, April 4, 2000, and in revised form, June 6, 2000  
Published, JBC Papers in Press, June 21, 2000, DOI 10.1074/jbc.M002858200

Panayiotis O. Vacratsis‡ and Kathleen A. Gallo§¶

From the Departments of ‡Biochemistry and §Physiology, Michigan State University, East Lansing, Michigan 48824

**Src homology 3 domain-containing proline-rich kinase (SPRK)/mixed lineage kinase-3 is a serine/threonine kinase that has been identified as an upstream activator of the c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathway. SPRK is capable of activating MKK4 by phosphorylation of serine and threonine residues, and mutant forms of MKK4 that lack the phosphorylation sites Ser<sup>254</sup> and Thr<sup>258</sup> block SPRK-induced JNK activation. A region of 63 amino acids following the kinase domain of SPRK is predicted to form a leucine zipper. The leucine zipper domain of SPRK has been shown to be necessary and sufficient for SPRK oligomerization, but its role in regulating activation of SPRK and downstream signaling remains unclear. In this study, we substituted a proposed stabilizing leucine residue in the zipper domain with a helix-disrupting proline to abrogate zipper-mediated SPRK oligomerization. We demonstrate that constitutively activated Cdc42 fully activates this monomeric SPRK mutant in terms of both autophosphorylation and histone phosphorylation activity and induces the same *in vivo* phosphorylation pattern as wild type SPRK. However, this catalytically active SPRK zipper mutant is unable to activate JNK. Our data show that the monomeric SPRK mutant fails to phosphorylate one of the two activating phosphorylation sites, Thr<sup>258</sup>, of MKK4. These studies suggest that zipper-mediated SPRK oligomerization is not required for SPRK activation by Cdc42 but instead is critical for proper interaction and phosphorylation of a downstream target, MKK4.**

Mitogen-activated protein kinases (MAPKs)<sup>1</sup> are serine/threonine kinases that are regulated by upstream kinase cascades. The MAPK pathways regulate many cellular processes including proliferation, differentiation, and gene expression. The best characterized MAPK pathways in mammalian cells are the

extracellular signal-regulated kinase, the reactivating kinase (p38/RK), and the c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathways (1, 2). In MAPK pathways, extracellular signals lead to the phosphorylation and activation of a MAPK kinase kinase. An activated MAPK kinase kinase can productively bind, phosphorylate, and, hence, activate a dual specific MAP kinase kinase (MAPKK), which in turn activates a MAPK by phosphorylating a threonine residue and a tyrosine residue within the conserved activation segment located between subdomains VII and VIII of its catalytic domain.

Activating phosphorylation sites for the MAPKK family have also been identified within kinase subdomains VII and VIII (3–5). For instance, the dual specific kinase, MAPK kinase-4 (MKK4/SEK1), which phosphorylates and activates JNK (6), requires phosphorylation on Ser<sup>254</sup> and Thr<sup>258</sup> for activation (4, 5). These residues are located within the conserved activation segment of kinase subdomains VII and VIII, and MKK4 mutants lacking these two phosphorylation sites fail to phosphorylate and activate JNK (3, 4, 7).

Src homology 3 domain-containing proline-rich protein kinase (SPRK), a member of the mixed lineage kinase family (also called MLK-3), has been identified as an upstream activator of the JNK pathway (4, 7, 8). SPRK is capable of activating MKK4 by phosphorylation of serine and threonine residues, and mutant forms of MKK4 that lack the phosphorylation sites, Ser<sup>254</sup> and Thr<sup>258</sup>, block SPRK-induced JNK activation (7, 8).

SPRK is an intracellular serine/threonine kinase with a predicted molecular mass of 93 kDa (9). In addition to the kinase domain, the sequence of SPRK encodes several domains that are predicted to be involved in protein-protein interactions, including an Src homology 3 domain, a leucine zipper domain, a Cdc42/Rac interactive binding motif and a COOH-terminal 220-amino acid region that is rich in proline, serine, and threonine residues.

Recently, it has been demonstrated that SPRK can associate with an activated form of Cdc42 (10, 11) and that this association requires a functional Cdc42/Rac-interactive binding motif (12). Coexpression of SPRK and activated Cdc42 in cells increases the catalytic activity of SPRK (11, 12). Interestingly, recombinant catalytically active SPRK is not activated further by the addition of purified GTP-bound Cdc42, suggesting the requirement of an additional cellular component for SPRK activation by Cdc42 (12). Furthermore, using *in vivo* labeling experiments and comparative two dimensional phosphopeptide mapping, we have shown that coexpression of SPRK with activated Cdc42 alters the *in vivo* phosphorylation pattern of SPRK (12).

A region of 63 amino acids following the kinase domain of

\* This work was supported by National Institutes of Health Grant CA76306. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Depts. of Physiology and Biochemistry, 108 Giltner Hall, Michigan State University, East Lansing, MI 48824. Tel.: 517-355-6475; Fax: 517-355-5125, E-mail: gallo@ppl.msu.edu.

<sup>1</sup> The abbreviations used are: MAPK, mitogen activated protein kinase; RK, reactivating kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MKK, MAPK kinase; SPRK, Src homology 3 domain-containing proline-rich kinase; MLK, mixed lineage kinase; HA, hemagglutinin; 3HA, triple HA; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MBP, maltose binding protein.

SPRK is predicted to be a leucine zipper domain. Leucine zippers mediate protein oligomerization by forming coiled coil structures. These structures are stabilized mainly by leucine residues spaced seven residues apart that interact at the interface of opposing helices (13–16). The heptad repeat is the name given to the notation of labeling the amino acids in the leucine zipper domain, *a* through *g*, with leucine residues predominately found at position *d* (17). In addition, electrostatic interactions in the form of salt bridges may also contribute to coiled coil stability and specificity (18, 19).

The leucine zipper domain of SPRK is necessary and sufficient to mediate homo-oligomerization (12, 20). Based on deletion studies, others have suggested that zipper-mediated SPRK oligomerization is required for its activation (20). We have examined the role of the leucine zipper of SPRK in more detail and arrive at a different conclusion. Rather than deleting the entire leucine zipper, we substituted a helix-disrupting proline for a leucine residue at one of the proposed *d* positions in the zipper motif of SPRK. Although this point mutant fails to oligomerize, constitutively activated Cdc42 fully activates this monomeric SPRK mutant in terms of both autophosphorylation and histone phosphorylation activity. Moreover, Cdc42 induces the same *in vivo* phosphorylation pattern of the SPRK zipper mutant and wild type SPRK. However, this catalytically active SPRK zipper mutant is unable to activate JNK. Our data show that SPRK oligomerization is necessary to phosphorylate Thr<sup>258</sup>, one of the two activating phosphorylation sites of MKK4. These studies suggest that zipper-mediated SPRK oligomerization is not required for the activation of SPRK by Cdc42 but is required for proper interaction and phosphorylation of a downstream target, MKK4.

#### EXPERIMENTAL PROCEDURES

**DNA Constructs and Mutagenesis**—Construction of the cytomegalovirus-based expression vectors carrying the cDNAs for wild type SPRK (pRK5-*sprk*) has been described elsewhere (9). The expression plasmid encoding NH<sub>2</sub>-terminal FLAG epitope-tagged constitutively active variant (pRK5-Nflag.cdc42<sup>Val-12</sup>) of Cdc42 was kindly provided by Avi Ashkenazi (Genentech, Inc.). A variant of SPRK containing a point mutation in the leucine zipper domain (SPRK L410P) was constructed using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA.), with the following oligonucleotides: 5'-CTTTTCCTTG-GCTCGCGGCTCGTCAAGAGACC-3' and 5'-GGTCTCTTCGACGAG-CCGCGAGCCAAAGAAAAG-3'. The presence of the desired mutation was confirmed by dideoxy DNA sequencing using Sequenase enzyme (Amersham Pharmacia Biotech) and the Sanger method. Construction of the COOH-terminal triple hemagglutinin (3HA) epitope-tagged variant (pRK5-C3HA-*sprk*) was generated by polymerase chain reaction amplification of the 3HA epitope from the CLN2T plasmid (21) using the following oligonucleotides: 5'-CGTGAGGTACCGGAAGCGGGCC-TTACCCATACGATGTTC-3' and 5'-CGAGGTCTAGATTAGCACTG-AGCAGCGTAATCTGG-3', followed by subcloning of the amplified fragment into the pRK5-*sprk* vector using *Nhe*I and *Xba*I. Dr. Ajay Rana (Massachusetts General Hospital, Boston, MA) kindly provided the pEBG-*sek/mkk4* expression plasmid encoding murine MKK4 fused to glutathione *S*-transferase (GST).

**Cell Culture, Transfections, and Lysis**—Human fetal kidney 293 cells were maintained in Ham's F-12 medium/low glucose Dulbecco's modified Eagle's medium (1:1) (Life Technologies, Inc.) supplemented with 8% fetal bovine serum (Life Technologies, Inc.), 2 mM glutamine, and penicillin/streptomycin (Life Technologies, Inc.). Plasmids (10 µg each for 100-mm dishes) were used to transfect 293 cells using the calcium phosphate technique. Cell monolayers were incubated with the DNA precipitate for 4 h, then washed once with phosphate-buffered saline, and cultured in the medium described above. Cells were harvested, washed with ice-cold phosphate-buffered saline, and lysed as described previously (12).

**Immunoprecipitations and GST Pulldown Assays**—The following antibodies against the proteins of interest were prebound to protein A-agarose beads: SPRK antiserum (0.25 µg/µl slurry), M2 monoclonal antibody (Kodak IBI) directed against the FLAG epitope (0.45 µg/µl slurry) and JNK C-17 antibody (Santa Cruz) (50 ng/µl slurry). Immunoprecipitation experiments were performed as described previously

(9). For the GST pulldown experiment, 293 cells were transiently co-transfected with pEBG-*sek/mkk4* or pEBG and expression vectors for SPRK variants. Clarified lysate (300 µl) was incubated with 20 µl of antibody bound protein A-agarose or 30 µl of glutathione-Sepharose resin for 90 min at 4 °C. Immunoprecipitates and the GST pulldowns were washed with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). Immunoprecipitates used for kinase assays were washed three times with HNTG buffer containing 1 M LiCl, three times with HNTG buffer, and twice with kinase assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MnCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>).

**Gel Electrophoresis and Western Blot Analysis**—Lysates and immunoprecipitates of proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (22). Proteins were transferred to nitrocellulose membranes and immunoblotted using SPRK antiserum (1 µg/ml), 16B12 HA monoclonal antibody (BabCo) (5 µg/ml), M2 FLAG monoclonal antibody (9 µg/ml), JNK C-17 antibody (0.5 µg/ml), MKK4 antibody (0.5 µg/ml), or phospho-MKK4 antibody (New England Biolabs) (0.5 µg/ml), followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Life Technologies, Inc.). Western blots were developed by chemiluminescence. Multiple exposures of the Western blots were developed, and densitometry (NIH Image) of unsaturated films was used to determine relative expression levels. Statistics were calculated using an unpaired Student's *t* test. A *p* value smaller than 0.05 was considered statistically significant.

**In Vitro Kinase Assays**—Kinase assays were performed in 20 µl of kinase assay buffer containing 50 µM ATP and 5 µCi of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol). For the SPRK kinase assay 10 µg of mixed histones (Roche Molecular Biochemicals) or 10 µg of murine GST-MKK4 was used as the substrate, and the reaction was carried out for 30 min at room temperature. GST-MKK4 was expressed from the pGEX-2T vector in *Escherichia coli* BL-21 cells and purified by glutathione-Sepharose chromatography. The reactions were terminated by the addition of an equal volume of 2× SDS sample buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 100 mM dithiothreitol, 1%  $\beta$ -mercaptoethanol) containing 50 mM EDTA, pH 8.0.

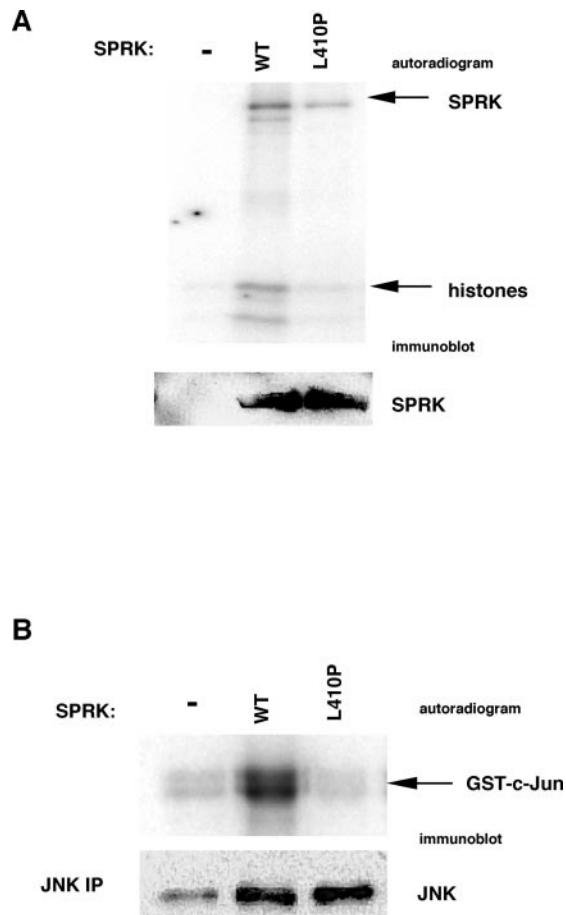
JNK assays were performed as described previously, using GST-c-Jun as the substrate (12). The pGEX-c-Jun (1–115) vector was kindly provided by Dr. Ajay Rana (Massachusetts General Hospital, Boston, MA). GST-c-Jun was expressed in *E. coli* XL-1 Blue cells and purified by glutathione-Sepharose chromatography. Following the kinase assay, proteins were separated by SDS-PAGE. Gels were rinsed in phosphate-buffered saline, dried, and incorporation of radioactivity into kinase or substrates was determined by phosphorimaging (Molecular Dynamics).

**Expression and Purification of MBP Fusion Proteins**—MBP fusion protein plasmid construction and protein expression were described previously (12). MBP fusion proteins were purified by amylose affinity chromatography according to the manufacturer's protocol. Fractions containing the MBP-zips or MBP-zips L410P, as determined by SDS-PAGE followed by Coomassie Blue staining, were pooled and concentrated to about 1 mg/ml using a Centrprep concentrator (Amicon).

**Size Exclusion Chromatography**—Gel filtration fast pressure liquid chromatography was used to analyze MBP-zips and MBP-zips L410P. The fusion proteins were applied to a Superose 6 HR 10/30 column (25 ml column volume) (Amersham Pharmacia Biotech). The void volume of the column was determined using blue dextran (2000 kDa). The column was calibrated with cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), amylase (200 kDa), apoferritin (445 kDa), and thyroglobulin (700 kDa) (Sigma). The fusion proteins were eluted with 250 mM sodium phosphate buffer, pH 7.2, containing 125 mM NaCl at room temperature. The flow rate was 0.5 ml/min, and the effluent was continuously monitored at 280 nm. Approximately 30 fractions of 1.0 ml each were collected, and the presence of MBP fusion proteins was assessed by SDS-PAGE followed by Coomassie Blue staining.

**Phosphopeptide Mapping**—After a 24-h transfection with pRK5-*sprk* or pRK5-*sprk* L410P in the presence or absence of pRK5-Nflag.cdc42<sup>Val-12</sup>, 293 cells were washed five times with phosphate-free medium (Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum (Summit Biotechnology) and incubated at 37 °C for 2 h. The cells were then incubated in phosphate-free medium containing 3 mCi/ml [<sup>32</sup>P]orthophosphate (NEN Life Science Products) for 4 h at 37 °C. Lysis and subsequent tryptic phosphopeptide mapping of labeled cells have been described in detail elsewhere (12).

**Phosphoamino Acid Analysis**—Following an *in vitro* kinase assay in which 10 µg of GST-MKK4 was used as a substrate, the proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Radiolabeled MKK4 bands were excised from the polyvi-

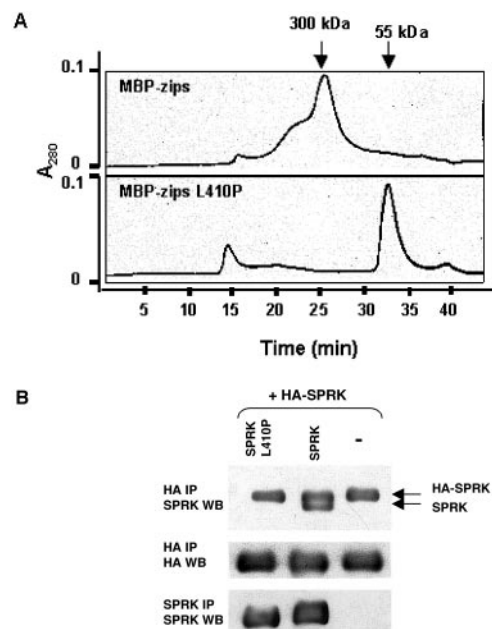


**FIG. 1. Effect of a point mutation in the SPRK zipper domain on catalytic activity and JNK activation.** Cells were transfected with expression vectors containing the cDNAs for wild type (WT) SPRK or SPRK L410P. A *minus sign* indicates that a control empty vector was transfected. The indicated SPRK variant or JNK was isolated from cellular lysates by immunoprecipitation (IP), and kinase activity was assessed *in vitro* as described under "Experimental Procedures." **A**, *in vitro* kinase assay of SPRK and SPRK L410P using histones as a substrate. Immunoprecipitated SPRK or SPRK L410P was subjected to an *in vitro* kinase assay. An autoradiogram with bands corresponding to SPRK autophosphorylation and histone phosphorylation is shown in the *top panel*. A Western blot of the immunoprecipitated samples using a SPRK antibody is shown in the *bottom panel*. The kinase assay shown is representative of five independent experiments. **B**, *in vitro* kinase assay of JNK using GST-c-Jun as a substrate. Endogenous JNK was immunoprecipitated from cellular lysates expressing SPRK or SPRK L410P and subjected to an *in vitro* kinase assay. An autoradiogram with bands corresponding to GST-c-Jun is shown in the *top panel*. An immunoblot for JNK from the same immunoprecipitated samples is shown in the *bottom panel*. The kinase assay shown is representative of three independent experiments.

nylidene difluoride membrane. After washing three times with methanol and three times with water, the radioactive piece of membrane was incubated in 200  $\mu$ l of 6 N HCl for 1 h at 100  $^{\circ}$ C. The hydrolyzed phosphoamino acids were concentrated in a SpeedVac. Unlabeled phosphoamino acid standards (Sigma) and xylene cyanol FF marker dye (Sigma) were added to each sample. The phosphoamino acids were separated by one-dimensional thin layer electrophoresis in pH 2.5 buffer (66.7% pH 3.5 buffer (glacial acetic acid/pyridine/water, 50:5:945 v/v/v) and 33.3% pH 1.9 buffer on 20  $\times$  20 cm cellulose TLC plates at 0  $^{\circ}$ C and 500 V for 1.5 h. The unlabeled phosphoamino acids were visualized by ninhydrin staining, and the  $^{32}$ P-labeled phosphoamino acids were visualized and quantitated by phosphorimaging.

## RESULTS

**Point Mutation in the Zipper Domain Decreases the *in Vitro* Kinase Activity of SPRK**—Previous studies have shown that SPRK with an intact zipper domain can self-associate and that large deletions in the zipper domain compromise the autophos-

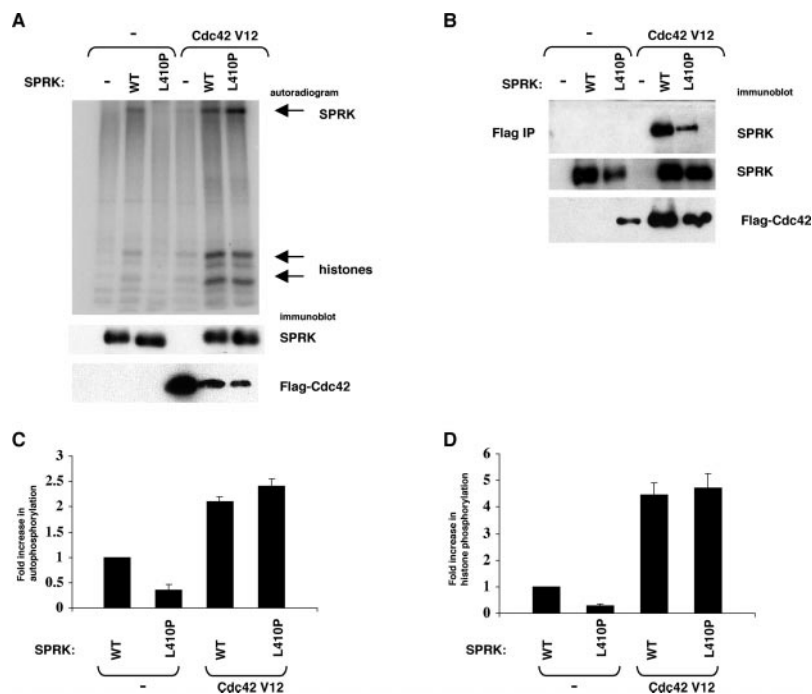


**FIG. 2. Size exclusion chromatography and coimmunoprecipitation analysis of the SPRK zipper point mutant.** **A**, size exclusion chromatographic analyses of MBP-zips and MBP-zips L410P. A Superose 6 HR 10/30 column was calibrated with cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), amylase (200 kDa), apoferritin (445 kDa), and thyroglobulin (700 kDa). The void volume of the column was determined with blue dextran (2000 kDa). Each fusion protein (100  $\mu$ g) was applied to the column and eluted with 250 mM sodium phosphate buffer, pH 7.2, containing 125 mM NaCl at room temperature. The flow rate was 0.5 ml/min, and the effluent was continuously monitored at 280 nm. **B**, coimmunoprecipitation of SPRK and SPRK L410P with 3HA-SPRK. Cellular lysates expressing the SPRK variants were immunoprecipitated (IP) with the HA antibody, and the presence of associated untagged SPRK or SPRK L410P was assessed by immunoblotting with a SPRK antibody. The SPRK antibody detects untagged SPRK as well as 3HA-SPRK, which migrates slower than untagged SPRK during SDS-PAGE. Immunoblots of 3HA-SPRK and SPRK from the immunoprecipitated samples are shown in the *middle and bottom panels* respectively. WB, Western blot.

phorylation activity of SPRK and abrogate its ability to activate JNK (12, 20). To more carefully probe the function of the zipper domain of SPRK, a leucine residue at position 410, which is predicted to reside at a coiled coil interface (23), was substituted with a proline residue using site-directed mutagenesis. The catalytic properties of this zipper mutant, SPRK L410P, were compared with those of wild type SPRK.

Cells transiently expressing SPRK or SPRK L410P were lysed, and the SPRK proteins were immunoprecipitated and used in an *in vitro* kinase assay with a mixture of histones as a substrate. Data from a representative experiment are shown in Fig. 1A. Based on three independent experiments, SPRK L410P has 35% of the autophosphorylation activity and 30% of the histone phosphorylation activity of wild type SPRK. Although expression of wild type SPRK activates JNK in 293 cells, SPRK L410P fails to induce JNK activation (Fig. 1B). These data suggest that the zipper domain of SPRK is important for its basal phosphorylation activity and for JNK activation.

**A SPRK Leucine Zipper Mutant Fails to Oligomerize**—Leucine zipper domains commonly dimerize or form higher order oligomers. To test whether SPRK L410P in the context of its zipper domain can oligomerize, fusion proteins consisting of the monomeric MBP from *E. coli*, and either the leucine zipper domain of wild type SPRK (MBP-zips) or that containing the proline mutation (MBP-zips L410P) were constructed and an-



**FIG. 3. Effect of SPRK L410P on Cdc42<sup>Val-12</sup> activation and binding of SPRK.** Cells were transfected with expression vectors containing the cDNAs for the indicated SPRK variant and/or Cdc42<sup>Val-12</sup> (V12). *In vitro* kinase assays and co-immunoprecipitation experiments were performed as described under "Experimental Procedures." *A*, *in vitro* kinase assay of SPRK and SPRK L410P using histones as a substrate. The *top panel* shows an autoradiogram with bands corresponding to SPRK autophosphorylation and histone phosphorylation as indicated. SPRK autophosphorylation and histone phosphorylation was quantitated by phosphorimaging and normalized to SPRK expression levels as described under "Experimental Procedures." The means  $\pm$  S.E. of three independent experiments are shown (*C* and *D*). Immunoblots of SPRK and Cdc42 from cellular lysates are shown in the *middle* and *bottom panels*, respectively. *B*, co-immunoprecipitation experiments of SPRK and SPRK L410P with Cdc42<sup>Val-12</sup>. FLAG epitope-tagged Cdc42<sup>Val-12</sup> was immunoprecipitated from cellular lysates using an antibody directed against the FLAG epitope. The presence of associated SPRK or SPRK L410P was determined by immunoblotting with a SPRK antibody (*top panel*). Immunoblots of SPRK and Cdc42 from cellular lysates are shown in the *middle* and *bottom panels*, respectively. WT, wild type.

alyzed by size exclusion chromatography (Fig. 2A). MBP-zips elutes as a high molecular mass complex corresponding to a molecular mass of approximately 300 kDa. However, MBP-zips L410P elutes predominantly as a single peak corresponding to a molecular mass of about 55 kDa, the expected size for the monomeric protein. These data demonstrate that the leucine zipper domain of SPRK is capable of forming multimers of MBP and that a point mutation of one leucine residue is sufficient to disrupt the oligomerization of the leucine zipper domain of SPRK.

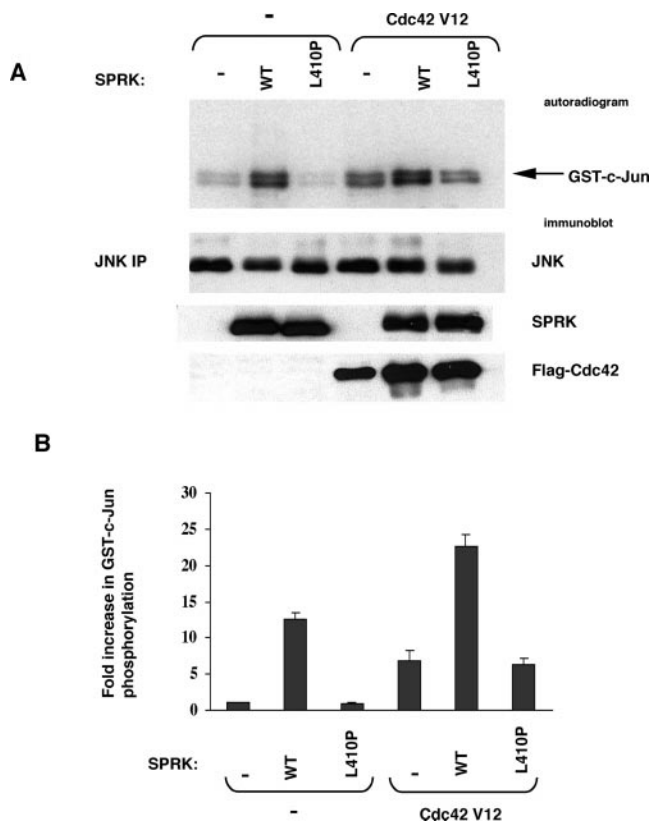
To examine whether the zipper point mutation effects association with full-length SPRK, we examined whether SPRK L410P or wild type SPRK could associate with epitope-tagged SPRK. After transient coexpression of SPRK L410P or wild type SPRK, with HA-tagged SPRK (3HA-SPRK), cellular lysates were immunoprecipitated with the HA antibody. The presence of associated untagged SPRK or SPRK L410P was assessed by Western blotting with a SPRK antibody (Fig. 2B). The triple HA epitope adds 27 amino acids to the COOH terminus of SPRK, allowing it to be distinguished from untagged SPRK by its slower mobility on SDS-polyacrylamide gels. Although wild type SPRK associates with 3HA-SPRK, SPRK L410P cannot form a detectable complex with 3HA-SPRK. These results, coupled with the gel filtration experiments, indicate that SPRK L410P behaves as a monomer.

**Activated Cdc42 Increases the *In Vitro* Catalytic Activity of Both Wild Type SPRK and SPRK L410P**—The GTPase Cdc42 in its active form can associate with SPRK and increase its catalytic activity (11, 12). This increased activity measured *in vitro* correlates with a change in the *in vivo* phosphorylation state of SPRK (12). We examined the effect of Cdc42<sup>Val-12</sup>, a mutant form of the GTPase that renders Cdc42 constitutively active, on the activity of SPRK L410P in an immunocomplex

kinase assay. SPRK or SPRK L410P, expressed in the presence or absence of Cdc42<sup>Val-12</sup>, was immunoprecipitated from cellular lysates of transiently transfected 293 cells and subjected to an *in vitro* kinase assay using a mixture of histones as a substrate. After coexpression with Cdc42<sup>Val-12</sup>, the *in vitro* autophosphorylation and histone phosphorylation activities of SPRK L410P are the same as those of wild type SPRK coexpressed with Cdc42<sup>Val-12</sup> (Fig. 3, A, C, and D).

To test the ability of the leucine zipper mutant to associate with activated Cdc42, SPRK L410P or wild type SPRK was coexpressed with FLAG-tagged Cdc42<sup>Val-12</sup> in 293 cells. As shown in Fig. 3B, SPRK L410P coimmunoprecipitated with Cdc42<sup>Val-12</sup>, although to a lesser extent than did wild type SPRK. These data suggest that SPRK L410P can bind and be fully activated by Cdc42<sup>Val-12</sup>.

**SPRK Oligomerization Is Necessary for SPRK-induced JNK Activation**—Because coexpression with Cdc42<sup>Val-12</sup> renders SPRK L410P fully active in *in vitro* kinase assays (Fig. 3, A, C, and D), we tested whether this zipper mutant, when coexpressed with the GTPase, was competent in downstream signaling. Cells expressing SPRK or SPRK L410P, in the presence or absence of Cdc42<sup>Val-12</sup>, were lysed, and the activity of endogenous JNK was measured in an immune complex *in vitro* kinase assay using GST-c-Jun as a substrate. Wild type SPRK, upon overexpression, activates JNK (Fig. 4, A and B, lane 2); Cdc42<sup>Val-12</sup> expressed alone moderately activates JNK (lane 4). Coexpression of Cdc42<sup>Val-12</sup> with SPRK further increases JNK activity (lane 5). However, SPRK L410P coexpressed with activated Cdc42 exhibits no increase in JNK activation over that of Cdc42<sup>Val-12</sup> alone (lanes 4 and 6). Thus, SPRK L410P, when coexpressed with Cdc42<sup>Val-12</sup>, fails to activate endogenous JNK even though under these conditions it exhibits wild type phosphotransfer activity *in vitro*. These findings suggest a role for

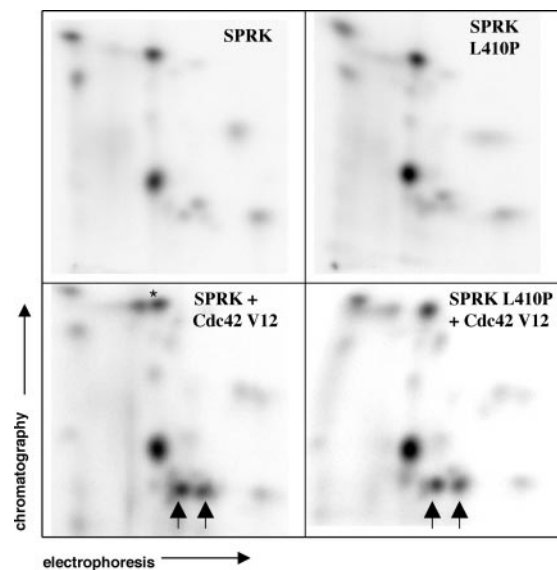


**FIG. 4. Effect of SPRK L410P on JNK activation.** Endogenous JNK was immunoprecipitated from cellular lysates expressing the indicated SPRK variant and/or Cdc42<sup>Val-12</sup> and subjected to an *in vitro* kinase assay using GST-c-Jun as a substrate. *A*, an autoradiogram with bands corresponding to phosphorylated GST-c-Jun indicated by an arrow is shown in the top panel. An immunoblot for JNK from the same immunoprecipitated samples, and immunoblots for SPRK and Cdc42<sup>Val-12</sup> from cellular lysates are shown below the autoradiogram. *B*, the means  $\pm$  S.E. for fold increase in GST-c-Jun phosphorylation from three independent experiments is shown. Only experiments with equal expression levels of the SPRK variants were included. GST-c-Jun phosphorylation was measured by phosphorimaging. WT, wild type.

leucine zipper-mediated SPRK oligomerization in downstream signaling events.

**Activated Cdc42 Changes the *in Vivo* Phosphorylation State of SPRK L410P**—We recently reported that coexpression with Cdc42<sup>Val-12</sup> induces a differential phosphorylation pattern of SPRK *in vivo* (12). To examine the effect of activated Cdc42 on the phosphorylation state of SPRK L410P *in vivo*, comparative two-dimensional tryptic phosphopeptide analyses of SPRK and SPRK L410P labeled *in vivo*, in the absence or in the presence of Cdc42<sup>Val-12</sup>, were performed. As shown in Fig. 5, in the absence of Cdc42<sup>Val-12</sup>, SPRK and SPRK L410P have similar phosphopeptide patterns. Cdc42<sup>Val-12</sup> induces a differential *in vivo* phosphorylation state of both SPRK and SPRK L410P, and the phosphopeptide patterns are alike. The two new phosphopeptides observed after coexpression of Cdc42<sup>Val-12</sup> with SPRK, and with SPRK L410P, are indicated by arrows in the lower panels of Fig. 5. These data strongly suggest that both SPRK and SPRK L410P undergo the same changes in phosphorylation upon coexpression with Cdc42<sup>Val-12</sup>. Furthermore, these changes in *in vivo* phosphorylation correlate with the increased *in vitro* phosphorylation activity of both SPRK and SPRK L410P (Fig. 3).

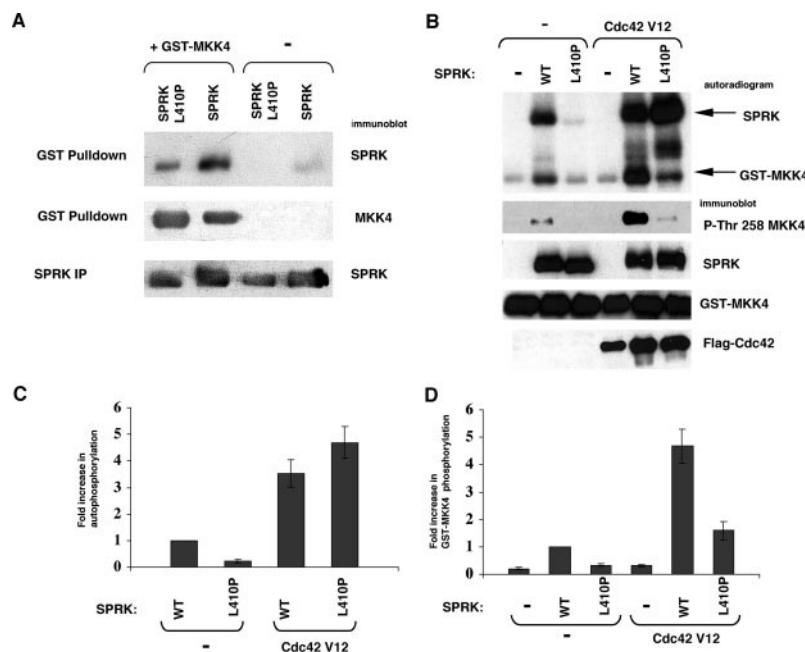
**The SPRK Leucine Zipper Mutant Has Reduced Ability to Phosphorylate MKK4**—Coexpression with Cdc42<sup>Val-12</sup> yields a fully active SPRK L410P as judged by *in vitro* catalytic activity (Fig. 3), yet SPRK L410P, even when coexpressed with



**FIG. 5. Two-dimensional maps of tryptic phosphopeptides derived from *in vivo* phosphorylated SPRK or SPRK L410P.** *A*, cells expressing the specified SPRK variants in the presence and absence of Cdc42<sup>Val-12</sup> were incubated with [<sup>32</sup>P]orthophosphate. SPRK variants were immunoprecipitated from cellular lysates, blotted onto a polyvinylidene difluoride membrane, and subjected to partial trypsin digestion. The resultant tryptic phosphopeptides were analyzed by comparative two-dimensional phosphopeptide analysis as described in detail previously (12). Short arrows indicate two new major tryptic phosphopeptides from SPRK, or SPRK L410P expressed in the presence of Cdc42<sup>Val-12</sup>. Phosphopeptides were detected by phosphorimaging. Long arrows indicate the direction of electrophoresis and chromatography. Because their chromatographic mobilities are essentially identical, the two spots indicated by an asterisk may result from differential trypsin digestion and may include the same phosphorylation site(s). Analogously, as discussed in Böck *et al.* (12), the two new major tryptic phosphopeptides (indicated by short arrows) that result from coexpression of activated Cdc42 of SPRK and SPRK L410P may also result from differential trypsin digestion and may include the same phosphorylation site(s) (12).

Cdc42<sup>Val-12</sup>, fails to activate JNK (Fig. 4). To explore these disparate findings we examined whether SPRK L410P might be defective in phosphorylating an established physiological substrate, MKK4, which can directly phosphorylate and activate JNK.

MKK4 was expressed in *E. coli* as a GST fusion and purified using a glutathione-Sepharose column. Primary data from an *in vitro* kinase assay using MKK4 as the substrate are shown in Fig. 6*B*, and quantitation of SPRK autophosphorylation and MKK4 phosphorylation is shown in Fig. 6*C* and *D*, respectively). MKK4 displays low basal autophosphorylation activity (Fig. 6*B*, top panel, lane 1). SPRK alone phosphorylates MKK4 (lane 2) but SPRK L410P alone does not (lane 3). Coexpression of Cdc42<sup>Val-12</sup> increases the phosphorylation of SPRK of MKK4 *in vitro* by ~4.5-fold. However, when coexpressed with Cdc42<sup>Val-12</sup>, the ability of SPRK L410P to phosphorylate MKK4 is less than that of wild type SPRK (lanes 5 and 6). Activation of murine MKK4 requires the phosphorylation of Ser<sup>254</sup> and Thr<sup>258</sup>. An immunoblot of the same gel (Fig. 6*B*, top panel) was probed with an antibody that recognizes phospho-Thr<sup>258</sup> of MKK4 (Fig. 6*B*, second panel). When coexpressed with activated Cdc42, the *in vitro* phosphorylation of Thr<sup>258</sup> of MKK4 by wild type SPRK is at least 6-fold greater than that of SPRK L410P (Fig. 6*B*, second panel, lanes 5 and 6). Thus after coexpression with the activated GTPase, the zipper mutant of SPRK, compared with wild type SPRK, has reduced MKK4 phosphorylation activity in an *in vitro* kinase assay, as judged by net phosphorylation of MKK4 as well as by phosphorylation of Thr<sup>258</sup> of MKK4.



**FIG. 6. Binding and phosphorylation of MKK4 by SPRK L410P *in vitro*.** Cells were transfected with expression vectors containing the cDNAs indicated above each lane. Co-immunoprecipitation experiments and *in vitro* kinase assays were performed as described under "Experimental Procedures." *A*, pull-down experiments of SPRK and SPRK L410P with GST-MKK4. Glutathione-Sepharose resin was incubated with cellular lysates containing SPRK or SPRK L410P, with or without GST-MKK4. The presence of associated SPRK or SPRK L410P was determined by immunoblotting with a SPRK antibody (*upper panel*). Immunoblots of recombinant GST-MKK4 and SPRK from cellular lysates are shown in the *middle* and *bottom panels*, respectively. *B*, SPRK *in vitro* kinase assay using 10  $\mu$ g of GST-MKK4 as a substrate. An autoradiogram with bands corresponding to SPRK autophosphorylation (*upper arrow*) and GST-MKK4 phosphorylation (*lower arrow*) is shown in the *top panel*. SPRK autophosphorylation and MKK4 phosphorylation was quantified by phosphorimaging and normalized to SPRK expression levels. The means  $\pm$  S.E. of three independent experiments are shown (*C* and *D*). The *second panel* from the *top* shows an immunoblot of the samples from the kinase assay using an antibody that recognizes phosphorylated Thr<sup>258</sup> of murine MKK4. Immunoblots of SPRK from cellular lysates, of recombinant GST-MKK4, and of Cdc42 from cellular lysates are shown in the *third*, *fourth*, and *bottom panels*, respectively. *IP*, immunoprecipitation; *WT*, wild type.

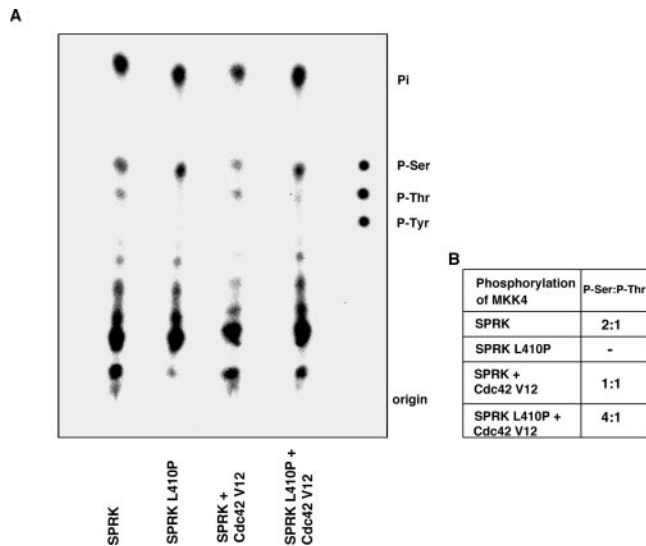
The ability of SPRK L410P to associate with MKK4 after coexpression was assessed in GST pull-down experiments with harvested cellular lysates from transiently transfected 293 cells (Fig. 6A). SPRK L410P associates with MKK4 but to a lesser extent than does wild type SPRK. This may suggest that SPRK oligomerization contributes to MKK4 binding.

MKK4 activation requires phosphorylation of Ser<sup>254</sup> and Thr<sup>258</sup> by its upstream activators MAPK/extracellular signal-regulated kinase kinase and SPRK (3, 7, 8). SPRK cannot phosphorylate a variant of MKK4 in which these two activating sites have been replaced by nonphosphorylatable residues (7, 8). Therefore, to compare the relative abilities of Cdc42<sup>Val-12</sup>-activated SPRK L410P to phosphorylate Ser<sup>254</sup> and Thr<sup>258</sup> of MKK4, phosphoamino acid analysis was performed. Following an *in vitro* kinase assay as shown in Fig. 6B, the radiolabeled MKK4 was subjected to acid hydrolysis and its phosphoamino acid content was analyzed by thin layer electrophoresis (Fig. 7A). The ratio of phospho-Ser to phospho-Thr in each sample as determined by phosphorimaging analysis is shown in Fig. 7B. Wild type SPRK coexpressed with Cdc42<sup>Val-12</sup> phosphorylates serine and threonine of MKK4 at a 1:1 ratio. This indicates that Cdc42<sup>Val-12</sup>-activated wild type SPRK phosphorylates serine and threonine of MKK4 to the same extent as would be predicted for full activation of MKK4. However SPRK L410P coexpressed with Cdc42<sup>Val-12</sup> phosphorylates serine and threonine of MKK4 at a 4:1 ratio. These data, together with the results from the phospho-Thr<sup>258</sup> blot, indicate that the monomeric SPRK zipper mutant is defective in phosphorylating Thr<sup>258</sup> of MKK4, suggesting that MKK4 would remain inactive and unable to activate JNK.

## DISCUSSION

Reversible protein phosphorylation is important in regulating virtually every physiological process. Thus it follows that the activities of the protein kinases and phosphatases that catalyze these events should also be tightly regulated. In response to a particular cellular signal, first a protein kinase is converted into an active form, and then the activated kinase can proceed to phosphorylate its physiological substrate. Although the catalytic domains of protein kinases share sequence and structural homology, considerable diversity exists outside of the kinase domains. These noncatalytic regions often mediate interactions with proteins, lipids, or small molecules, which modify the activity of the protein kinase itself or contribute to the binding and phosphorylation of its physiological substrates. We report herein that activation of SPRK by Cdc42 is independent of zipper-mediated oligomerization, whereas proper phosphorylation of a downstream substrate by Cdc42-activated SPRK depends on zipper-mediated oligomerization.

Numerous intracellular serine/threonine protein kinases have been identified that contain leucine zipper-like motifs that may serve as oligomerization domains, including type I and II cGMP-dependent protein kinases (24), Protein kinase C-related kinase N (25), Tausel kinase (26), and the human centrosomal kinase, Nek-2 (27). The predicted leucine zippers of the mixed lineage kinases, MLK-1 (28), MST/MLK-2 (29, 30), and SPRK/MLK-3, are relatively dissimilar to those of the more distantly related DLK/MUK/ZPK (31–33) and LZK (34). In fact, the zippers of SPRK/MLK-3 and DLK fail to interact (35), suggesting that zipper-mediated heterodimerization *in vivo* is unlikely. SPRK can homo-oligomerize through its zipper and



**FIG. 7. Phosphoamino acid analysis of MKK4 phosphorylated by SPRK variants.** Following a SPRK *in vitro* kinase assay using GST-MKK4 as a substrate as described in Fig. 6, proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. *A*, radiolabeled bands corresponding to GST-MKK4 were excised from the membrane and hydrolyzed in 6 N HCl for 1 h at 100 °C. The phosphoamino acids were analyzed using one-dimensional thin layer electrophoresis on TLC plates and visualized by phosphorimaging. The autoradiogram shown is representative of three independent experiments. The positions of the phosphoamino acid standards, as well as the position of free inorganic phosphate ( $P_i$ ), are indicated. *B*, the ratio of phosphoserine to phosphothreonine of MKK4 was calculated using Image Quant software (Molecular Dynamics).

large deletions of 32–60 amino acids of the zipper domain result in a kinase with reduced autophosphorylation and JNK activity (12, 20). Based on deletion studies, it has been suggested that oligomerization is required for SPRK/MLK-3 activation (20). To more precisely decipher the role of the zipper domain of SPRK while keeping SPRK intact, we engineered a monomeric form of SPRK that contains a single point mutation in its leucine zipper domain.

Leucine zippers are  $\alpha$ -helical coiled coil structures characterized by the presence of leucine or another nonaromatic hydrophobic amino acid at every seventh position. Although proline residues are almost always absent from short helices or coiled coils, a single proline can be tolerated in some long  $\alpha$ -helices, albeit with disruption of local helical geometry (36–38). We replaced the leucine residue at position 410 with a proline to deliberately disrupt (at least the local)  $\alpha$ -helical structure of the zipper with the aim of destabilizing SPRK oligomerization.

The MBP fusion system (39) has been successfully used to biophysically characterize multimerization of leucine zipper domains (40, 41). Thus fusion proteins between the wild type SPRK zipper, or the SPRK L410P mutant zipper, and the monomeric MBP of *E. coli* were constructed, and their native molecular masses were estimated using size exclusion chromatography. The leucine zipper of SPRK is capable of forming oligomers of MBP (Fig. 2A), whereas appending of the mutant zipper yields a monomeric MBP fusion protein. These data indicate that substitution of a single conserved leucine in the zipper domain with proline adequately disrupts zipper-mediated oligomerization. Furthermore, using the full-length kinase in coimmunoprecipitation experiments, SPRK L410P was unable to form a stable complex with wild type SPRK (Fig. 2B). Taken together, these results argue that SPRK L410P behaves as a monomeric protein. Thus we have constructed an oligomeric loss-of-function SPRK point mutant by substituting a helix disrupting proline residue within the leucine zipper

domain.

In addition to its leucine zipper motif, SPRK contains a Cdc42/Rac-interactive binding motif that is required for binding and activation by the small GTPase Cdc42 (12). We asked whether SPRK oligomerization is required for its activation by Cdc42. Coexpression of the monomeric SPRK L410P with activated Cdc42 resulted in a fully active SPRK as judged by *in vitro* autophosphorylation and histone phosphorylation (Fig. 3A). Upon coexpression with Cdc42, wild type SPRK undergoes additional phosphorylation event(s) *in vivo* that correlate with increased SPRK activity (12). Comparative phosphopeptide mapping experiments presented here indicate that SPRK L410P undergoes those same changes in *in vivo* phosphorylation upon coexpression with activated Cdc42 (Fig. 5). Taken together these findings demonstrate that zipper-mediated SPRK oligomerization is not required for activation by Cdc42. Furthermore, the leucine to proline substitution in SPRK does not compromise the catalytic integrity of the mutant monomeric SPRK, suggesting that the structure of the catalytic domain of the SPRK L410P is not perturbed.

In accord with the ability of Cdc42<sup>Val-12</sup> to activate SPRK L410P, SPRK L410P does indeed coimmunoprecipitate with activated Cdc42 but in lesser amounts than does wild type SPRK (Fig. 3B). This may suggest a decreased affinity of Cdc42<sup>Val-12</sup> for SPRK L410P. Alternatively, a single Cdc42 molecule may bind an oligomer of wild type SPRK but only a monomer of SPRK L410P. Without knowledge of the stoichiometry of SPRK oligomerization or the stoichiometry of the SPRK-Cdc42<sup>Val-12</sup> interaction, we cannot distinguish between these possibilities. Regardless, it is apparent that the *in vivo* affinity of Cdc42<sup>Val-12</sup> for SPRK L410P is sufficient for full activation of the kinase.

As a MAPK kinase, SPRK activates the JNK pathway through phosphorylation of MKK4 or MKK7. Interestingly, both in the absence or presence of activated Cdc42 (Fig. 6), SPRK L410P is unable to activate endogenous JNK in 293 cells. These findings indicate that zipper-mediated SPRK oligomerization is critical for downstream signaling events that culminate in JNK activation. Even though the monomeric SPRK zipper mutant, when coexpressed with activated Cdc42, has high autophosphorylation and histone phosphorylation activities (Fig. 3A), we determined that its *in vitro* activity toward a physiological substrate MKK4 has been compromised (Fig. 6B). Interestingly, it has been reported that a monomeric cGMP-dependent protein kinase mutant is capable of autophosphorylation and histone phosphorylation but displays a reduced ability to phosphorylate a physiological substrate vimentin (42).

Protein kinases of the MKK family are activated by dual phosphorylation of two conserved serine/threonine residues in their activation loops. Activation of murine MKK4 requires phosphorylation of both Ser<sup>254</sup> and Thr<sup>258</sup>, and mutation of these to nonphosphorylatable amino acids blocks SPRK activation of JNK (3, 7, 8). Determination of the ratio of serine to threonine phosphorylation, in combination with the use of an antibody that recognizes phosphorylated Thr<sup>258</sup> of MKK4, leads us to conclude that the Cdc42-activated zipper mutant of SPRK has some serine phosphorylation activity but is defective in phosphorylating Thr<sup>258</sup> of MKK4. These results imply that SPRK L410P cannot activate JNK because the zipper mutant fails to properly phosphorylate and activate its physiological substrate, MKK4.

Using a monomeric point mutant of SPRK, we have been able to dissociate two discrete steps in the regulation of SPRK. Our results clearly demonstrate that zipper-mediated SPRK oligomerization is not required for Cdc42 to induce a catalytic-

cally active SPRK. However, zipper-mediated SPRK oligomerization is critical for phosphorylating downstream signaling targets and is specifically crucial for full activation of MKK4. The mechanistic rationale of why the zipper mutant has a reasonable ability to phosphorylate Ser<sup>254</sup> of MKK4 but not Thr<sup>258</sup> is not clear from these studies. It is possible that monomeric SPRK has lower affinity for serine phosphorylated MKK4 or that a SPRK oligomer containing more than one catalytic domain may be needed to bind and phosphorylate both sites of MKK4. Interestingly, wild type SPRK that has not been activated by Cdc42 prefers serine over threonine phosphorylation of MKK4 (Fig. 7, lane 1). In Ferrell's distributive model of the activation of extracellular signal-regulated kinase by dual phosphorylation, the rate-limiting phosphorylation of Thr<sup>183</sup> (43–47) is proposed to impart specificity, albeit at the expense of sensitivity. Similarly, phosphorylation of Ser<sup>254</sup> of MKK4 by wild type SPRK may be kinetically favored, and phosphorylation of Thr<sup>258</sup>, which requires a fully active SPRK oligomer, may provide specificity in the activation of MKK4 under physiological conditions.

**Acknowledgments**—We are grateful to Barbara Böck for construction of the mammalian 3HA-SPRK expression vector; Pierfrancesco Vianello for expression and purification of recombinant GST-MKK4; Avi Ashkenazi (Genentech, Inc.) for the mammalian Cdc42 expression vector; and Ajay Rana (Massachusetts General Hospital) for the mammalian MKK4 expression vector and the GST-c-Jun expression plasmid. We thank Donald B. Jump for valuable discussions and critical reading of the manuscript.

#### REFERENCES

- Fanger, G. R., Gerwins, P., Widmann, C., Jarpe, M. B., and Johnson, G. L. (1997) *Curr. Opin. Genet. Dev.* **7**, 67–74
- Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) *Physiol. Rev.* **79**, 143–180
- Deacon, K., and Blank, J. L. (1997) *J. Biol. Chem.* **272**, 14489–14496
- Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372**, 798–800
- Zheng, C. F., and Guan, K. L. (1994) *EMBO J.* **13**, 1123–1131
- Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **372**, 794–798
- Rana, A., Gallo, K., Godowski, P., Hirai, S., Ohno, S., Zon, L., Kyriakis, J. M., and Avruch, J. (1996) *J. Biol. Chem.* **271**, 19025–19028
- Tibbles, L. A., Ing, Y. L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J. R., and Lassam, N. J. (1996) *EMBO J.* **15**, 7026–7035
- Gallo, K. A., Mark, M. R., Scadden, D. T., Wang, Z., Gu, Q., and Godowski, P. J. (1994) *J. Biol. Chem.* **269**, 15092–15100
- Burbelo, P. D., Drechsel, D., and Hall, A. (1995) *J. Biol. Chem.* **270**, 29071–29074
- Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T., and Gutkind, J. S. (1996) *J. Biol. Chem.* **271**, 27225–27228
- Böck, B. C., Vacratsis, P. O., Qamirani, E., and Gallo, K. A. (2000) *J. Biol. Chem.* **275**, 14231–14241
- O'Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) *Science* **254**, 539–544
- Hu, J. C., O'Shea, E. K., Kim, P. S., and Sauer, R. T. (1990) *Science* **250**, 1400–1403
- Hodges, R. S., Zhou, N. E., Kay, C. M., and Semchuk, P. D. (1990) *Pept. Res.* **3**, 123–137
- Hodges, R. S., Semchuk, P. D., Taneja, A. K., Kay, C. M., Parker, J. M., and Mant, C. T. (1988) *Pept. Res.* **1**, 19–30
- Hodges, R. S., Saund, A. K., Chong, P. C., St-Pierre, S. A., and Reid, R. E. (1981) *J. Biol. Chem.* **256**, 1214–1224
- O'Shea, E. K., Rutkowski, R., and Kim, P. S. (1992) *Cell* **68**, 699–708
- Hu, J. C., Newell, N. E., Tidor, B., and Sauer, R. T. (1993) *Protein. Sci.* **2**, 1072–1084
- Leung, I. W., and Lassam, N. (1998) *J. Biol. Chem.* **273**, 32408–32415
- Tyers, M., Tokiwa, G., Nash, R., and Fletcher, B. (1992) *EMBO J.* **11**, 1773–1784
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Lupas, A. (1996) *Methods. Enzymol.* **266**, 513–525
- Gamm, D. M., Francis, S. H., Angelotti, T. P., Corbin, J. D., and Uhler, M. D. (1995) *J. Biol. Chem.* **270**, 27380–27288
- Mukai, H., and Ono, Y. (1994) *Biochem. Biophys. Res. Commun.* **199**, 897–904
- Roe, J. L., Durfee, T., Zupan, J. R., Repetti, P. P., McLean, B. G., and Zambryski, P. C. (1997) *J. Biol. Chem.* **272**, 5838–5845
- Fry, A. M., Arnaud, L., and Nigg, E. A. (1999) *J. Biol. Chem.* **274**, 16304–16310
- Dorow, D. S., Devereux, L., Dietzsch, E., and De Kretser, T. (1993) *Eur. J. Biochem.* **213**, 701–710
- Katoh, M., Hirai, M., Sugimura, T., and Terada, M. (1995) *Oncogene* **10**, 1447–1451
- Dorow, D. S., Devereux, L., Tu, G. F., Price, G., Nicholl, J. K., Sutherland, G. R., and Simpson, R. J. (1995) *Eur. J. Biochem.* **234**, 492–500
- Hirai, S., Izawa, M., Osada, S., Spyrou, G., and Ohno, S. (1996) *Oncogene* **12**, 641–650
- Holzman, L. B., Merritt, S. E., and Fan, G. (1994) *J. Biol. Chem.* **269**, 30808–30817
- Reddy, U. R., and Pleasure, D. (1994) *Biochem. Biophys. Res. Commun.* **202**, 613–620
- Sakuma, H., Ikeda, A., Oka, S., Kozutsumi, Y., Zanetta, J. P., and Kawasaki, T. (1997) *J. Biol. Chem.* **272**, 28622–28629
- Nihalani, D., Merritt, S., and Holzman, L. B. (2000) *J. Biol. Chem.* **275**, 7273–7279
- Sankararamkrishnan, R., and Vishveshwara, S. (1990) *Biopolymers* **30**, 287–298
- Chang, D. K., Cheng, S. F., Trivedi, V. D., and Lin, K. L. (1999) *J. Struct. Biol.* **128**, 270–279
- Chakrabarti, P., and Chakrabarti, S. (1998) *J. Mol. Biol.* **284**, 867–873
- Blondel, A., and Bedouelle, H. (1991) *Protein. Eng.* **4**, 457–461
- Shugars, D. C., Wild, C. T., Greenwell, T. K., and Matthews, T. J. (1996) *J. Virol.* **70**, 2982–2991
- Chen, S. S., Lee, C. N., Lee, W. R., McIntosh, K., and Lee, T. H. (1993) *J. Virol.* **67**, 3615–3619
- MacMillan-Crow, L. A., and Lincoln, T. M. (1994) *Biochemistry* **33**, 8035–8043
- Robbins, D. J., and Cobb, M. H. (1992) *Mol. Biol. Cell* **3**, 299–308
- Haystead, T. A., Dent, P., Wu, J., Haystead, C. M., and Sturgill, T. W. (1992) *FEBS Lett.* **306**, 17–22
- Scott, A., Haystead, C. M., and Haystead, T. A. (1995) *J. Biol. Chem.* **270**, 24540–24547
- Butch, E. R., and Guan, K. L. (1996) *J. Biol. Chem.* **271**, 4230–4235
- Ferrell, J. E., Jr., and Bhatt, R. R. (1997) *J. Biol. Chem.* **272**, 19008–19016