Cdc42-inducedActivation of the Mixed-LineageKinase

SPRK in Vivo

REQUIREMENT OF THE Cdc42/Rac INTERACTIVE BINDING MOTIF AND CHANGES IN PHOSPHORYLATION*

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Src homology 3 domain (SH3)-containing proline-rich protein kinase (SPRK)/mixed-lineage kinase (MLK)-3 is a serine/threonine kinase that upon overexpression in mammalian cells activates the c-Jun NH2-terminal kinase pathway. The mechanisms by which SPRK activity is regulated are not well understood. The small Rho family GTPases, Rac and Cdc42, have been shown to bind and modulate the activities of signaling proteins, including SPRK, which contain Cdc42/Rac interactive binding motifs. Coexpression of SPRK and activated Cdc42 increases SPRKs activity. SPRKs Cdc42/Rac interactive binding-like motif contains six of the eight consensus residues. Using a site-directed mutagenesis approach, we show that SPRK contains a functional Cdc42/Rac interactive binding motif that is required for SPRKs association with and activation by Cdc42. However, experiments using a SPRK variant that lacks the COOH-terminal zipper region/basic stretch suggest that this region may also contribute to Cdc42 binding. Unlike the PAK family of protein kinases, we find that the activation of SPRK by Cdc42 cannot be recapitulated in an in vitro system using purified, recombinant proteins. Comparative phosphopeptide mapping demonstrates that coexpression of activated Cdc42 with SPRK alters the in vivo serine/threonine phosphorylation pattern of SPRK suggesting that the mechanism by which Cdc42 increases SPRKs catalytic activity involves a change in the in vivo phosphorylation of SPRK. This is, to the best of our knowledge, the first demonstrated example of a Cdc42-mediated change in the in vivo phosphorylation of a protein kinase. These studies suggest an additional component or cellular environment is required for SPRK activation by Cdc42.

The vast majority of mammalian protein kinases catalyze the transfer of the γ-phosphate of ATP to serine, threonine, or tyrosine residues of their target proteins. Phosphorylation is rendered reversible in vitro by the action of protein phosphatases. Since phosphorylation is highly regulated in virtually all physiological processes, it follows that the activities of the protein kinases, themselves, should be highly regulated. Binding of activating or inhibitory molecules, including lipids, cyclic nucleotides, or proteins, can modulate the activity of protein kinases. In addition, post-translational modifications, such as phosphorylation and proteolysis, can regulate protein kinase activity. These regulatory events may alter the specific activity of a protein kinase or may change its stability. Finally, access to physiological substrates may be limited by restricted subcellular localization or translocation of a protein kinase.

Small GTPases regulate certain protein kinases. For instance, by binding and recruiting the serine/threonine kinase Raf to the plasma membrane, GTP-bound, farnesylated Ras contributes to the activation of Raf, and consequently activates the extracellular-regulated protein kinase (ERK)1-mitogen-activated protein kinase (MAPK) pathway (1). Rho family GTPases, which include Rho, Rac, and Cdc42, play crucial roles in diverse cellular processes (2, 3), including cytoskeletal rearrangements (4–6), cell cycle progression (7), cellular transformation (8–14), and nuclear signaling (15–20). They can also function as protein kinase activators. One well-characterized target of Cdc42 and Rac is the p21-activated kinase (PAK) (21–23). The interaction between Cdc42 and the serine/threonine kinase PAK requires the CRIB (Cdc42/Rac interactive binding) motif (26), a 14–16-amino acid sequence containing eight consensus amino acids. The structural determinants required for GTPase binding and the mechanism of activation of multiple PAK isoforms have been extensively investigated (21, 24, 25, 32–34). CRIB-dependent interaction of PAK with GTP-bound Rac/Cdc42 induces PAK autoprophosphorylation and activation both in vitro and in vivo.

Diverse proteins, including the tyrosine kinase, activated Cdc42HS-associated kinase (ACK) (27), and the non-kinase, Wiskott-Aldrich Syndrome protein (WASP) (28, 29), also contain CRIB motifs, suggesting that mechanistically diverse regulatory pathways may share this common structural motif. Likewise, not all protein kinases which interact with Cdc42 and Rac do so through a CRIB motif. For instance, both the 70-kDa ribosomal S6 kinase (30) and MAPK kinase-1 (31), which lack CRIB motifs, have been shown to interact with GTP-bound Cdc42 and Rac. Furthermore, MAPK kinase-4 contains a modified CRIB motif whose deletion only partially diminishes binding to Cdc42 and Rac, indicative of a CRIB-independent GTPase binding determinant (31). Thus the

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† The abbreviations used are: ERK, extracellular regulated protein kinase; MAPK, mitogen-activated protein kinase; PAK, p21-activated kinase; CRIB, Cdc42/Rac-interactive binding; ACK, activated Cdc42HS-associated kinase; WASP, Wiskott-Aldrich Syndrome protein; SPRK, SH3 domain-containing proline-rich kinase; SH3, Src-homology 3; MLK, mixed-lineage kinase; JNK, c-Jun NH2-terminal kinase; MKK, MAPK kinase; JIP; JNK interacting protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; MBP, maltose-binding protein; GTPyS, guanosine 5’-3-O-(thio)triphosphate.
role of CRIB motifs and the mechanisms by which many protein kinases are activated by small GTPases remains largely unexplored.

Src homology 3 domain (SH3)-containing proline-rich protein kinase (SPRK) (35), also called mixed-lineage kinase-3 (MLK-3) (36), or protein-tyrosine kinase-1 (37), is a member of the so-called “mixed-lineage” kinases. SPRK contains a CRIB motif bearing six of the eight consensus amino acids, as well as other domains that may mediate protein-protein interactions including an NH2-terminal SH3 domain, a leucine/isoleucine zipper motif, and a large COOH-terminal region that is rich in serine, threonine, and proline residues (Fig. 1).

Upon overexpression in mammalian cell lines SPRK activates c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase through phosphorylation and activation of the dual specificity kinase, MAPK kinase-4 (MKK-4/SEK1) (38) or MKK-7 (39), and binds the JNK scaffold proteins, JNK interacting protein (JIP)-1 and JIP-2 (39, 43, 44). In some cell types, SPRK (39), and binds the JNK scaffold proteins, JNK interacting protein (JIP)-1 and JIP-2 (39, 43, 44). In some cell types, SPRK (39), and binds the JNK scaffold proteins, JNK interacting protein (JIP)-1 and JIP-2 (39, 43, 44). In some cell types, SPRK (39), and binds the JNK scaffold proteins, JNK interacting protein (JIP)-1 and JIP-2 (39, 43, 44).

Comparative phosphopeptide mapping revealed that an additional cellular component is required for kinase activity. These studies represent the first case where a Cdc42-mediated change in the activity of purified, catalytically active SPRK, suggest-
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 mM LiCl, three times with HNTG 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, and 1 mM PMSF) for 30 min at room temperature. Independent experiments showed that the reaction was carried out for 30 min at room assay 10 (3000 Ci/mmol) (NEN Life Science Products). For the SPRK kinase assay, pGEX-c-Jun was expressed in XL-1 Blue (Massachusetts General Hospital, Harvard Medical School, Boston, MA). GST-c-Jun was expressed in BL21. JNK C-17 antibody (0.5 μg/ml) and JNK 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 compiled 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 [γ-³²P]ATP (3000 Ci/nmol) (NEN Life Science Products). For the SPRK kinase assay 10 μg of mixed histones (Roche Molecular Biochemicals) was used as the substrate and the reaction was carried out for 30 min at room temperature. Independent experiments showed that the reaction was linear within this time frame. The reaction was terminated by the addition of the reaction volume of 2 × SfSE buffer and the mixture was transferred to a polyethylene difluoride membrane. Radial immunoblot blots comigrating with SPRK, as judged by Western blotting, were excised from the polyvinylidene difluoride membrane. After washing three times with 1× SSC and two times with water, the radioactive piece of membrane was blocked with 1ml of 0.5% polyvinylpyrrolidone-360 (Sigma) containing 100 μM acetic acid for 30 min at 37 °C, and then washed five times with water. Tryptic digestion was performed with 10 μg of sequencing grade trypsin (Roche Molecular Biochemicals) for 2 h in 200 μl of 50 mM NH₄HCO₃ (pH 8.3) at 37 °C. An additional 10 μg of trypsin was added and the digestion mixture was incubated for an additional 2 h at 37 °C. The membrane was then sonicated for 3 min in 300 μl of water to remove additional tryptic peptides. The solution containing the released tryptic peptides was concentrated in a SpeedVac (Savant Instruments).

The peptides were separated on cellulose thin layer chromatography (TLC) plates (Kodak, 20 × 20 cm) by thin layer electrophoresis (TLE) in the first dimension in pH 1.9 buffer (formic acid (88% w/v)/glacial acetic acid/water, 25:78:897, v/v) at 0 °C and 1000 V for 30 min, and in the second dimension by TLC in phosphochromatography buffer (n-butanol/pyridine/glacial acetic acid/water, 15:10:3:12, v/v). Radio-labeled phosphopeptides were visualized and quantitated using a PhosphorImager.

Phosphoamino Acid Analysis—The tryptic peptides were hydrolyzed in 200 μl of 6 M HCl for 1 h at 100 °C. The phosphoamino acids were concentrated in a SpeedVac. Unlabeled phosphoamino acid standards (Sigma) and xylene cyanol FF maker dye (Sigma) were added to each sample. Phosphoamino acids were separated by one-dimensional TLE in pH 2.5 buffer (66.7% pH 3.5 buffer (glacial acetic acid/water, 50:5:945, v/v), 50:5:945, v/v) and 33.3% pH 1.9 buffer on cellulose TLC plates at 0 °C and 500 V for 1.5 h. Unlabeled phosphoamino acid standards were visualized by ninhydrin staining and the ³²P-labeled phosphoamino acids derived from the SPRK peptides were visualized by PhosphorImaging.

**Results**

**Association of Activated Cdc42 with SPRK Does Not Require SPRK Kinase Activity**—Recently Hall and co-workers have shown that several proteins, including SPRK, associate with GTP-bound Cdc42 and Rac in filter binding assays (26). All of these proteins contain a 14–16-amino acid sequence that includes eight consensus residues, which has been coined the CRIB motif. The CRIB motif of SPRK contains six of the eight consensus amino acids (Fig. 2). In this study we examined the structural requirements and mechanism of Cdc42 binding and activation of SPRK.

SPRK and Flag epitope-tagged Cdc42 expression vectors were transiently transfected in 293 cells. To mimic the GTP-bound state of Cdc42 we used a constitutively active mutant of the GTPase, i.e. Cdc42Val-12. Cellular lysates from 293 cells were immunoprecipitated with the Flag antibody and the presence of MBP Denaturing Assay—One day following transfection of 293 cells with cDNAs encoding SPRK and SPRKzip, the cells were lysed in 1 ml of lysis buffer as described above. Amylose resin (30 μl) was incubated with 10 μg of purified MBP or MBP-zips for 60 min at 4 °C, and then washed twice with column buffer (20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM MgCl₂, 1 mM EGTA). Cell lysates were added to the amylose resin and incubated at 4 °C with mixing for 90 min. After removal of the lysate, the resin was washed twice with HNTG buffer. The binding of SPRK or SPRKzip to MBP or MBP-zips was analyzed by SDS-PAGE followed by Western blotting using the SPRK antisera. Coomassie Blue staining verified equal amounts of MBP containing proteins in the assays.
with active and inactive SPRK, and Cdc42 Val-12, demonstrate kinase assay (35).

SPRK. Amino acid numbers are indicated to the et al. (35), the constitutively active form of Cdc42, but
iments (Fig. 3 first panel). We have not observed association of SPRK with the dominant negative variant Cdc42 Asn-17 but, in our hands, expression of this Cdc42 variant has been low. Expression levels of SPRK and Cdc42 in cellular lysates were determined by Western blot analysis (Fig. 3A, lower panels). These data indicate that SPRK preferentially associates with the GTP-bound form of Cdc42.

SPRK can autophosphorylate in vitro (35). Although SPRKs postulated site of interaction with Cdc42 is not within the catalytic domain, it is plausible that either SPRK autophosphorylation or SPRK phosphorylation of another interacting molecule might modulate the Cdc42-SPRK interaction. To examine whether SPRKs catalytic activity effects its ability to bind to Cdc42, the kinase-defective SPRK variant (SPRK K144A) was tested for its ability to associate with Cdc42 Val-12. The SPRK K144A variant shows no autophosphorylation in an in vitro kinase assay (35).

Co-transfection and co-immunoprecipitation experiments with active and inactive SPRK, and Cdc42 Val-12, demonstrate that constitutively active Cdc42 associates equally well with wild type SPRK and the kinase-defective variant SPRK K144A (Fig. 3B, upper panel). This indicates that SPRKs catalytic activity is not required for its association with Cdc42. The retarded mobility of wild type SPRK, as compared with that of inactive SPRK, suggests a more highly phosphorylated form of SPRK that is likely due, at least in part, to autophosphorylation (Fig. 3B, upper and middle panels).

To determine whether association with Cdc42 effects SPRKs catalytic activity, an in vitro kinase assay for SPRK was developed. While its kinase domain shares overall sequence similarity with both serine/threonine and tyrosine kinases, SPRK shows high amino acid sequence identity with the serine/thre- onine kinase B-Raf in a small region of subdomain VIII and VIII that is important for substrate specificity and catalytic activity (48). In addition, both Raf and SPRK appear to function as MKKs adding primarily to the activation of ERK and JNK, respectively. Since histones are commonly employed as exoge- nous substrates for Raf in in vitro kinase assays (49, 50), a mixture of histones was tested as an exogenous substrate for SPRK. SPRK, immunoprecipitated from cellular lysates of transiently transfected 293 cells, exhibits basal autophosphorylation as well as phosphorylation of the histones H3 and H4 (Fig. 3B). In contrast, the kinase-defective SPRK shows no autophosphorylation or histone phosphorylation, indicating that the phosphorylation events observed in these assays are attributable to SPRK, rather than to contaminating kinases.

The expression of Cdc42 Val-12 in transiently transfected 293 cells increases the autophosphorylation activity as well as the substrate phosphorylation activity of SPRK about 3–5-fold (Fig. 3C). No background phosphorylation is observed with the kinase-defective SPRK variant in the presence or absence of Cdc42 Val-12. Interestingly, under these in vitro kinase assay conditions, we do not detect Cdc42 associated with SPRK by Western blotting (data not shown).

SPRKs CRIB Motif Is Necessary for Association with Cdc42 and for Cdc42-induced Activation of SPRK—To test whether SPRKs potential CRIB motif actually functions in the binding of Cdc42 we took a site-directed mutagenesis approach. Three different SPRK variants were generated by mutating conserved amino acids in the CRIB motif to alanine residues: SPRK F498A, SPRK H500A, and SPRK I492A/S493A (Fig. 2). While we cannot absolutely rule out the possibility that introduction of these mutations might alter SPRKs conformation, the expression levels of the SPRK CRIB mutants in transient transfections of 293 cells are at least as high as that of wild type SPRK (Fig. 4A), suggesting that these variants are stable. The wild type SPRK coexpressed with Cdc42 Val-12, none of the SPRK CRIB mutants detectably associates with the activated GTPase (Fig. 4A).

If Cdc42-induced activation of SPRK is mediated through its interaction with SPRKs CRIB motif, one would expect that the SPRK CRIB mutants should exhibit a defect in Cdc42-induced activation. Accordingly, cells were transiently transfected with cDNAs encoding wild type SPRK or SPRK I492A/S493A, in the presence or absence of Cdc42 Val-12. The activity of the immunoprecipitated SPRK or SPRK CRIB mutant was measured in an in vitro kinase assay. In the absence of Cdc42 Val-12, both wild type SPRK and the SPRK CRIB variant show similar levels of autophosphorylation and substrate phosphorylation (Fig. 4, B and C). In the absence of Cdc42 Val-12, the differences in the activities of SPRK and the SPRK CRIB variants were not statistically significant. This further supports the idea that the mutations in the CRIB motif do not grossly perturb SPRKs structure or inherent catalytic activity. However, both autophosphorylation and substrate phosphorylation of the CRIB variant is markedly lower (3-fold) than that of wild type SPRK, when each is coexpressed with the activated GTPase (Fig. 4, B and C). The small increase in the catalytic activity of the SPRK CRIB mutant when activated Cdc42 is coexpressed may be due to Cdc42-activated endogenous SPRK. Alternatively there may be residual binding of the CRIB variant to activated Cdc42 in vitro, which we do not detect in our in vitro co-immunoprecipitation assay. Taken together these results demonstrate that SPRK does contain a functional CRIB motif, and that Cdc42-induced activation of SPRK is mediated via association with this CRIB motif.

Effects of Deleting the COOH-terminal Portion of SPRKs Zipper Domain—in WASP (51), PAK (32, 52), and ACK (27) the CRIB motif is necessary but not sufficient for GTPase binding. Outside of the CRIB motif SPRKs shares no sequence similarity with the minimal GTPase-binding domains that have been defined for these proteins. Instead, SPRK contains two closely spaced leucine/isoleucine zipper motifs spanning amino acids 400–462, COOH-terminal to the CRIB motif (Fig. 1). Considering the close vicinity in linear sequence of the zippers and the CRIB motif we asked whether the zipper motif might contrib-
Cdc42-induced Activation and in Vivo Phosphorylation of SPRK

immunoblots of SPRK and Cdc42 Val-12 from cellular lysates, respectively. The kinase assay shown in Fig. 5, middle panel, was determined by immunoblotting with a SPRK antibody (upper panel). B, coimmunoprecipitation experiments of SPRK and SPRK K144A with Cdc42 Val-12. Cdc42 Val-12 was immunoprecipitated and the presence of bound SPRK or SPRK K144A was determined by immunoblotting as described above. The middle and lower panels show immunoblots of SPRK and Cdc42 from cellular lysates, respectively. C, in vitro kinase assay of SPRK and SPRK variants. SPRK was immunoprecipitated and subjected to an in vitro kinase assay using histones as a substrate. To eliminate contaminating (non-SPRK) kinase activity in the immune complex in vitro kinase assay, SPRK immunoprecipitates were stringently washed. The top panel shows an autoradiogram with bands corresponding to SPRK autophosphorylation and histone phosphorylation indicated by arrows. The middle and lower panels show immunoblots of SPRK and Cdc42 Val-12 from cellular lysates, respectively. The kinase assay shown in C is representative of five independent experiments.

FIG. 3. Expression, coimmunoprecipitation, and SPRK in vitro kinase activity from cellular lysates expressing variants of SPRK and Cdc42. Cells were transfected with expression vectors containing the cDNAs indicated above each figure. A minus sign indicates that a control empty vector was transfected. Transient transfections of 293 cells, SDS-PAGE, coimmunoprecipitation experiments, immunoblotting, and in vitro kinase assays were performed as described under “Experimental Procedures.” A, coimmunoprecipitation experiments of SPRK with wild type Cdc42 and the constitutively active variant Cdc42 Val-12. Cdc42 or Cdc42 Val-12 was immunoprecipitated (IP) from cellular lysates using an antibody directed against the Flag epitope which is appended to the NH₂ termini of the expressed Cdc42 and Cdc42 variant. The presence of bound SPRK was determined by immunoblotting with a SPRK antibody (upper panel). The middle and lower panels show immunoblots of SPRK and Cdc42, respectively, from cellular lysates. B, coimmunoprecipitation experiments of SPRK and SPRK K144A with Cdc42 Val-12. Cdc42 Val-12 was immunoprecipitated and the presence of bound SPRK or SPRK K144A was determined by immunoblotting as described above. The middle and lower panels show immunoblots of SPRK and Cdc42 from cellular lysates, respectively. C, in vitro kinase assay of SPRK and SPRK variants. SPRK was immunoprecipitated and subjected to an in vitro kinase assay using histones as a substrate. To eliminate contaminating (non-SPRK) kinase activity in the immune complex in vitro kinase assay, SPRK immunoprecipitates were stringently washed. The top panel shows an autoradiogram with bands corresponding to SPRK autophosphorylation and histone phosphorylation indicated by arrows. The middle and lower panels show immunoblots of SPRK and Cdc42 Val-12 from cellular lysates, respectively. The kinase assay shown in C is representative of five independent experiments.

ute to the binding of SPRK to Cdc42 Val-12. Accordingly, a variant of SPRK, SPRK Δzip, which lacks amino acids 430–486, as shown in Fig. 1, was constructed. This deletion removes the second half of the zipper region as well as 22 COOH-terminal amino acids which includes a short basic region, but leaves the entire CRIB motif intact.

SPRK Δzip is expressed in transiently transfected 293 cells at levels comparable to that of wild type SPRK (Fig. 5A). The ability of Cdc42 Val-12 to associate with SPRK Δzip was tested in coimmunoprecipitation experiments with cellular lysates harvested from transiently transfected 293 cells. The deletion of the second zipper/basic stretch greatly diminishes the ability of SPRK to bind to Cdc42 Val-12, despite the presence of the complete CRIB motif (Fig. 5A). Based on these and our previous results, both the CRIB motif and the second half of the zipper region and a stretch of basic amino acids may contribute to Cdc42 binding. Alternatively, the COOH-terminal zipper/basic stretch may not directly interact with Cdc42, but may be required for the proper presentation and binding of the CRIB motif to the GTPase.

SPRK Δzip has approximately 70% of the basal autophosphorylation activity of wild type SPRK (Fig. 5, B and C). However, in contrast to wild type SPRK, there is no Cdc42-induced increase in autophosphorylation of SPRK Δzip, consistent with the finding that SPRK Δzip binds Cdc42 Val-12 only very weakly. In an in vitro kinase assay, SPRK Δzip expressed with or without Cdc42 Val-12 (Fig. 5, B and D) lacks the ability to phosphorylate histones. To address whether the lack of histone phosphorylation might be due to some unique feature of histones we performed the same experiment with myelin basic protein as a substrate and obtained the analogous results (data not shown). These data suggest that the zipper domain/basic stretch may be fundamentally required for substrate phosphorylation.

SPRK Δzip Fails to Activate JNK—SPRK has been identified as an upstream activator of the JNK/stress-activating protein kinase pathway (38, 40, 42). JNK activity was measured after immunoprecipitation of endogenous JNK from cellular lysates of transiently transfected 293 cells in an immune complex in vitro kinase assay using GST-c-Jun as the substrate. Overexpression of SPRK in 293 cells leads to a 5-fold increase in JNK activity over the basal activity in vector control-transfected cells (Fig. 6, A and B). Transient expression of Cdc42 Val-12 increases JNK activity some 2–3-fold. Although coexpression of SPRK and Cdc42 Val-12 increases SPRK catalytic activity, as measured in an in vitro kinase assay, the observed SPRK-induced JNK activation with coexpressed Cdc42 Val-12 over that of SPRK alone does not reach statistical significance. A likely explanation for this finding is that the activity of overexpressed
SPRK alone is sufficient to maximally activate the endogenous JNK in 293 cells. SPRK\textsubscript{D\textsubscript{zip}} is unable to phosphorylate an exogenous substrate in an \textit{in vitro} kinase assay, and, indeed, we have found that it completely lacks the ability to activate the JNK pathway (Fig. 6, \textit{A} and \textit{B}). Thus, it appears that the zipper motif is critical for substrate phosphorylation by SPRK both \textit{in vitro} and \textit{in vivo}.

The Leucine Zipper Domain Is Sufficient for SPRK Oligomerization—Despite its substantial autophosphorylation activity, SPRK\textsubscript{D\textsubscript{zip}} fails to activate JNK. To characterize the oligomerization properties of SPRK\textsubscript{D\textsubscript{zip}}, we engineered a cDNA construct encoding amino acids 386–477 of SPRK fused to the coding sequence of the monomeric MBP of \textit{E. coli}, designated MBP-zips, and tested purified MBP and MBP-zips for their ability to associate with full-length SPRK and SPRK\textsubscript{D\textsubscript{zip}}. As shown in Fig. 7 \textit{A}, full-length SPRK binds MBP-zips but not MBP, as judged by immunoblotting. Furthermore, SPRK\textsubscript{D\textsubscript{zip}} fails to bind either MBP-zips or MBP. Equal amounts of MBP or MBP-zips in the assays were verified by Coomassie Blue staining of a duplicate gel (Fig. 7 \textit{B}). Western blotting of cellular lysates using a SPRK antibody shown in Fig. 7 \textit{C}, reveals that full-length SPRK and SPRK\textsubscript{D\textsubscript{zip}} were expressed at similar levels. These data provide direct evidence that the leucine zipper/basic stretch of SPRK is capable of protein-protein interactions and is sufficient to mediate SPRK homo-oligomerization.

**Activated Cdc42 Fails to Activate SPRK \textit{in Vitro}**—The small GTPases Rac and Cdc42 can stimulate the autophosphorylation activity of the CRIB-containing serine/threonine kinase PAK-2 \textit{in vitro} (23). To determine whether Cdc42-induced activation of SPRK can be recapitulated in a completely \textit{in vitro} system, hexahistidine NH\textsubscript{2}-terminal tagged SPRK was expressed using the baculovirus system, and purified by metal-chelate chromatography. GST-Cdc42 was expressed in and purified from \textit{E. coli}. The purified SPRK is catalytically active as judged by its basal autophosphorylation activity. GTP\textsubscript{gS}- or GDP-loaded GST-Cdc42 was incubated with purified SPRK or PAK-2 in an \textit{in vitro} kinase assay (Fig. 8 \textit{B}). While GTP\textsubscript{gS}-loaded Cdc42 activates purified PAK-2, it fails to activate purified SPRK. Likewise, SPRK immunoprecipitated from transfected 293 cells cannot be activated \textit{in vitro} by the addition of GTP\textsubscript{yS}-loaded Cdc42 (Fig. 8 \textit{A}). These data support the requirement of a cellular context or coactivator for SPRK activation by Cdc42.

Cdc42 Alters the \textit{in Vivo} Phosphorylation Pattern of SPRK—As described above, co-immunoprecipitation experiments and \textit{in vitro} kinase assays show that Cdc42\textsubscript{Val-12} when coexpressed with SPRK can associate with SPRK and modulate its catalytic activity. However, purified, activated Cdc42 cannot stimulate the autophosphorylation of SPRK \textit{in vitro}. In order to determine if the presence of activated Cdc42 alters SPRK phosphorylation \textit{in vivo}, two-dimensional phosphopeptide analysis...
of SPRK labeled in vivo, in the absence and presence of Cdc42Val-12, was performed.

The net incorporation of radiolabel into SPRK increased approximately 3-fold when SPRK was coexpressed with Cdc42Val-12. Two-dimensional TLE/TLC revealed that while the basic pattern of phosphopeptides from the two samples is similar (Fig. 9A), there are notable differences. The major changes are observed in the triangular cluster of phosphopeptides, b, c, and d. Phosphopeptide a predominates in both samples. Phosphopeptides b and c are detected in the triangular cluster of the SPRK map, but are low in abundance relative to peptide a. In the corresponding map of SPRK that had been expressed in the presence of Cdc42Val-12, however, phosphopeptide b is not detected. Instead, phosphopeptide c is the prominent phosphopeptide in the triangular cluster, with 70% of the radioactivity of phosphopeptide a (Fig. 9B). Furthermore, phosphopeptide d, nearly undetectable in the map of SPRK, appears at high levels in the map of SPRK expressed with Cdc42Val-12. For comparison, the level of another peptide (x) relative to peptide a is essentially constant in both maps. The labeling and mapping from three independent experiments yielded the same results. These data indicate that the presence of activated Cdc42 changes the in vivo phosphorylation state of SPRK, which correlates with an increase in SPRK catalytic activity. Phosphoamino acid analysis of the pooled tryptic phosphopeptides (Fig. 9C) reveals predominantly serine phosphorylation, some threonine phosphorylation, and no tyrosine phosphorylation. The ratios of phosphoserine to phosphothreonine in the two samples are not substantially different.

**DISCUSSION**

Small GTPases of the Ras superfamily have been shown to regulate protein kinases. PAK has emerged as the paradigm CRIB-containing serine/threonine kinase that is activated by GTP-bound Cdc42 and/or Rac. The PAKs play roles in diverse processes, including apoptosis, modulation of actin cytoskeleton, gene transcription, and cell cycle (53). SPRK is a member of the so-called mixed-lineage kinases. Except for the presence of a loosely conserved CRIB motif, SPRK differs dramatically from the mammalian PAKs. SPRKs catalytic domain is just 20% similar to those of the mammalian PAKs. SPRK differs dramatically from the PAKs, both structurally and functionally. While the mammalian PAK-1, -2, and -3 share 95% sequence similarity in their catalytic domains, SPRKs catalytic domain is just 20% similar to those of the mammalian PAKs. The CRIB motif of the PAKs is found NH2-terminal to the catalytic domain, whereas SPRKs CRIB motif is COOH-terminal to the catalytic domain. Flanking SPRKs catalytic domain is an NH2-terminal SH3 domain and a COOH-terminal leucine zipper motif, both lacking in the PAKs. The only well established function thus far ascribed to SPRK is as an M KK in the activation of the JNK pathway. Because the MLKs are so different from the PAKs, it is important to determine whether the structural features of their binding to and the mechanisms of activation by Cdc42 and/or Rac also differ from that of the PAKs.

Whereas the three mammalian PAK isoforms contain perfect consensus CRIB motifs, as defined by Burbelo et al. (26), SPRKs CRIB motif contains only six of the eight consensus residues (Fig. 2). We show that mutations in conserved residues of SPRKs CRIB motif disrupt the ability of the Cdc42 to bind to and activate SPRK, indicating that SPRK does indeed contain a functional CRIB motif. WASP and ACK, two other
proteins whose CRIB-dependent binding to Cdc42 has been well established, also contain less than perfect CRIB motifs (Fig. 2), with WASP (28, 29), and ACK (27) containing 7 of the 8 consensus residues, and 6 of the 8 consensus residues, respectively. It may well be that the CRIB consensus motif is biased toward PAK, due to the large number of PAK isoforms that have been identified.

SPRK and the closely related MLK-2 lack the second of the two conserved histidine residues of the consensus CRIB motif (Fig. 2). We show here that mutation of the first conserved histidine in SPRK to an alanine residue (H500A) disrupts binding to Cdc42. The fact that both SPRK (26) and MLK-2 (26, 54) have been demonstrated to bind Cdc42 may indicate that the second of the two conserved histidines in the consensus CRIB motif in other CRIB-containing proteins is not required for binding to Cdc42. Further support for this notion is provided by the finding that the conserved Asp in Cdc42 interacts primarily with the first of the two conserved histidine residues (H520) in ACK (55). In addition, mutation of the first of the two conserved histidine residues in N-WASP to aspartate (H208D) decreases the in vitro binding affinity for Cdc42 and Rac, as well as the activity of N-WASP in vivo and in vitro (56). The regions outside of the CRIB motifs of ACK and WASP exhibit low sequence similarity, and, perhaps not surprisingly, low structural similarity when bound to Cdc42 (55, 57). It is likely that the GTPase-binding domain of SPRK, with the exception of the CRIB motif, will differ structurally from those of both WASP and ACK.

Because of the proximity of SPRKs zipper and CRIB motifs in linear sequence, and because sequences flanking the CRIB motif in other proteins contribute to Cdc42/Rac binding, we tested whether deletion of the COOH-terminal half of the zipper motif affects Cdc42 binding. The binding of SPRKΔzip (Fig. 1), which contains an intact CRIB motif, to activated Cdc42 is reduced more than 10-fold, suggesting that, in addition to SPRKs CRIB motif, the zipper domain or the following short basic region of SPRK may contribute to Cdc42 binding. Interestingly, the basal autophosphorylation activity of SPRKΔzip is about 70% that of wild type SPRK. This may indicate some intramolecular autophosphorylation activity. Alternatively, SPRKΔzip may homo-oligomerize and undergo intermolecular autophosphorylation. Leung and Lassam (58) recently reported a very large reduction in GST-SPRK/MLK-3 autophosphorylation activity in vitro upon deleting the entire zipper region, but leaving the basic stretch intact.

Recent site-directed mutagenesis studies indicate that the serine/threonine kinase PAK contains a basic stretch consisting of three contiguous lysine residues upstream of the CRIB motif, whose charge is important for binding to Rac1 and Rac2, and whose presence is required for efficient PAK-1 activation by Rac1, Rac2, and Cdc42 (33). SPRK contains four contiguous arginine residues between the zipper domain and the CRIB.

**Fig. 6. Effect of SPRKΔzip on JNK activity.** A, endogenous JNK-1 was immunoprecipitated from cellular lysates that had been transiently transfected with cDNAs encoding the specified SPRK and/or Cdc42 variants. An in vitro immune complex assay for JNK activity was performed using GST-c-Jun as a substrate. The uppermost panel shows an autoradiogram with bands corresponding to GST-c-Jun indicated by an arrow. The second panel shows a JNK immunoblot of the same immunoprecipitated samples from the in vitro kinase assay. The third panel shows a SPRK immunoblot of cellular lysates. The lowest panel shows an immunoblot for Cdc42 from cellular lysates. B, quantitation of in vitro kinase assays of JNK. GST-c-Jun phosphorylation was measured by PhosphorImaging and normalized to SPRK expression levels as described under “Experimental Procedures.” The mean ± S.E. for fold-increase in GST-c-Jun phosphorylation in three independent experiments are graphed.
motif. These basic amino acids are deleted in the SPRK\textsubscript{zip} variant, which exhibits greatly reduced binding to activated Cdc42. Thus, it is plausible that the arginine tract in SPRK

![Figure 7](Image)

**FIG. 7.** Assay for association of the zipper/basic stretch of SPRK with SPRK and SPRK\textsubscript{zip} in vitro. Lysates from 293 cells expressing SPRK or SPRK\textsubscript{zip} were incubated with amylose resin to which purified MBP or MBP-zips had been prebound. A, after washing of the resin, the presence of bound SPRK or SPRK\textsubscript{zip} was assessed by immunoblotting with the SPRK antibody. B, equal loading of MBP and MBP-zips on the amylose resin was confirmed by Coomassie staining. C, expression of SPRK and SPRK\textsubscript{zip} was assessed by immunoblotting of cellular lysates with a SPRK antibody.

![Figure 8](Image)

**FIG. 8.** In vitro kinase assays using purified Cdc42. A, SPRK was immunoprecipitated from cellular lysates that had been transiently transfected with cDNA encoding SPRK and was incubated in an in vitro kinase assay in the presence of 4 \( \mu \)g of GST-Cdc42 preloaded with either GTP\textsubscript{S} or GDP. The top panel shows an autoradiogram with bands corresponding to SPRK autophosphorylation indicated by arrows. The lower panel shows an immunoblot of SPRK from cellular lysates. B, purified SPRK and PAK-2 (1 and 2 \( \mu \)g, respectively) were incubated in an in vitro kinase assay in the presence of 4 \( \mu \)g of GST-Cdc42 preloaded with either GTP\textsubscript{S} or GDP. Shown is an autoradiogram with bands corresponding to SPRK autophosphorylation and PAK-2 autophosphorylation indicated by arrows.

![Figure 9](Image)

**FIG. 9.** Phosphopeptide mapping and phosphoamino acid analysis of tryptic peptides derived from in vivo phosphorylated SPRK. See “Experimental Procedures” for detail. A, two-dimensional phosphopeptide mapping of \( ^{32} \)P-labeled SPRK from 293 cells transfected with expression vectors for SPRK (top) or for SPRK and Cdc42\textsubscript{Val-12} (bottom). SPRK was immunopurified from cellular lysates, blotted onto a polyvinylidene difluoride membrane, and subjected to partial trypsin digestion. Equal amounts of radioactivity, as determined by Cerenkov counting of the resultant tryptic peptides, were analyzed by TLE in the first dimension and TLC in the second dimension. The direction of the electrophoresis and chromatography are indicated by long arrows. Phosphopeptides were visualized by PhosphorImaging. The phosphopeptides of interest are alphabetically labeled. Shown is a representative map from three independent experiments. B, the percent radioactivity of the indicated phosphopeptides compared with phosphopeptide a. Calculated as: [(volume – background)/phosphopeptide a] \( \times \) 100, using Image Quant software (Molecular Dynamics). C, phosphoamino acid analysis of in vivo \( ^{32} \)P-labeled SPRK. Phosphoamino acids derived from the SPRK tryptic peptides in A were separated by TLE and visualized by PhosphorImaging. The third lane (Stds) shows the positions of phosphoamino acid standards. The position of free inorganic phosphate (Pi) is also indicated.
may contribute to Cdc42 binding or activation of SPRK. Currently we are defining a minimal Cdc42-binding domain of SPRK and are assessing the relative contributions of various amino acids within this domain to Cdc42 binding and Cdc42-induced SPRK activation.

We show that SPRK and activated Cdc42 can be co-immunoprecipitated from cellular lysates. However, under the conditions of our in vitro kinase assay, which clearly show a Cdc42-induced increase in SPRK autophosphorylation and histone phosphorylation, Cdc42 is not detected. Possibly once SPRK is activated by Cdc42, SPRK has a reduced affinity for the GTPase as has been demonstrated with PAK-2 (21). Furthermore, the sites of in vitro autophosphorylation of SPRK expressed with and without Cdc42, and isolated from mammalian cells, are essentially identical as judged by mapping of tryptic phosphopeptides (data not shown). The mechanism by which the highly conserved PAK family members (PAK-1, -2, and -3) are activated by Rac and Cdc42 has been well studied. Increased autophosphorylation activity is observed upon incubation of purified activated Cdc42 with purified PAK. In contrast to PAK, purified, catalytically active SPRK cannot be further activated in vitro by the addition of GTP-bound Cdc42. These data are consistent with a catalytic model in which Cdc42 activates SPRK in vivo, but is not required to maintain SPRK in its activated state. We therefore decided to assess whether Cdc42 induces differential phosphorylation of SPRK in vivo.

Phosphoamino acid analysis of in vivo labeled SPRK revealed serine and threonine, but no tyrosine, phosphorylation. Two-dimensional tryptic phosphopeptide mapping studies of in vivo labeled SPRK, expressed with or without constitutively active Cdc42, showed similar phosphopeptide maps, with major differences observed in a triangular cluster of phosphopeptides b, c, and d (Fig. 9A). In the map of SPRK alone, phosphopeptides b and c are present, but at low levels. When activated Cdc42 is coexpressed with SPRK, phosphopeptide b is not detected and phosphopeptides c and d appear at high levels. Since the change in in vitro phosphorylation of SPRK with Cdc42 correlates with increased SPRK activity, it is likely that phosphopeptides c and d contain activating phosphorylation sites. Phosphopeptides c and d may be distinct phosphopeptides. However, since their chromatographic mobilities are essentially identical, it is also possible that phosphopeptides c and d result from differential trypsin digestion. Phosphopeptides b and c lie on a diagonal which slopes toward the anode, characteristic of peptides that are phosphoisoformers. Upon phosphorylation, the negative charge and polar nature of the added phosphate group reduces a peptides mobility in both the electroforetic and chromatographic dimensions (59). Therefore, phosphopeptide c may differ from phosphopeptide b by the addition of a phosphate group(s). This is consistent with the observation that when activated Cdc42 is coexpressed with SPRK, phosphopeptide c emerges while phosphopeptide b disappears.

Cdc42 may induce differential SPRK autophosphorylation in vivo or, alternatively, another SPRK-activating kinase may be responsible for the in vivo change in SPRK phosphorylation. Because Cdc42 is geranylgeranylated (60) and has been localized to cellular membranes (61, 62, 63) as well as to cytoskeletal elements (64), it is possible that Cdc42 recruits SPRK to the vicinity of an activating kinase. SPRK and the serine/threonine kinase Raf both appear to function as MKKKs, activating the JNK and ERK pathways, respectively. The idea that Cdc42 may recruit SPRK to an activating kinase is reminiscent of the proposed mechanism by which the small GTPase Ras activates Raf. Analogous to our findings with Cdc42 and SPRK, the addition of purified, activated Ras to Raf in vitro is not sufficient for full activation of Raf. However, appending a membrane targeting motif to the COOH terminus of Raf causes Raf translocation and activation in the absence of activated Ras (1).

It has been recently shown that PAK-3 phosphorylates Raf in vivo and in vitro leading to an increase in Raf activity (65), although it has yet to be determined whether this event requires Raf translocation. In yeast, the PAK homolog STE20 functions as an M KK in the activation of a yeast MAPK pathway. By analogy, potential SPRK-activating kinases may include PAK-related kinases (53) such as hematopoietic protein kinase-1. In coexpression studies hematopoietic protein kinase-1 binds to SPRKs SH3 domain and phosphorylates SPRK, but the effect on SPRK activity is not reported (66). Interestingly, unlike PAK-1, -2, and -3, hematopoietic protein kinase-1 lacks a CRIB motif. Our finding that catalytically active SPRK cannot be further activated in vitro by GTP-bound Cdc42 suggests that the GTPase activates SPRK differently than the PAKs. Whereas the PAKs can be activated in vitro by interaction with unprenylated GTP-bound Cdc42, the activation of SPRK by Cdc42 appears to require prenylation of Cdc42, the cellular environment, or an as yet unidentified cellular component. Our data supports a model in which the in vivo CRIB-dependent interaction of SPRK and Cdc42 either allows SPRK to adopt a conformation that leads to autophosphorylation or recruits SPRK to the vicinity of a serine/threonine kinase that phosphorylates and activates SPRK. Determination of the precise sites of Cdc42-induced phosphorylation of SPRK, coupled with subcellular localization studies, should shed further light on the detailed mechanism of SPRK activation by Cdc42.

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Cdc42-induced Activation of the Mixed-Lineage Kinase SPRK\textit{in Vivo}:
REQUIREMENT OF THE Cdc42/Rac INTERACTIVE BINDING MOTIF AND
CHANGES IN PHOSPHORYLATION

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