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# The Mixed Lineage Kinase SPRK Phosphorylates and Activates the Stress-activated Protein Kinase Activator, SEK-1*

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**Ajay Rana‡§, Kathleen Gallo¶, Paul Godowski‖, Shu-ichi Hirai**, Shigeo Ohno**, Leonard Zon‡‡, John M. Kyriakis‡, and Joseph Avruch‡**

*From the ‡Diabetes Unit and Medical Service, Massachusetts General Hospital, Boston, Massachusetts 02129 and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, the ¶Department of Physiology, Michigan State University, East Lansing, Michigan 48824, the ‖Department of Molecular Biology, Genentech, Inc., South San Francisco, California 94080, the **Department of Molecular Biology, Yokohama City University School of Medicine, 3-9 Fuku-ura Kanazawa-ku, Yokohama 236, Japan, and the ‡‡Howard Hughes Medical Institute and Division of Hematology/Oncology, Children's Hospital and Dana Farber Cancer Institute, Boston, Massachusetts 02115*

**SPRK (also called PTK-1 and MLK-3), a member of the mixed lineage kinase subfamily of (Ser/Thr) protein kinases, encodes an amino-terminal $SH_3$ domain followed by a kinase catalytic domain, two leucine zippers interrupted by a short spacer, a Rac/Cdc42 binding domain, and a long carboxyl-terminal proline-rich region. We report herein that SPRK activates the stress-activated protein kinases (SAPKs) but not ERK-1 during transient expression in COS cells; the p38 kinase is activated modestly (1.3–2 fold) but consistently. SPRK also activates cotransfected SEK-1/MKK-4, a dual specificity kinase which phosphorylates and activates SAPK. Reciprocally, expression of mutant, inactive SEK-1 inhibits completely the basal and SPRK-activated SAPK activity. Immunoprecipitated recombinant SPRK is able to phosphorylate and activate recombinant SEK-1 *in vitro* to an extent comparable to that achieved by MEK kinase-1. These results identify SPRK as a candidate upstream activator of the stress-activated protein kinases, acting through the phosphorylation and activation of SEK-1.**

The stress-activated protein kinases SAPKs[1] (1), also known as Jun amino-terminal kinases, JNKs (2), and p38 (3), also known as reactivating kinase, RK (5, 6), and CSBPs (7) are two recently described subclasses of the ERK family of protein kinases. In contrast to the p44/p42 MAP kinases (ERK-1 and -2, respectively), which are central elements in mitogenic signal transduction downstream of Ras (8), the SAPKs and p38 are activated most vigorously by the inflammatory cytokines, tumor necrosis factor $\alpha$ (1) and interleukin 1$\beta$ (6), and by a diverse array of cellular stresses, such as heat shock, UV, and ionizing radiation (1–3, 5, 6), and during the recovery from anoxia (9). The immediate downstream targets include a variety of transcription factors, notably c-Jun (10), ATF-2 (11), and Elk-1 (12) for the SAPKs, as well as the transcription factor, Max (4), and other protein kinases (*e.g.* MAPKAP kinase-2) for p38 (5). The cellular responses that commonly accompany SAPK and p38 activation include reparative and/or apoptotic programs (13), although a proliferative response may occur in certain cell backgrounds.

Considerable progress has been achieved in the identification of some of the upstream elements that couple the SAPKs/p38 to these numerous stimuli; like the MAPKs, the SAPKs and p38 are activated by dual phosphorylation of a threonine and tyrosine residue, situated in the motif T*X*Y in catalytic subdomain VIII (where *X* = Pro for the SAPKs and Gly for p38), a reaction catalyzed by novel members of the MAPK kinase (MEK) family. Thus, SEK-1/MKK-4 catalyzes the phosphorylation and activation of SAPK (14, 15), and (at least *in vitro*) p38 as well (15), whereas MKK-3 phosphorylates and activates p38 but not SAPKs (15). The element immediately upstream of MKK-3/p38 has not yet been identified; however, the protein kinase MEKK-1 is capable of directly phosphorylating and activating SEK-1/MKK-4 *in vitro*; *in situ*, low level expression of a constitutively active MEKK-1 results in the selective activation of the SAPK pathway, whereas ERK-1/2 activation is evident only with marked MEKK overexpression, and p38 activation is not observed (16). Recently, another (Ser/Thr) kinase, MUK (17), also known as DLK (18) or ZPK (19), has also been shown to activate SAPK (17). Structural similarity of MUK/ZPK/DLK to the kinase family called mixed lineage kinases (MLKs) led us to examine the ability of SPRK (20)/PTK-1 (21)/MLK-3 (22), another mixed lineage kinase, to activate SAPKs. This report shows that SPRK is a candidate regulator of the SAPK pathway; further, we show that SPRK is acting through the phosphorylation and activation of the dual specificity kinase, SEK-1/MKK-4 *in vitro* and *in situ*.

## MATERIALS AND METHODS

The cDNAs encoding rat p54SAPK$\alpha$1 and p46SAPK$\beta$, rat p44ERK-1 (14), human p38, and SEK-1AL(S220A,T224L) were tagged at their amino terminus with a nine amino acid epitope from the influenza hemagglutinin (HA epitope) and inserted into the mammalian expression vector pMT2. SPRK was tagged at its carboxyl terminus with the "Flag" epitope (DYKDDDDK) and inserted into the cytomegalovirus-based vector pRK5 (20). The MEKK-1 catalytic domain (carboxyl-terminal 320 amino acids), tagged at its amino terminus with the EE epitope (16), was inserted into pCMV5. BXB-Raf is an amino-terminally truncated c-Raf1 polypeptide that is constitutively active when expressed in COS cells (23). SEK-1 and the inactive mutants SEK-1(Lys → Arg) and SEK-1AL were expressed as glutathione *S*-transferase (GST) fusion proteins in COS cells using the vector pEBG (14). Prokaryotic recombinant SAPKs and GST-Jun were expressed and purified as before (14). COS cells were transfected by the DEAE-dextran method using the amounts of DNA indicated; empty vector was used to achieve

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§ To whom correspondence should be addressed: Diabetes Unit, Massachusetts General Hospital, 149 13th St., Charlestown, MA 02129. Tel.: 617-726-6909; Fax: 617-726-5649; E-mail: rana@helix.mgh.harvard.edu.

[1] The abbreviations used are: SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MLK, mixed lineage kinase; HA, hemagglutinin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

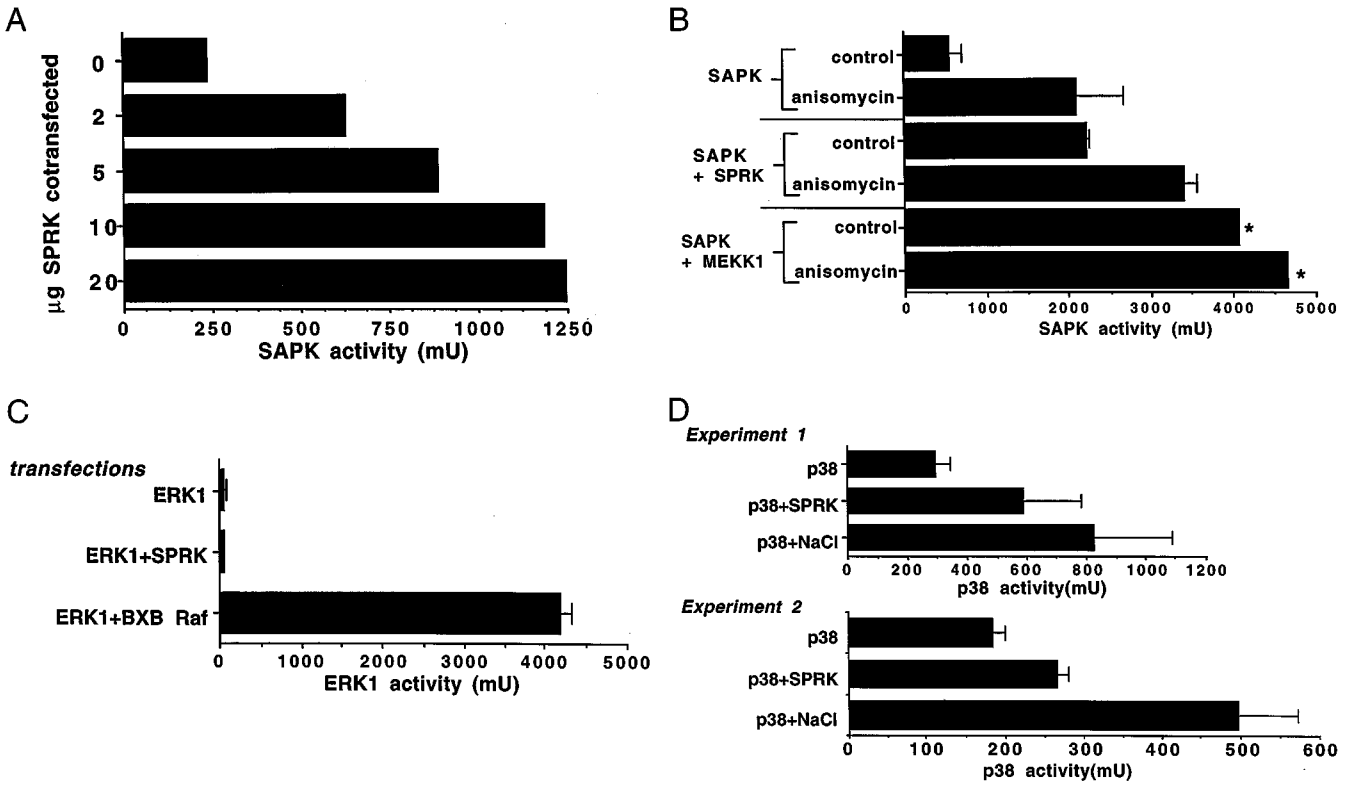


FIG. 1. **SPRK activates SAPK but not MAPK (ERK-1) on cotransfection.** *A*, COS cells were transfected with a fixed amount (2 $\mu$g) of HA-p46SAPK$\beta$ and increasing amounts of SPRK as indicated; 12CA5 immunoprecipitates were assayed for GST-Jun kinase activity. The average of duplicate assays is shown. *B*, comparison of SPRK with MEKK-1 as SAPK activators. COS cells were transfected with HA-SAPK (2 $\mu$g) and SPRK, MEKK-1, or empty vector (10 $\mu$g). After 48 h, cells were treated with carrier or anisomycin (10 $\mu$g/ml); HA-SAPK was immunoprecipitated and assayed for GST-Jun kinase activity. The * indicates the average of duplicate assays. *C*, SPRK does not activate MAPK (ERK-1). HA-ERK-1 (10 $\mu$g) was cotransfected with SPRK or BXB-Raf-1 (10 $\mu$g), an amino-terminally truncated constitutively active Raf mutant. The 12CA5 immunoprecipitate was assayed for myelin basic protein kinase. *D*, p38 kinase is slightly activated by SPRK. Two representative experiments comparing SPRK with 0.7 M NaCl (10 min) on p38 MBP kinase are shown. In experiment 1, 10 $\mu$g of DNA encoding p38 were cotransfected with 10 $\mu$g of pRK5-Flag SPRK or pRK5 alone (vector); the difference in p38 activity, measured after immunoprecipitation from each of three plates, was not different between vector and SPRK cotransfections ($0.05 < p < 0.1$). In experiment 2, 10 $\mu$g of p38 DNA was coexpressed with 2 $\mu$g of pRK5 or pRK5-Flag-SPRK. The difference in p38 activity was statistically significant $0.002 < p < 0.005$.

equal amounts of each vector in each transfection. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 48 h prior to extraction. When present, anisomycin was added to a final concentration of 10 $\mu$g/ml 15 min prior to harvest; NaCl was added to 0.7 M 10 min prior to extraction. Cell lysis and immunoprecipitation was carried out as before (1, 14). HA-tagged polypeptides were complexed with the monoclonal antibody 12CA5, Flag-tagged SPRK with the M2 monoclonal anti-Flag monoclonal, and the EE-tagged MEKK-1 with the anti-EE monoclonal antibody. Immune complexes were recovered with Protein A-Sepharose and washed four times in assay buffer (1, 14) containing 1 M LiCl, followed by two washes in assay buffer. GST-SEK-1 polypeptides were recovered on glutathione GSH-agarose, washed as above, eluted with 0.1 M Tris-Cl, pH 8, 0.12 M NaCl, 20 mM reduced glutathione, 5 mM dithiothreitol, and dialyzed into assay buffer.

SAPK activity was assayed as the rate of phosphorylation of bacterial GST-Jun; 1 unit equals 1 pmol of $PO_4$ incorporated $min^{-1}$. MAPK and p38 activity were measured as the rate of phosphorylation of myelin basic protein; 1 unit equals 1 pmol of $^{32}P$ incorporation $min^{-1}$. SEK-1 activity in the experiment shown in Fig. 2*A* was measured by the incorporation of $^{32}P$ into bacterial SAPK. Kinase results are shown as mean ± S.D. of triplicate determinations, except where indicated; each experiment was performed three or more times.

In the experiments examining the activation of GST-SEK-1 *in vitro*, the soluble GST-SEK-1 polypeptide (approximately 0.2 $\mu$g) or buffer was added to Protein A-Sepharose beads containing normal IgG or the immobilized SPRK and MEKK-1 polypeptides, at 30 °C for 15 min in the presence of 10 mM Mg- and 0.1 mM [$\gamma$-$^{32}P$]ATP). The reaction was terminated by sedimentation of the beads and removal of an aliquot of the supernatant to an incubation containing bacterial SAPK or buffer. After 15 min, GST-Jun polypeptide was added; the incubation was continued for an additional 15 min and terminated with SDS.

Immunoblotting was carried out after SDS-PAGE and transfer of polypeptides to polyvinylidene difluoride membranes; bound antibodies were detected by enhanced chemiluminescence (Amersham).

## RESULTS

Coexpression of HA-SAPK p46$\beta$1 with a full-length SPRK cDNA in COS cells results in a 4–6-fold activation of the c-Jun kinase activity of immunoprecipitated SAPK (Fig. 1, *A* and *B*) despite a substantial dose-dependent inhibition of SAPK expression by cotransfected SPRK cDNA (not shown). This extent of SAPK activation *in situ* is at least 50% of that obtained by cotransfection with a constitutively active MEKK (Fig. 1*B*). Neither the expression nor activity of recombinant ERK-1 (10 $\mu$g of DNA) is altered by cotransfection with SPRK (10 $\mu$g; Fig. 1*C*). As regards p38, SPRK (10 $\mu$g of DNA) induced a 2-fold increase in p38 activity (10 $\mu$g of DNA; Fig. 1*D*, experiment 1) as compared with the 4–6-fold activation of SAPK (Fig. 1, *A* and *B*). As with SAPK, p38 expression was inhibited by coexpression with SPRK, and the overall p38 activation using 10 $\mu$g of SPRK DNA was variable and not statistically significant. A lesser amount of SPRK (2 $\mu$g of DNA) resulted in more consistent p38 expression; p38 activation, although now statistically significant, remained <2-fold (Fig. 1*D*, experiment 2). Thus, we conclude that overexpression of SPRK results in a statistically significant activation of p38 of modest magnitude, substantially less than that observed for SAPK.

SAPKs are activated by the dual phosphorylation of a threonine and tyrosine residue in the motif TPY, situated in catalytic subdomain VIII, just amino-terminal to the highly conserved APE motif. Several chromatographically dis-

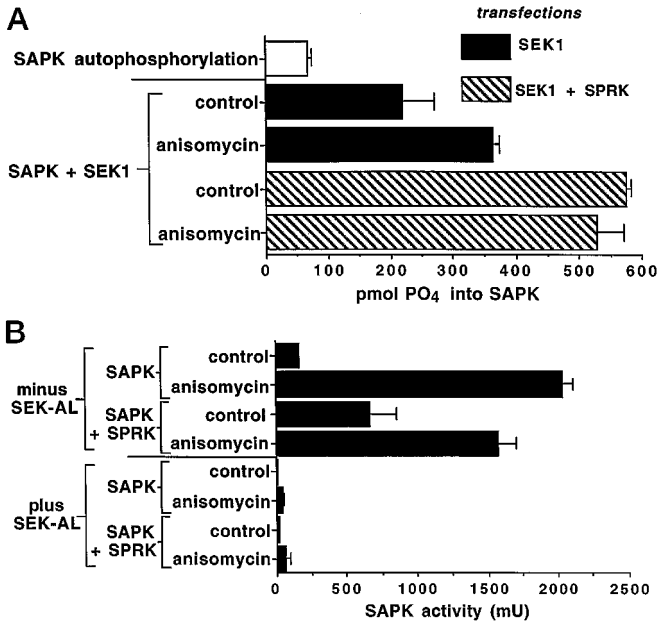


FIG. 2. **Regulation of SEK-1 by SPRK *in situ*.** *A*, activation of SEK-1 upon coexpression with SPRK. COS cells were transfected with 10 $\mu$g of pEBG-SEK-1 and either 10 $\mu$g of pRK5-SPRK (*hatched*) or empty plasmid (*black bars*). Some cells were treated with anisomycin prior to harvest. GST-SEK-1 was purified from cell lysates using glutathione-agarose beads and assayed for its ability to phosphorylate bacterial SAPK-p54$\alpha$I *in vitro*. *B*, dominant inhibitory SEK-1 (SEK-AL) abolishes SPRK activation of SAPKs. COS cells were transfected with 2 $\mu$g of HA-SAPK-p46$\beta$ plus 10 $\mu$g of either pRK5-SPRK or pRK5 vector and 10 $\mu$g of either pMT2 HA-SEK-AL (a nonphosphorylatable dominant inhibitory SEK-1) or $PMT_2$. After treatment with carrier or anisomycin, 12CA5 immunoprecipitates were assayed for GST-Jun kinase activity.

tinguishable peaks of SAPK activating kinase activity can be discerned (at least in response to hyperosmolarity), one of which corresponds to the MEK-like enzyme designated SEK-1/MKK-4 (24). Coexpression of SPRK with SEK-1 in COS cells results in a 4-fold increase in SEK-1 activity, as measured by the ability of the COS recombinant SEK-1 to phosphorylate a prokaryotic recombinant p54SAPK$\alpha$ polypeptide *in vitro* (Fig. 2*A*). SEK-1 is also known to be activated by phosphorylation in catalytic subdomain VIII, at a serine and a threonine residue in the motif -SIAKT-; mutation of this serine and threonine to alanine and leucine, respectively, produces SEK-AL, a catalytically inactive polypeptide which is not activable by upstream elements (14). Overexpression of SEK-AL results in a potent inhibition of the activity of coexpressed SAPK, both in the basal state and in response to SAPK activators, including coexpressed MEKK. SEK-AL also suppresses the SPRK-induced activation of SAPK (Fig. 2*B*, with no change in SPRK or SAPK polypeptide expression (not shown)). This finding suggests that SEK-AL sequesters elements necessary for the SPRK activation of SAPK and implies that SEK-1 itself or a closely related kinase participates in the activation of SAPK by SPRK. This possibility led us to determine whether SPRK itself can directly activate SEK. Epitope-tagged versions of SPRK and a constitutively active catalytic fragment of MEKK were transiently expressed in COS cells and isolated from cell extracts by immunoprecipitation. After extensive washing, the immobilized kinases were incubated with a purified, soluble recombinant GST-SEK-1 polypeptide (*e.g.* see Fig. 3*B*, *middle panel*) in the presence of MgATP. Incubation was terminated by removal of an aliquot of the soluble GST-SEK-1; the ability of GST-SEK-1 to activate prokaryotic recombinant SAPK was measured as the GST-SEK-induced activation of the SAPK-catalyzed phos-

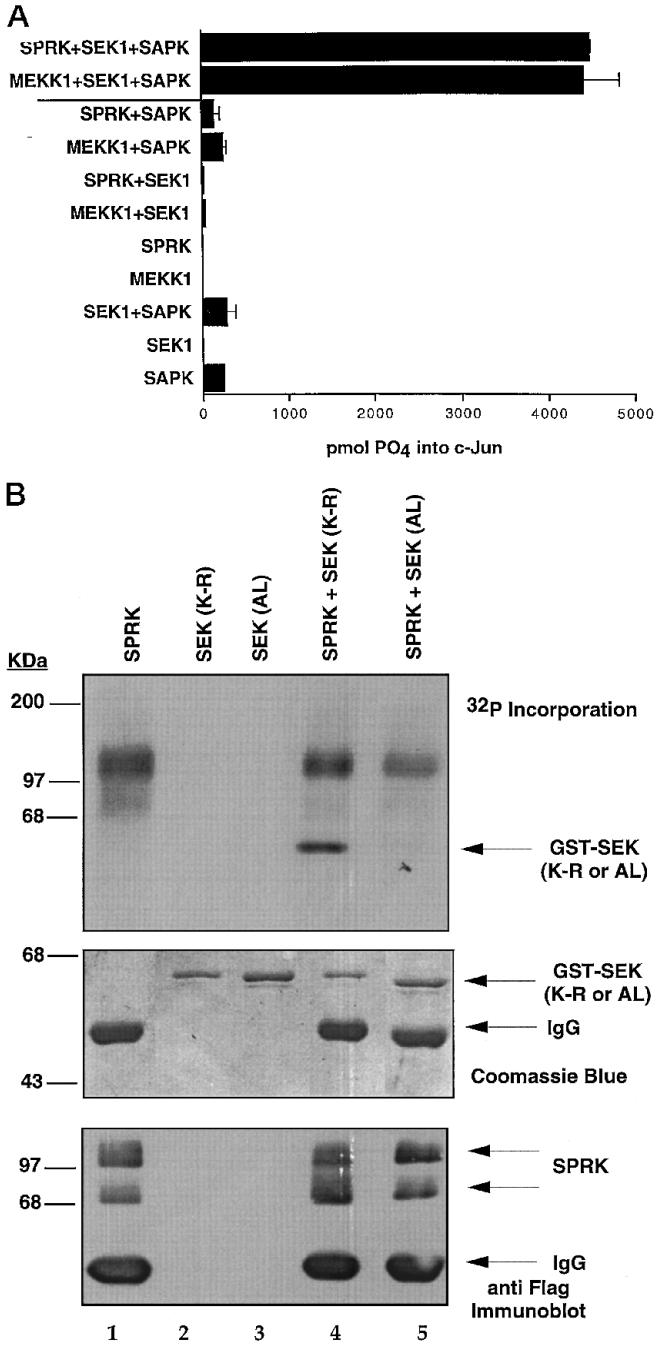


FIG. 3. **SPRK phosphorylates and activates GST-SEK *in vitro*.** *A*, activation of SEK-1 by SPRK *in vitro*. COS cells were transfected with pRK5-Flag-SPRK (10 $\mu$g) or pCMV-EE-MEKK-1 (10 $\mu$g) and harvested after 48 h. Flag-SPRK was immunoprecipitated using M2 Flag monoclonal antibody and EE-MEKK with the anti-EE monoclonal antibody; the kinases were recovered using Protein A-Sepharose beads. The immobilized kinases were washed four times in SAPK assay buffer with 1 M LiCl and two times with assay buffer. The GST-SEK-1 polypeptides (wild-type, Lys $\rightarrow$ Arg, and AL mutants) were expressed in COS cells with the pEBG-vector, purified on GSH-agarose, eluted with 20 mM GSH, pH 8, and dialyzed into assay buffer. After incubation with immobilized SPRK or MEKK, the activity of GST-SEK-1 was measured in a coupled assay using bacterial SAPK and GST-Jun, as described under "Materials and Methods." Values are mean $\pm$ S.D. for triplicates. *B*, SPRK phosphorylates GST-SEK *in vitro*. Solubilized COS recombinant GST-SEK-1(Lys $\rightarrow$ Arg) or GST-SEK-1AL (approximately 1.2 $\mu$g each) were incubated with immobilized Flag-SPRK or control beads in the presence of Mg- and [$\gamma$-$^{32}$P]ATP for 15 min at 30 °C. After SDS-PAGE, the Coomassie Blue-stained polypeptides (*middle panel*) were subjected to autoradiography (*upper panel*) and immunoblotted for Flag-SPRK (*lower panel*).

phorylation of GST-c-Jun. The SPRK and MEKK immunoprecipitates are themselves devoid of SEK (SAPK kinase) or SAPK (c-Jun kinase); however, both SPRK and MEKK increase the catalytic activity of recombinant GST-SEK-1 by 15–20-fold (Fig. 3*A*). Concomitantly, the SPRK catalyzes the phosphorylation of a catalytically inactive GST-SEK-1(Lys → Arg) polypeptide (to an approximate stoichiometry of 1 pmol of phosphate/20 pmol of GST-SEK(Lys → Arg) in 15 min) but is unable to catalyze significant phosphorylation of an equivalent amount of GST-SEK-1AL (Fig. 3*B*).

## DISCUSSION

The present results indicate that SPRK, a member of the mixed lineage subfamily of protein (Ser/Thr) kinases is a candidate upstream regulator of the SAPK pathway and, to a modest extent, perhaps the p38 kinases; SPRK overexpression does not affect the Ras-regulated MAPK pathway. The magnitude of SAPK activation observed on coexpression with SPRK was at maximum 4–8-fold and usually about 50% that elicited by MEKK. Wild-type SPRK, however, substantially suppressed the expression of recombinant SAPK polypeptide, an effect not observed with MEKK. Thus, the actual extent of SAPK activation by SPRK is underestimated by these data. Moreover, the MEKK employed in this comparison is an amino-terminally truncated, presumably activated MEKK mutant; in contrast, the full-length SPRK polypeptide was employed in these studies, and, although full-length SPRK clearly has substantial basal activity, the extent to which SPRK can be further activated is unknown. Consequently, the potency of SPRK as a SAPK activator *in situ* is substantial, and it is likely that SPRK, or other members of the MLK subfamily, are among the physiologic regulators of the SAPK pathway. Nevertheless, considerably more SPRK cDNA is necessary for SAPK activation during transient expression than is required for MEKK-1, and the potency of SPRK polypeptide relative to other MLKs and to MEKK remains to be defined more fully.

The proximate biochemical mechanism by which SPRK activates SEK-1 appears to be direct phosphorylation of the SEK-1 polypeptide at one or both of the sites mutated in SEK-AL. The related mixed lineage kinase, DLK/MUK, has also been reported to activate the SAPKs on cotransfection (17) and is thus likely to also act directly on SEK-1. The mixed lineage kinases are so called because the conserved amino acid sequences in catalytic subdomains I-VII resemble most closely those of the Ser/Thr kinases, particularly those in the MEKK family (MEKK1, Ste11, byr2, etc.) whereas subdomains VIII through XI are most similar to those of the receptor tyrosine kinases, particularly the FGFR and HER4. The SPRK/MLK-3 catalytic domain is about 75% identical to those of MLK-1 and MLK-2; all three kinases have considerable similarity in amino acid sequence through their noncatalytic domains as well, although MLK-1 lacks $SH_3$ and Cdc42/Rac binding domains. MUK/DLK, while similar in domain structure to SPRK including $SH_3$ and Cdc42/Rac-binding domains, is only 36% identical to SPRK, in catalytic domain sequence, and may represent another subgroup within the MLKs. Thus, the mixed lineage kinases are the second kinase family, along with the MEKK subfamily (including MEKK-1 and TAK-1, Ref. 25) shown to be capable of directly phosphorylating and activating SEK-1.

Several questions concerning the function of SPRK as a SAPK regulator remain to be more fully addressed. The key physiologic question is the nature of the stimuli that recruit SPRK to an active state and utilize it to activate the SAPK pathway. The development of an assay for SPRK activity will enable a preliminary analysis of SPRK regulation, so as to determine whether the activity of endogenous SPRK is constitutive or regulated and whether the high activity of recombinant SPRK reflects a distortion introduced by transfection and overexpression. The reported ability of Cdc42/Rac to bind directly to SPRK (26) suggests that stimuli which promote the GTP charging of these small G-proteins may recruit the SPRK; no information is available as to whether binding to Cdc42/Rac will directly activate SPRK kinase, as occurs with the Ste20 homolog, PAK-1 (27). In turn, it will be necessary to address whether SPRK is recruited by other candidate upstream activators of the SAPK pathway, namely PAK-1 (28), and the GC kinase (29), enzymes whose immediate substrates are not known. Inasmuch as SPRK and PAK-1 are likely to be competitors for the Rac1/Cdc42 effector domains, these two kinases may represent alternate effectors in the GTPase activation of the SAPK pathway (30, 31), whose contribution is determined primarily by input collateral to GTPase activation. The existence of multiple independent inflows to the SAPK pathway is not surprising, in view of the numerous perturbations that result in activation of the SAPKs.

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