SPAK, a STE20/SPS1-related kinase that activates the p38 pathway

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We have cloned a member of the STE20/SPS1 protein kinase family from a transformed rat pancreatic beta cell line. SPAK (STE20/SPS1-related, proline alanine-rich kinase) belongs to the SPS1 subfamily of STE20 kinases and is highly conserved between species. SPAK is expressed ubiquitously, although preferentially in brain and pancreas. Biochemical characterization of SPAK catalytic activity demonstrates that is a serine/threonine kinase that can phosphorylate itself and an exogenous substrate *in vitro*. SPAK is immunoprecipitated from transfected mammalian cells as a complex with another, as yet uncharacterized, serine/threonine kinase which is capable of phosphorylating catalytically-inactive SPAK and myelin basic protein in an *in vitro* kinase assay. SPAK specifically activates the p38 pathway in cotransfection assays. Like MST1 and MST2, SPAK contains a putative caspase cleavage site at the junction of the catalytic domain and the C-terminal region. Full-length SPAK is expressed in the cytoplasm in transfected cells, while a mutant corresponding to caspase-cleaved SPAK is expressed predominantly in the nucleus. The similarity of SPAK to other SPS1 family members, its ability to activate the p38 pathway, in addition to its putative caspase cleavage site, provide evidence that SPAK may act as a novel mediator of stress-activated signals. *Oncogene* (2000) **19**, 4290–4297.

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Introduction

Mammalian cells respond to extracellular stimuli by activation of the mitogen activated protein kinase (MAPK) signalling cascades, leading to a diverse range of physiological effects including cell growth, cell cycle arrest, apoptosis and changes in cytoskeletal organization. The MAPK superfamily comprises at least three parallel yet interwoven cascades, the ERK, JNK/SAPK and p38 pathways. Each cascade comprises a module of three kinases which are activated in series, with the terminal enzyme, the MAPK, activated by a MAPKK which in turn is activated by a MAPKKK (for review see Robinson and Cobb, 1997). Although the MAPK modules themselves are relatively well defined, the events leading to activation of the MAPKKKs are less well understood.

We previously analysed protein kinase expression in a transformed rat pancreatic beta cell line and identified signalling molecules involved in β-cell

development (DeAizpurua et al., 1997). During these studies a novel kinase sequence was identified which displays similarity to members of the STE20 serine/threonine kinase family. Studies in yeast and in mammalian cells have indicated that STE20-related kinases function upstream of MAPKKKs, where they regulate the activation of the stress-responsive kinases, particularly the JNK/SAPK pathway in mammals (Brown et al., 1996). In Saccharomyces cerevisiae, STE20 was originally identified as essential for pheromone response, where it was placed downstream of the heterotrimeric G-protein and upstream of the MAPKKK STE11 (Leberer et al., 1992; Ramer and Davis, 1993).

A number of kinases have been assigned to the family of STE20 homologues and can be divided into two groups. The first group includes yeast STE20 and the mammalian and Drosophila PAKs and is characterized by a C-terminal catalytic domain and an N-terminal domain with Rac1/Cdc42 binding motifs. The second group has homology within the catalytic domain to STE20, but is more similar in structure to another yeast kinase, SPS1 (Friesen et al., 1994). Kinases in this group have N-terminal catalytic domains and extensive C-terminal regulatory domains and are exemplified by the mammalian homologue GCK (Katz et al., 1994).

Members of the SPS1 subfamily can be divided into two broad groups based on their structure and function (Kyriakis, 1999). The first group contains GCK (Katz et al., 1994) and the closely related kinases GCKR (Shi and Kehrl, 1997), GLK (Diener et al., 1997), HPK1 (Hu et al., 1996; Kiefer et al., 1996) and NIK (Su et al., 1997). These kinases are closely related by phylogenetic analysis, have a C-terminal regulatory region and have been shown to selectively activate the JNK pathway. The second group is comprised of SPS1 (Friesen et al., 1994), SOK-1 (Pombo et al., 1996), MST1/Krs2 (Creasy and Chernoff, 1995a; Taylor et al., 1996), MST2/Krs1 (Creasy and Chernoff, 1995b; Taylor et al., 1996), MST3 (Schinkmann and Blenis, 1997), LOK (Kuramochi et al., 1997) and severin kinase (Eichinger et al., 1998). A number of these kinases have been shown to be activated by environmental stress (Pombo et al., 1996; Taylor et al., 1996). However, with the exception of MST1, these kinases do not appear to regulate any of the known MAPK cascades. Although these proteins share significant homology within their catalytic domains, there are no obvious common binding motifs in their C-terminal regions.

Here we report the identification and characterization of a new member of the STE20/SPS1-related

kinase family. SPAK (STE20/SPS1-related proline alanine-rich kinase) is a ubiquitously expressed serine/threonine kinase, which is physically associated with another, as yet uncharacterized kinase. SPAK specifically activates the p38 pathway. Truncation of the C-terminus of SPAK at a putative caspase cleavage site critically alters the intracellular localization of the protein. These studies suggest that SPAK may act as a novel mediator of stress activated signals in mammals.

Results

Cloning and sequencing of SPAK

In a previous study (DeAizpurua *et al.*, 1997), we sought to identify protein kinases expressed in pancreatic β-cells. One of the sequences detected corresponded to a novel kinase with homology to the STE20 kinase family. Screening of a human brain cDNA library with the cDNA fragment revealed a sequence of 3215 nucleotides with the putative initiation codon represented by the most 5′ ATG occurring within a strong Kozak consensus sequence (Figure 1).

To isolate mouse and rat homologues of SPAK, a mouse brain λ gt11 library and a rat insulinoma RIN-5AH cDNA library were screened with the original rat cDNA fragment and full-length sequences obtained (data not shown). Full-length sequences were submitted to the Genbank database. Mouse and rat SPAK show 95% and 96% amino acid identity to the human sequence, respectively.

SPAK is a STE20-related kinase

Human SPAK encodes a 547 amino acid protein with a predicted molecular mass of 59,641 Da. Using the BLAST program to determine sequence homologies, SPAK was identified as a member of the STE20 family of kinases. The catalytic domain, containing all the conserved features of a serine/threonine kinase, is located at the N-terminus of the protein (Figure 2a), placing SPAK in the SPS1/GCK subfamily of STE20 kinases. The amino terminus of the protein contains a region of proline and alanine repeats (PAPA box). The c-terminus of the protein contains a polybasic sequence (RAKKVRR) which has homology to the canonical nuclear localization signal of the SV40 T antigen. There is no sequence identity with other members of the STE20 family within the C-terminus of SPAK. However, there is a highly acidic region at the junction of the catalytic domain and the C-terminal tail which is also reported in the PAKs, STE20, MST1, MST2 and SOK-1. Within this acidic region there is a putative consensus caspase cleavage recognition motif (DEMD), similar in location and sequence to the motif described in MST1. The SPAK catalytic domain is most similar to that of MST3 (46%) (Schinkmann and Blenis, 1997), SOK-1 (46%) (Pombo *et al.*, 1996), severin kinase (44%) (Eichinger *et al.*, 1998) and MST1 (40%) (Creasy and Chernoff, 1995a), as illustrated by phylogenetic analysis of the catalytic domains of the STE20-related kinases (Figure 2b).

SPAK is ubiquitously expressed

Northern blot analysis using poly(A+)RNA from multiple human tissues demonstrated that the SPAK transcript is ubiquitously expressed. Using a probe specific to a unique region of the SPAK catalytic domain (nucleotides 619–759), a 3.8 kb transcript was identified in all tissues examined, with relatively abundant expression in the brain and pancreas (Figure 3a). To examine the expression of the endogenous SPAK protein, a polyclonal antibody raised to the C-terminus of SPAK was used to immunoblot lysates from various mouse tissues (Figure 3b). This antibody specifically recognized a protein of approximately 60 kDa in all mouse tissues examined.

SPAK is a serine/threonine kinase

Recombinant GST–SPAK phosphorylated itself and also phosphorylated the substrate MBP in an *in vitro* kinase assay (Figure 4a). GST–SPAK fusion protein is purified from bacteria as both a full-length and a proteolytically cleaved product. This cleavage is unaffected by expression of the construct in protease deficient bacteria. A catalytically inactive mutant of SPAK (GST–SPAKK/E) did not autophosphorylate or phosphorylate substrate. The phosphoamino acid analysis of *in vitro* phosphorylated GST–SPAK detected phosphorylated serine and threonine residues only, establishing SPAK as a serine/threonine kinase (Figure 4b).
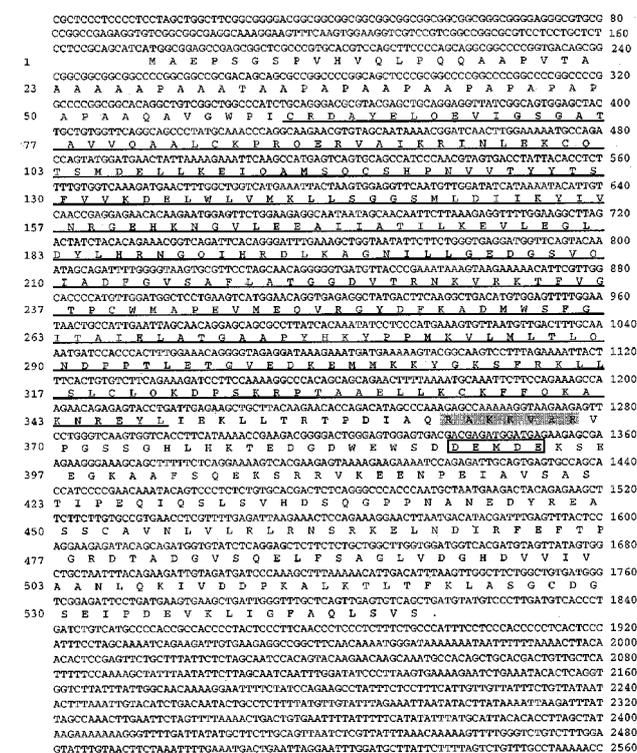
Figure 1 Nucleotide sequence and deduced amino acid sequence of human SPAK. The start codon is indicated in bold. The kinase domain is underlined. Numbering of amino acids and nucleotides is indicated on left and right margins, respectively. The putative caspase cleavage consensus sequence is boxed. The putative nuclear localization signal is shaded

A.

B.
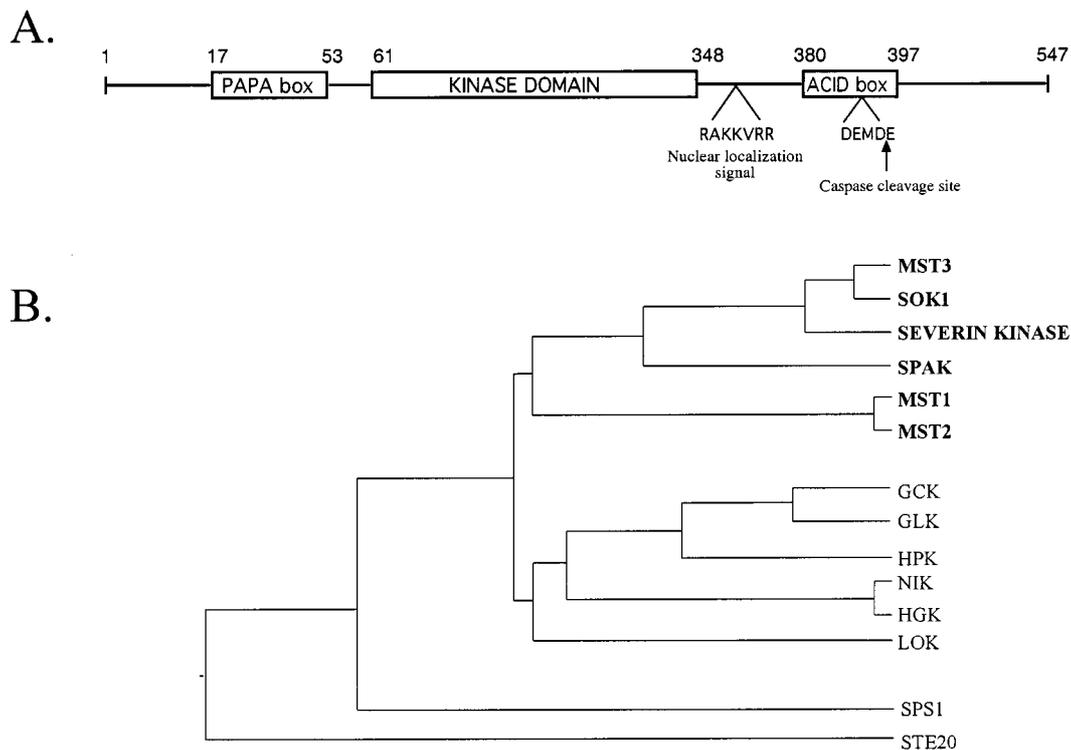
Figure 2 Putative domain structure of SPAK and phylogenetic position. (**a**) Schematic of the structure of human SPAK. The locations of the putative nuclear localization signal and the caspase cleavage site are indicated. (**b**) A multiple sequence alignment of the catalytic domains of the SPS1-related kinases was performed using DNASTAR, and used to construct a phylogenetic tree. Kinases most closely related to SPAK are shown in bold

SPAK is immunoprecipitated as a complex from mammalian cells

Both GFP–SPAK and its catalytically inactive variant GFP–SPAKK/E as well as the substrate MBP are phosphorylated in an *in vitro* kinase assay after transient expression and immunoprecipitation from COS7 cells (Figure 5, left). However, when SPAK and its catalytically inactive variant are purified as GST-fusion proteins from bacteria and included in an *in vitro* kinase assay, GST–SPAK is autophosphorylated and phosphorylates MBP, while GST–SPAKK/E has no catalytic activity (Figure 5, middle). Phosphorylation in an *in vitro* kinase assay of catalytically inactive SPAK after immunoprecipitation from mammalian cells implies that another kinase is co-immunoprecipitated with SPAK. After preincubation with COS7 cell lysate and following extensive high stringency washing, GST-SPAKK/E is phosphorylated in an *in vitro* kinase assay, implying that a kinase present in COS7 cells can physically associate with and phosphorylate SPAK (Figure 5, right). Further, phosphorylation of SPAK by this kinase does not occur on tyrosine residues, as determined by Western blotting with anti-phosphotyrosine antibodies (data not shown).

SPAK activates the p38 MAP kinase pathway

To determine whether SPAK can activate the ERK, p38 or JNK pathways, COS7 cells were co-transfected with mammalian expression vectors encoding wild-type or catalytically inactive SPAK along with HA-tagged ERK2, p38 or JNK2. The MAP kinases were then immunoprecipitated from cell lysates and their activities assayed with the appropriate exogenous substrate. Coexpression of wild-type SPAK (GFP–SPAK), catalytically inactive SPAK (GFP–SPAKK/E) and a C-terminal truncation of SPAK (GFP–SPAKΔC), truncated at the putative caspase cleavage site, induced no increase in the ability of ERK or JNK to phosphorylate substrate. (Figure 6, left and middle). In comparison, expression of catalytically active GFP-SPAK and its truncation mutant GFP-SPAKΔC specifically activated the p38 pathway, as demonstrated by the ability of immunoprecipitated p38 to phosphorylate its substrate ATF2 (Figure 6, right). The level of activation of p38 after coexpression with GFP–SPAK and GFP–SPAKΔC was comparable to that induced by anisomycin treatment. In contrast, no detectable activation of p38 was observed after coexpression of catalytically inactive SPAK.

C-terminally truncated SPAK is expressed in the nucleus

Members of the SPS1 subfamily of STE20 kinases (MST3 and SOK-1) are localized to the cytosol of transfected cells (Pombo *et al.*, 1996; Schinkmann and Blenis, 1997). It has been hypothesized that caspase-mediated cleavage may affect the subcellular localization of another SPS1 kinase, MST1 (Graves *et al.*, 1998). SPAK contains a DEMD^{392}E amino acid sequence that closely resembles the caspase cleavage consensus sequences found in MST1 and MST2 (Figure 7a). To determine if the subcellular distribution of SPAK was affected by the loss of the region C-terminal to the putative caspase cleavage site, COS7

A.

B.
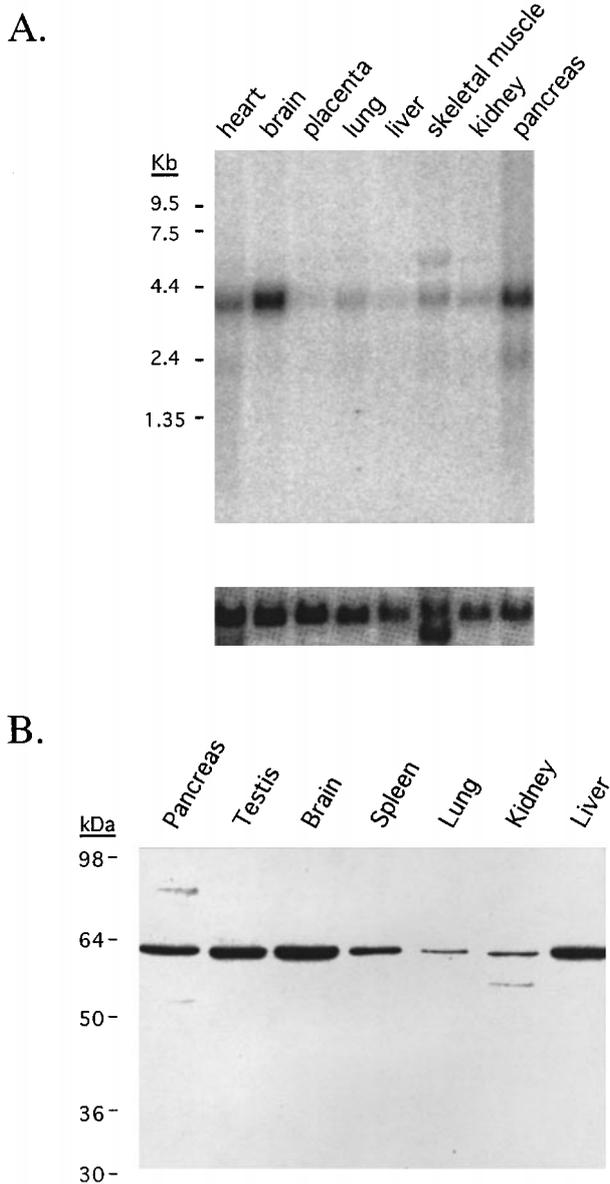
Figure 3 Expression of SPAK. (**a**) Northern blot analysis of SPAK mRNA. Poly(A+)RNA from human tissues was probed with a radiolabelled-SPAK cDNA fragment (nucleotides 619–759) (top). RNA size markers are shown on the left. The same membrane was probed with radiolabelled β-actin cDNA (bottom) (**b**) Western blot analysis of endogenous SPAK protein in mouse tissues. Lysate from the indicated mouse tissues (50 μg) was resolved by SDS–PAGE and immunoblotted with antibodies raised to the C-terminus of SPAK

Figure 4 SPAK is a serine/threonine kinase. (**a**) Kinase assays were performed with equal amounts of wild-type GST–SPAK, catalytically inactive GST–SPAKK/E and GST using MBP as substrate. A Coomassie-stained SDS-gel shows expression levels of GST-fusion proteins (lower panel) (**b**) Phosphoamino acid analysis of *in vitro* phosphorylated SPAK. The positions of phosphorylated serine, threonine and tyrosine are indicated by arrows
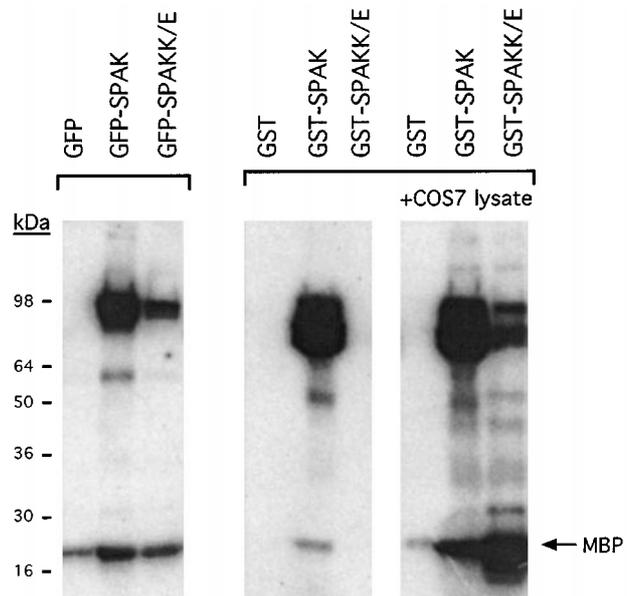
Figure 5 SPAK is physically associated with another kinase in mammalian cells. *In vitro* kinase assays were performed using MBP as substrate with immune complexes precipitated from COS7 cells transfected with GFP, GFP-SPAK, GFP-SPAKK/E (left). Identical assays were performed with equal amounts of GST, GST-SPAK and GST-SPAKK/E purified on glutathione-Sepharose either alone (middle), or after preincubation with lysate from COS7 cells (right)

cells were transiently transfected with the GFP vector, with GFP–SPAK or with the equivalent of caspase-cleaved SPAK, GFP–SPAKΔC. When examined by fluorescence microscopy after 48 h, cells transfected with wild-type GFP displayed a bright green fluorescence distributed throughout the cell (Figure 7b, left). In contrast, GFP-SPAK was localized predominantly in the cytosol of transfected cells (Figure 7b, middle), whereas GFP–SPAKΔC was localized predominantly in the nucleus (Figure 7b, right). We have obtained the identical result with transiently transfected FLAG-tagged constructs and also in stably transfected RIN–5AH cells (data not shown). To confirm this finding, transfected cells were lysed to obtain a cytosolic fraction, a nuclear fraction, and a particulate fraction.

GFP–SPAKΔC was detected in the nuclear, as well as cytosolic and particulate fractions, whereas GFP–SPAK was detected only in the cytosolic fraction (Figure 7c).
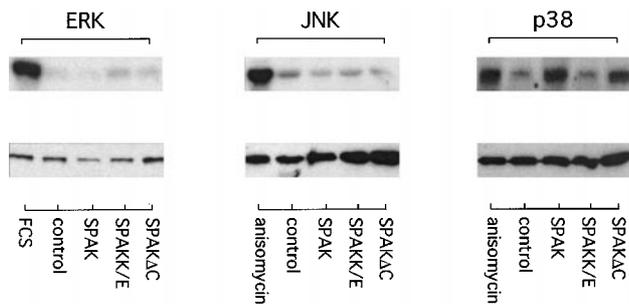
Figure 6 SPAK activates the p38 pathway in COS7 cells. COS7 cells were transfected with either empty vector, wild-type SPAK (SPAK), catalytically inactive SPAK (SPAKK/E) or truncated SPAK (SPAKΔC) in addition to epitope-tagged ERK2, JNK2 or p38 MAP kinases. Kinase activities were determined by immune complex kinase assays with the appropriate substrates as described in 'Materials and methods' (upper panels). The effects of 10% FCS on the activity of ERK2 and of 1 μg/ml anisomycin on the activity of JNK2 and p38 are shown for comparison. Western blotting with anti-epitope antibodies shows expression levels of the MAPKs (lower panels)

Discussion

We have cloned and characterized a kinase, designated SPAK (STE20/SPS1-related proline-alanine rich kinase) that belongs to the SPS1 subfamily of STE20 kinases. SPAK sequences are highly conserved between mouse, rat and human. The rat sequence has recently been published elsewhere (Ushiro *et al.*, 1998). SPAK shares sequence homology with members of the SPS1 subfamily of kinases, which includes MST1, MST2, MST3, SOK-1 and severin kinase. SPAK lacks the Cdc42/Rac1 binding motifs found in the PAK family of kinases, as well as the PEST motifs and distinctive C-terminal domain of some of the other STE20 family members.

SPAK is most similar in sequence to MST3, but the degree of similarity between SPAK and members of the SPS1 subfamily is reduced due to two intriguing features. First, the catalytic domain of SPAK contains several amino acid insertions which render it larger

A.
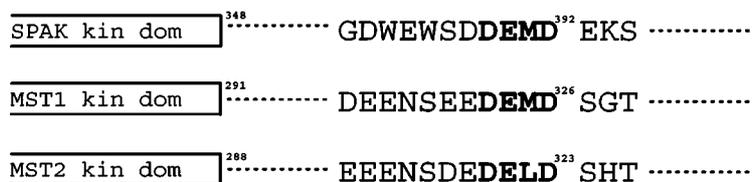
B.
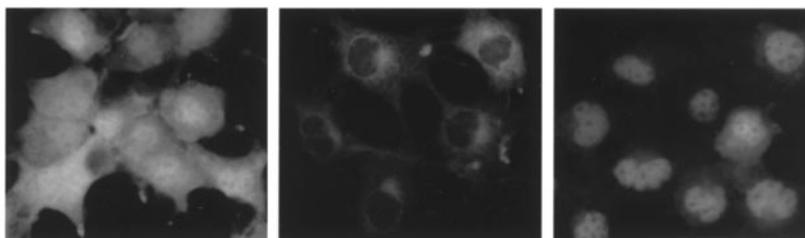
C.
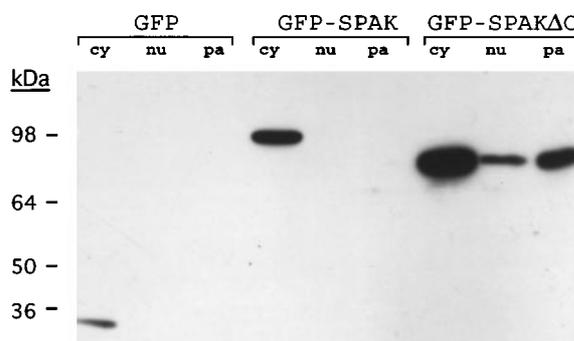
Figure 7 Subcellular localization of SPAK (**a**) Alignment of the predicted caspase cleavage consensus sequence (in bold) of SPAK with those of MST1 and MST2. (**b**) COS7 cells were transiently transfected with GFP fusion proteins encoding wild-type GFP (left), GFP–SPAK (middle) or a C-terminal truncation of SPAK, GFP–SPAKΔC (right). Forty-eight hours after transfection, cells grown on coverslips were fixed and mounted in aqueous mounting medium, and GFP localization visualized with a fluorescence microscope. (**c**) 48 h after transfection with GFP, GFP–SPAK or GFP–SPAKΔC, cells were harvested and fractionated, as described in 'Materials and methods', to yield cytoplasmic (cy), nuclear (nu) and particulate fractions (pa). Lysates were separated by 10% SDS–PAGE and immunoblotted with a polyclonal anti-GFP antibody

than the kinase domain of other SPS1 kinases. Second, the catalytic domain of SPAK is uncharacteristically distant from the N-terminus of the protein (61 amino acids compared to 19 amino acids for MST3 and 15 amino acids for SOK-1), due to the presence of a region of proline and alanine repeats (PAPA box). Such a region is found in a number of other proteins, including β1 crystallin (Hejtmancik et al., 1986), E-MAP-115 (Masson and Kreis, 1993), myosin light chain kinase (Frank and Weeds, 1974) and p57 (KIP2) (Lee et al., 1995), as well as the 'orphan' MAPK ERK5 (Zhou et al., 1995), but is not found within any other STE20 family members. The proline-alanine repeats of myosin light chain kinase target the kinase to a specific subcellular location by facilitating a direct interaction with actin (Williamson, 1994). Accordingly, the PAPA box may allow SPAK to take part in intermolecular interactions with cytoskeletal structures such as actin, or actin-like proteins.

Our in vitro phosphorylation studies show that wild-type SPAK can phosphorylate the substrate MBP in immune complex kinase assays. Interestingly, when expressed as a GFP-fusion protein in COS7 cells, SPAK is immunoprecipitated as a complex with another, tightly associated kinase. This unidentified kinase phosphorylates MBP more efficiently than SPAK, and is capable of phosphorylating catalytically-inactive SPAK. Catalytically-inactive mutants missing the PAPA box and C-terminal region display activity towards MBP (data not shown), consistent with the coprecipitation of another kinase which physically associates with the catalytic domain of SPAK. The consequences of the association and subsequent phosphorylation of SPAK by the co-associated kinase remain to be clarified.

SPS1 family members most closely related to GCK activate the mammalian JNK pathway. Activation of the known MAP kinase pathways by the other SPS1 family members, with the exception of MST1, has not been demonstrated and it is presumed that these kinases act in novel stress response pathways. However, overexpression of MST1 in 293T cells has been shown to activate the p38 and JNK kinase pathways (Graves et al., 1998). Our co-expression data indicate that like MST1, SPAK and its truncation mutant are capable of activating the p38 pathway when expressed in COS7 cells. However, we could not demonstrate activation of the JNK pathway by either wild-type SPAK or C-terminally truncated SPAK. This is the first report of a SPS1-related kinase which specifically activates the p38 pathway, and implicates this kinase in cellular responses to stress.

One of the cellular responses to stress is the induction of apoptosis. Activation of the JNK and p38 MAPK pathways have been shown to correlate with apoptosis (Graves et al., 1996; Wilson et al., 1996; Xia et al., 1995) and conversely, caspase inhibitors can block activation of the stress-activated protein kinase pathways by apoptosis-inducing agents (Cahill et al., 1996; Juo et al., 1997). The activation of caspases is central to the apoptotic pathway (Nagata, 1997) and leads to the cleavage of a variety of cellular substrates. The SPS1 kinases MST1 and MST2 are cleaved and activated by caspase-3 during apoptosis and it has been suggested that caspase cleavage of these proteins may affect their subcellular localization (Graves et al., 1998;

Lee et al., 1998). Caspase cleavage sites in both MST1 and MST2 are located at the junction of the catalytic domain and the C-terminal regulatory domain. Rat, mouse and human SPAK all contain a DEMD amino acid sequence that closely resemble the caspase cleavage consensus sequences found in MST1 and MST2, as well as in other caspase-3 substrates. As is the case for MST1 and MST2, this putative caspase cleavage site juxtaposes the kinase domain and is located within an acidic-rich region. We show that a C-terminal truncation mutant (GFP–SPAKΔC), equivalent to the putative caspase-3 cleavage product of SPAK, is expressed predominantly in the nucleus, while wild-type SPAK is cytosolic. Further, SPAK contains a putative nuclear localization signal, RAKKVRR, which has homology with the canonical nuclear localization signal of SV40 T antigen (reviewed in Boulikas, 1993). These data raise the possibility that endogenous SPAK may be similarly capable of translocation to the nucleus. Such translocation may occur through unmasking of the nuclear localization signal by caspase-mediated cleavage, or by conformational changes mediated by phosphorylation events. Changes in subcellular localization may provide a means of regulating the activity of SPAK, whereby translocation to the nucleus may expose the kinase to specific substrates, which the cytosolically expressed, full-length protein cannot access.

The SPS1-related kinases are involved in the response of cells to environmental stress. MST1 and MST2 are activated by heat shock as well as by treatment of cells with high concentrations of staurosporine, okadaic acid, or sodium arsenite (Taylor et al., 1996), while SOK-1 is strongly activated by oxidative stress (Pombo et al., 1996). Conditions that activate SPAK are not yet known. However, the similarity of SPAK with other SPS1 family members, its putative caspase cleavage site and its ability to activate the p38 MAPK pathway, suggest that it is also involved in stress responses. Understanding how SPAK interacts with other signaling pathway components may shed light on the mechanisms by which cells respond to stress.

Materials and methods

cDNA cloning and sequencing

Degenerate oligonucleotide primers corresponding to conserved regions VIb and IX within the protein kinase catalytic domain and modified to include BamHI and EcoRI restriction sites respectively, were used to amplify 200–210 bp kinase specific DNA fragments from RNA of cultured rat insulinoma RIN 5AH cells, as described previously (DeAizpurua et al., 1997). Fragments were cloned into the puc19 plasmid (Promega) and sequenced by dideoxy terminator sequencing using a 320A sequenator (Applied Biosystems). The BLAST algorithm was used to scan databases for protein and DNA homologies. One clone which encoded a kinase domain fragment with homology to the STE20 family of kinases was used to screen a human brain 5'-STRETCH PLUS cDNA library (Clontech). Following purification, positive clones were sequenced as above using primers generated to 5' and 3' flanking regions of the vector. To isolate mouse and rat homologues of SPAK, a mouse brain λ gt11 library and a rat insulinoma RIN–5AH phagemid library in λZAP Express (Stratagene) were screened

with the original rat cDNA fragment and full-length sequences were obtained.

Northern blot analysis of SPAK

A human multiple tissue Northern blot (Clontech) was probed with a ^{32}P-labelled cDNA probe corresponding to a unique region within the SPAK catalytic domain, encompassing nucleotides 619 to 759. Hybridization was performed at 68°C in ExpressHyb Hybridization Solution (Clontech), as per the manufacturer's instructions. Blots were exposed for 24 h at −70°C.

Plasmids

Full-length SPAK was cloned into the eukaryotic expression vector pEGFP–C1 (Clontech) to generate N-terminally tagged GFP–SPAK. Catalytically inactive GFP–SPAK (GFP–SPAKK/E) was generated by mutating a conserved lysine (K^{94} for human SPAK, K^{101} for rat SPAK) in the catalytic domain to a glutamic acid using site-directed polymerase chain reaction-based mutagenesis. For the generation of C-terminally truncated SPAK (GFP–SPAKΔC), a fragment encompassing the catalytic domain and the putative caspase cleavage site was amplified by PCR using oligonucleotide primers 5′-tacgagctccaggaggtta-3′ and 5′-gagtgatgatgagatggat-3′. The blunt-ended fragment was then digested with *Kpn*I and inserted into GFP–SPAK digested with *Kpn*I and *Sma*I. Full-length and catalytically-inactive SPAK were cloned in frame into pGEX-3T (Pharmacia Biotech Inc) to create GST–SPAK and GST–SPAKK/E. GST-c-*jun* and HA-tagged expression constructs ERK2, JNK2 and p38 were kindly provided by Dr Donna Dorow (Peter MacCallum Research Institute, Melbourne, Australia).

Cell culture and transfection

RIN-5AH cells were cultured in RPMI-1640 medium supplemented with 10% FCS and antibiotics. COS7 cells were maintained in Dulbecco's modified Eagles medium with 10% FCS and antibiotics. COS7 cells plated at a density of 1×10^5 per 35 mM well were transfected within 24 h of plating with 1 μg of DNA using FuGENE (Roche), according to the manufacturer's instructions.

GST fusion protein purification, in vitro kinase assays

For GST fusion protein purification, bacterial expression constructs GST–SPAK and GST–SPAKK/E were used to transform competent *E. coli*. GST fusion proteins were produced by inducing 500 ml log phase cultures with 1 mM IPTG, followed by lysis of bacteria by sonication. Following centrifugation, lysates were incubated with glutathione-Sepharose beads (Pharmacia) according to the manufacturer's specifications. For immunoprecipitations from mammalian cells, cells were lysed 48 h after transfection in Triton X-100 lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl$_2$, 1 mM EGTA) supplemented with 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM PMSF, 1 μg/ml aprotinin and 5 mM DTT. Lysates were clarified by centrifugation at 12 000 *g* for 10 min at 4°C. Pre-cleared lysates from transiently transfected cells were incubated with 5 μl of anti-GFP antibody (Clontech) or anti-HA (12CA5) antibody for 2 h at 4°C. Protein-G Sepharose beads (Pharmacia) were added and the mixture was incubated for an additional 45 min at 4°C. For *in vitro* kinase assays, proteins immobilized on Sepharose were washed three times with kinase buffer (25 mM HEPES, pH 7.5, 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 5 mM MnCl$_2$, 1 mM DTT, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 mM PMSF, and 1 μg/ml aprotinin). Sepharose beads were resuspended in 30 μl kinase buffer containing

20 μM ATP, 1 μg of the indicated substrate and 1 μCi of γ-^{32}P-ATP and incubated for 20 min at 30°C. For MAPK activation assays, MBP (Sigma) was used as a substrate for ERK2, ATF-2 (Santa Cruz) for p38, and GST-c-*jun* for JNK2. The reaction was terminated by addition of reducing SDS sample buffer and resolved by SDS–PAGE on a 10–20% Tris-glycine gel. Phosphorylated products were visualized by autoradiography. For detection of the SPAK-binding kinase, GST, GST–SPAK and GST–SPAKK/E proteins immobilized on glutathione-Sepharose were preincubated with lysate from 10^6 COS7 cells for 30 min at 4°C, washed three times with lysis buffer, three times with lysis buffer containing 500 mM NaCl, and three times with kinase buffer. Kinase assays were performed as described.

Antibody production

To generate polyclonal antibodies to the C-terminus of SPAK (amino acids 507–547), purified GST fusion proteins were eluted from glutathione Sepharose beads by addition of 50 mM Tris-HCl (pH 8.0)/5 mM reduced glutathione. GST-fusion protein (0.5 mg) was emulsified with Freund's complete adjuvant and injected into two individual rabbits at multiple intramuscular sites. Following two booster immunizations 6 weeks and then 2 weeks after the primary inoculation, rabbits were bled, serum collected and stored at −20°C. Serum was tested for SPAK-specific antibodies after each of the booster immunizations by testing for reactivity against immunoprecipitated GFP-tagged SPAK by Western blot analysis.

Western blot analysis

For Western blot analysis, proteins from cell lysates or immunoprecipitations were resolved by SDS–PAGE, electroblotted onto nitrocellulose membrane (Schleicher and Schuell) and blocked overnight at 4°C in 1% skim milk, 1% bovine serum albumin and 0.05% Tween-20 in TBS, pH 7.4. Membranes were incubated with anti-GFP (Clontech), anti-HA (12CA5) or anti-pTyr (UBI) for 1 h at room temperature. Following four washes in TBS/0.05% Tween-20, anti-rabbit (for anti-GFP, anti-HA) or anti-mouse (for anti-pTyr) HRP-immunoglobulin was added and incubated for 45 min. After washing, blots were developed using Lumi-light Western Blotting substrate (Roche Molecular Biochemicals).

Subcellular localization

For cell fractionation, transiently transfected cells were lysed in Triton X-100 buffer and clarified by centrifugation at 12 000 *g* and the supernatant retained as the cytoplasmic fraction. The pellet was resuspended in lysis buffer with 500 mM NaCl to lyse nuclear membranes. After clarification of the nuclear lysate, the pellet was dissolved in SDS sample buffer to give the particulate fraction. Lysates were separated by SDS–PAGE, transferred to nitrocellulose and membranes were immunoblotted with a polyclonal anti-GFP antibody. To visualize intracellular SPAK localization, cells were grown on coverslips and transfected as described with GFP, GFP–SPAK and GFP–SPAKΔC. Cells were fixed with 4% paraformaldehyde 48 h after transfection, mounted on slides and GFP fluorescence was visualised using a fluorescence microscope (Zeiss).

Phosphoamino acid analysis

Phosphorylated SPAK was resolved using a 10% SDS-polyacrylamide gel, and transferred to PVDF membrane. After autoradiography, radioactive bands were excised. The PVDF membrane was soaked in methanol, then distilled water. Proteins were hydrolysed in 6 N HCl for 1 h at 110°C.

The acid containing the hydrolysed phosphoamino acids was lyophilized, then washed with distilled water. The dried pellets were resuspended in 5.9% glacial acetic acid, 0.8% formic acid, 0.3% pyridine and 0.3 mM EDTA, pH 2.5, with a tracer amount of phosphorylated serine, threonine and tyrosine standards, and spotted onto a TLC plate, then separated at 20 mA for 2 h. The standards were visualized by spraying with 0.3% ninhydrin in acetone. The labelled residues were detected by autoradiography.

Abbreviations

MAP kinase, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N terminal kinase; MAPKK, mitogen activated protein kinase kinase; MAPKKK, mitogen activated protein kinase kinase kinase; PAK, p21-activated kinase; GCK, germinal center kinase; GCKR, germinal center kinase-related; GLK, GCK-like kinase; HPK1, hematopoietic progenitor kinase 1; NIK, NCK-interacting kinase; SOK-1, STE20/ oxidant stress responsive kinase-1; MST, mammalian sterile 20-like; LOK, lymphocyte oriented kinase; SPAK, STE20/SPS1-related proline alanine-rich kinase; RIN, rat insulinoma; BLAST, basic local alignment search tool; GFP, green fluorescent protein; PCR, polymerase chain reaction; GST, glutathione S-transferase; HA, hemagglutinin; FCS, fetal calf serum; IPTG, isopropyl-B-D-thiogalactoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MBP, myelin basic protein; HRP, horseradish peroxidase; TBS, tris-buffered saline; PVDF, polyvinylidene difluoride.

Accession numbers

The nucleotide sequences reported in this paper have been submitted to the GenBank™/EBI Data Bank with accession numbers AF099988, AF099990 and AF099989 for mouse, rat and human SPAK, respectively.

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