Prostate-derived Sterile 20-like Kinase 2 (PSK2) Regulates Apoptotic Morphology via C-Jun N-terminal Kinase and Rho Kinase-1*

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We have reported previously that human prostate-derived sterile 20-like kinase (PSK) 1 alters actin cytoskeletal organization and binds to microtubules, regulating their organization and stability. We have shown a structurally related protein kinase PSK2, which lacks a microtubule-binding site, activated c-Jun N-terminal kinase (JNK), and induced apoptotic morphological changes that include cell contraction, membrane blebbing, and apoptotic body formation. Apoptotic stimuli increased the catalytic activity of endogenous PSK2 and JNK, and dominant negative JNK or a physiological inhibitor of JNK blocked these apoptotic morphological responses to PSK2, demonstrating a requirement for JNK. PSK2 also stimulated the cleavage of Rho kinase-1 (ROCK-I), and the activity of ROCK-I was required for PSK2 to induce cell contraction and membrane blebbing. The activation of caspases was also needed for the induction of membrane blebbing by PSK2, which was itself a substrate for caspase 3. PSK2 therefore regulates apoptotic morphology associated with the execution phase of apoptosis, which involves dynamic reorganization of the actin cytoskeleton, via downstream targets that include JNK and ROCK-I. Our findings suggest that PSKs form a subgroup of sterile 20 (STE20)-like kinases that regulate different cytoskeletal processes.

Prostate-derived sterile 20-like kinase 2 (PSK2)3 is a member of a family of more than 30 mammalian sterile 20 (STE20)-like kinases, which regulate a diverse array of processes that include gene transcription, stress responses, cytoskeletal organization, and apoptosis (1). STE20s have been divided into the p21-activated kinases (PAKs) or the germinal-center kinase (GCK)-like kinases according to their structure and function, and PSK2 belongs to the GCK-like class of STE20s that possess an N-terminal catalytic domain and a putative C-terminal regulatory domain (1). Most STE20s can act upstream of the mitogen-activated protein kinase (MAPK) signaling pathways and regulate different cytoskeletal processes.

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3 The abbreviations used are: PSK, prostate-derived sterile 20-like kinase; STE20, sterile 20; ROCK-I, Rho kinase-1; MAP, mitogen-activated protein; MAPK, MAP kinase; GCK, germinal center kinase; PAK, p21-activated kinase; JNK, C-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MBP, myelin basic protein; MST, mammalian STE20-like kinase; Z, benzoyloxycarbonyl; FMK, fluoromethyl ketone; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate.

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caspase activity is required for the induction of membrane blebbing by PSK2, which is itself a substrate for caspase 3.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—PRK5-Myc-PSK2 was prepared by PCR using the human cDNA clone KIAA1361 (gift from Takahiro Nagase, Kazusa DNA Research Institute) and appropriate oligonucleotides. PRK5-Myc-PSK2 (1–416) was prepared by PCR from this construct, and pRK5-Myc-PSK2 (K57A) and pRK5-PSK2 (D376N) were prepared from pRK5-Myc-PSK2 using the QuickChange mutagenesis kit (Stratagene). Colleagues donated the following plasmids: pCMV-FLAG-JNK1 (APF) (Dr. R. Davis, University of Massachusetts), pGEX-c-Jun (Dr. J. Ham, Eisai London Research Laboratories), and dominant negative pCDNA3-FLAG-JNK1 (APF) (Dr. R. Davis, University of Massachusetts). The caspase inhibitor Z-VAD-FMK and inhibitors for ROCK-I (23, 24) were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-caspase 3 and mouse anti-ROCK-I (G6) antibody was obtained as described previously (24). Mouse anti-ROCK-I (G6) antibody was obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-caspase 3 and mouse anti-PSK2/TAO1 were obtained from AbCam and BD Transduction Laboratories.

Immunoblotting—Cultures were lysed in 200 μl of lysis buffer (1% Nonidet P-40, 130 mM NaCl, 1 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM NaF, 0.1 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris, pH 7.4). 100 μg of total protein were mixed with Laemmli denaturing buffer, separated by SDS-PAGE (8–15%), and transferred to nitrocellulose. Immunoblotting was carried out as described previously (24). Mouse anti-ROCK-I (G6) antibody was obtained from Sigma. Rabbit anti-caspase 3 and mouse anti-PSK2/TAO1 were obtained from Calbiochem. The p38 inhibitor SB202190 was obtained from Sigma.

Transient Cell Expression—COS-1 cells and human non-small cell lung carcinoma cells (H1299) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (10% CO2, 37 °C). For transfection, 4 × 105 or 8 × 105 cells were seeded onto 35- or 60-mm Petri dishes, respectively. After 16 h, the indicated plasmids (1.5 or 2.5 μg of DNA) were transfected into COS-1 cells or H1299 cells using Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen).

Immunoprecipitation and in Vitro Kinase Assays—For immunoprecipitation, 400 μg of protein were made up to a final volume of 1 ml in binding buffer (130 mM NaCl, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM NaF, 0.1 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris, pH 7.4). 100 μg of total protein were mixed with Laemmli denaturing buffer, separated by SDS-PAGE (8–15%), and transferred to nitrocellulose. Immunoblotting was carried out as described previously (24). Mouse anti-ROCK-I (G6) antibody was obtained from Sigma. Rabbit anti-caspase 3 and mouse anti-PSK2/TAO1 were obtained from AbCam and BD Transduction Laboratories, respectively.

Immunoprecipitation and in vitro Kinase Assays—For immunoprecipitation, 400 μg of protein were made up to a final volume of 1 ml in binding buffer (130 mM NaCl, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM NaF, 0.1 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl, pH 7.6). Samples were incubated with 3 μg of anti-Myc 9E10 mouse monoclonal antibody (Sigma), 3 μg of anti-FLAG mouse M2 monoclonal antibody (Sigma), or 2 μg of anti-PSK2 mouse monoclonal antibody for 1 h at 4 °C before the addition of 100 μl of 10% (v/v) protein G-Sepharose beads (Sigma, 1 h at 4 °C).

For in vitro kinase assays, beads were washed three times, pelleted by centrifugation, and placed in 30 μl of kinase buffer (20 mM MgCl2, 2 mM MnCl2, 30 mM Tris-HCl, pH 7.6) containing 20 μM ATP, 5 μCi of [γ-32P]ATP (MP Biomedicals, 3000 Ci/mmole), and 1 μg of glutathione S-transferase-c-Jun protein or 1 μg of myelin basic protein added where indicated and incubated for 45 min at 37 °C. Kinase assays were terminated in gel sample buffer, and proteins were separated by SDS-PAGE for transfer to nitrocellulose and analysis by immunoblotting or phosphorimaging (Fuji FLA-2000). Protein normalization and expression were confirmed for each sample by immunoblotting total cell lysates.

Immunofluorescence and Confocal Microscopy—H1299 cells (2 × 105) were seeded onto 22-mm glass coverslips (VWR Scientific Products) in 30-mm Petri dishes and transfected with the indicated plasmids as described above. At the appropriate times, cultures were washed with phosphate-buffered saline (PBS) and fixed for 20 min in PBS containing 3.7% formaldehyde for 15 min at room temperature, and cells were permeabilized with 0.2% Triton X-100 in PBS. To detect Myc-tagged PSKs, cells were incubated with either 1:400 anti-Myc rabbit polyclonal antibody or 1:400 mouse 9E10 antibody (Santa Cruz Biotechnology, Inc.) in blocking buffer (PBS containing 20% goat serum, Sigma) for 1 h at room temperature followed by 1:400 FITC-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch) in blocking buffer. To detect FLAG-tagged JNK, cells were incubated with 1:200 anti-FLAG mouse M2 monoclonal antibody in blocking buffer (1 h at room temperature) followed by 1:400 Alexa Fluor 633-conjugated goat anti-mouse IgG (Molecular Probes) in blocking buffer. To detect polymerized actin (F-actin), cells were incubated with 0.1 μg/ml TRITC-conjugated phallolidin (Sigma) in PBS. Cells were imaged with a Bio-Rad MRC-1024 confocal laser-scanning microscope. The percentages of transfected cells undergoing cell contraction or membrane blebbing were determined by examining more than 100 cells in triplicate using confocal microscopy.

In Vitro Caspase Cleavage Assays—PSK2 was subcloned into pcDNA4-His (Invitrogen) and [35S]methionine-labeled PSK2 synthesized using an in vitro transcription and translation kit according to the manufacturer’s instructions (Promega). Labeled His-tagged PSK2 in binding buffer (130 mM NaCl, 2 μg/ml aprotinin, 0.1 mM EDTA, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 20 mM Tris, pH 7.4) was immunoprecipitated from the reaction mixture using mouse anti-His monoclonal antibody and protein G-Sepharose (Sigma). PSK2 attached to beads was washed with binding buffer lacking protease inhibitors and incubated in 50 μl of caspase reaction buffer (100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 20 mM PIPES, pH 7.2) for 60 min at 37 °C. Recombinant caspase 3 (100 ng, BD Transduction Laboratories) and Z-VAD-FMK (50 μM) were added where indicated. Reactions were terminated with sample buffer, and proteins were separated by SDS-PAGE (10%) and transferred to nitrocellulose for analysis using a phosphorimaging device (Fuji).

RESULTS

PSK2 Activates JNK—We have shown previously that the GCK-like kinase PSK1 activates the JNK MAPK signaling pathway via its N-terminal catalytic domain and contains a C-terminal microtubule-binding and regulatory domain (23, 24). PSK2 is highly homologous to PSK1 over its N-terminal kinase domain but does not possess a microtubule-binding domain, and we therefore set out to determine whether this protein could also activate JNK (Fig. 1, A and B). An expression vector for Myc-tagged PSK2 was prepared from the human cDNA clone KIAA1361 (Kazusa DNA Research Institute) as well as an additional expression construct for its kinase-defective form PSK2 (K57A). Myc-tagged PSK2 or PSK2 (K57A) were expressed in COS-1 cells and immune complexes of each protein prepared from cell lysates. In vitro kinase assays were carried out using the immunoprecipitates and myelin basic protein (MBP) as a substrate, and PSK2 underwent autophosphorylation and phosphorylated MBP (Fig. 1C). In contrast, immune complexes prepared from cells that expressed its kinase-defective counterpart, PSK2 (K57A), showed that this protein was unable to phosphorylate itself or MBP due to the lack of a crucial lysine residue in the second kinase subdomain that is required for ATP binding (Fig. 1C). PSK2 is therefore a functional kinase, and so we examined the effects of these proteins on the JNK signaling pathway. Myc-tagged PSK2 or PSK2 (K57A) were co-transfected with FLAG-tagged JNK into COS-1 cells and immune complexes of JNK prepared from cell lysates for in vitro kinase assays using recombinant c-Jun as a substrate. Transfected PSK2 activated JNK and stimulated the phosphorylation of c-Jun, whereas PSK2 (K57A) was unable to increase the levels of phosphorylated c-Jun above those obtained in control cells transfected with vector alone (Fig. 1D). We also expressed the
N-terminal kinase domain of PSK2-(1–416) in cells and found that this protein could also activate JNK (data not shown).

PSK2 Induces Cell Contraction, Membrane Blebbing, and Cleavage of ROCK-I—Since JNK activation has been shown to play important roles in the regulation of apoptosis and some STE20-like kinases can regulate cytoskeletal organization, we examined whether PSK2 could stimulate changes in cell morphology that are associated with apoptosis (2, 24–26). Human non-small cell lung carcinoma cells (H1299) were transfected with PSK2, and the expression of this protein induced a marked reduction in the cell spread area and caused cell rounding in 70.5 ± 10.8% of transfected cells. Cell contraction correlated with a reorganization of actin into a cortical membrane-associated ring, which we observed in contracted cells. Contraction of this ring results in blebbing, and PSK2 induced substantial blebbing of the plasma membrane in 64.3 ± 10.1% of transfected cells (Fig. 2, A and B). Membrane blebbing is finally followed by dynamic protrusion and retraction of the plasma membrane, resulting in condensation of the cell into apoptotic bodies correlating with dissolution of polymerized actin. We observed apoptotic body formation in 36 ± 10.4% of transfected cells (Fig. 2, A and B). In contrast, kinase-defective PSK2 (K57A) was diffusely spread throughout the cytoplasm, as was wild type PSK2, before the onset of apoptotic morphological changes (data not shown) and caused no significant reduction in spread area or membrane blebbing (Fig. 2, A and B). These results demonstrate a requirement for the catalytic activity of PSK2 to induce these apoptotic morphological alterations.

The actin-myosin system is thought to provide the contractile force that drives the formation of membrane blebs. Cleavage and activation of the Rho effector ROCK-I by caspase 3 appears to be a crucial event in the formation of blebs in apoptotic cells (19, 20). To test whether PSK2 could cause cleavage of ROCK-I, we expressed PSK2 or PSK2 (K57A) in cells and examined their effects on endogenous ROCK-I. Immunoblotting of cell lysates with an antibody raised against the C terminus of ROCK-1 (amino acids 755–840) detected full-length ROCK-I at 160 kDa, and we observed cleavage of ROCK-I at p160 and p130 in the presence of PSK2 (K57A) (Fig. 2, C). Membrane blebbing is finally followed by dynamic protrusion and retraction of the plasma membrane, resulting in condensation of the cell into apoptotic bodies correlating with dissolution of polymerized actin. We observed apoptotic body formation in 36 ± 10.4% of transfected cells (Fig. 2, A and B). In contrast, kinase-defective PSK2 (K57A) was diffusely spread throughout the cytoplasm, as was wild type PSK2, before the onset of apoptotic morphological changes (data not shown) and caused no significant reduction in spread area or membrane blebbing (Fig. 2, A and B). These results demonstrate a requirement for the catalytic activity of PSK2 to induce these apoptotic morphological alterations.

FIGURE 1. A, diagram illustrating regions of homology between human PSK2 and PSK1. MT, microtubule; KD, kinase domain. B, the PSK2 locus. PSK2 is generated from a single transcript on chromosome 17 (GenBankTM ID 57551; locus tag, HGCN, 29259; location, 17q11.2), comprising 20 exons with the start codon located on exon 2. UTR, untranslated region. C and D, PSK2 has in vitro kinase activity and stimulates JNK. Growing COS1 cells were transfected with pRK5-Myc vector, pRK5-Myc-PSK2, or pRK5-Myc-PSK2 (K57A) with (D) or without (C) CMV-FLAG-JNK. After 48 h, Myc-tagged PSKs (C) or FLAG-tagged JNK (D) were immunoprecipitated from cell lysates and taken for in vitro kinase assays using MBP (C) or c-Jun (D) as the substrate. MBP phosphorylated by PSKs or c-Jun phosphorylated by JNK was detected using a phosphorimaging device. The expression of Myc-PSKs or FLAG-JNK was confirmed by immunoblotting total cell lysates.

FIGURE 2. PSK2 induces cell contraction, membrane blebbing and cleavage of ROCK-I. A, growing H1299 lung carcinoma cells were transfected with pRK5-Myc-PSK2 or pRK5-Myc-PSK2 (K57A), and after 36 h, cultures were fixed and co-stained with anti-Myc 9E10/FITC (left column) and TRITC-phalloidin (red) (right column). Yellow arrowheads denote membrane bleb morphology, and green arrowheads indicate apoptotic bodies. Red arrowheads highlight cortical actin in contracted cells. Scale bar, 20 μm. B, the percentages of transfected cells undergoing cell contraction (black bars) or membrane blebbing (white bars) after transfection of the indicated plasmids were determined after examining more than 100 cells in each of three independent experiments using confocal microscopy. The percentages of transfected cells with apoptotic body morphology are also shown for PSK 2 (gray bars). Standard deviations are shown. GFP, green fluorescent protein. C, growing H1299 cells were transfected as indicated, and after 30 h, total cell lysates were immunoblotted with anti-Myc-PSK2 or anti-ROCK-I antibodies.
kDa in control cells as well as an additional N-terminal fragment of ROCK-I at 130 kDa in cells that were transfected with PSK2 (Fig. 2C). In contrast, expression of PSK2 (K57A) did not cause a significant increase in the 130-kDa form of ROCK-I, consistent with the inability of this mutated protein to induce blebbing or contraction of cells (Fig. 2C).

Since the cleavage of ROCK-I has been shown to result in its activation, we also tested whether the cell-permeable ROCK-I inhibitor Y27632 could affect the apoptotic morphological response of cells to PSK2 (19, 20). We found that Y27632 prevented blebbing and contraction of cells that expressed PSK2 (Fig. 3, A and B). Interestingly, 27 ± 10.2% of PSK2-transfected cells showed accumulation of cortical actin in the presence of Y27632.

JNK Activity Is Required for the Induction of Apoptotic Morphology by PSK2—The results presented above demonstrated that the catalytic activity of PSK2 is important for its stimulation of cell shrinkage and membrane blebbing as well as the cleavage of ROCK-I. To determine whether the activation of JNK by PSK2 was involved in the stimulation of apoptosis, we tested the effects of dominant negative JNK. Expression of dominant negative JNK inhibited the induction of cell contraction and membrane blebbing by PSK2 (Fig. 4, A and B). We also investigated the effects of the cell-permeable and selective JNK inhibitor SP600125 on both of these apoptotic morphological responses. In contrast, the cell-permeable and selective p38 inhibitor SB203580 was unable to block PSK2-induced contraction or blebbing of cells (data not shown).

Apoptotic Stimuli Activate Endogenous PSK2—To determine whether the catalytic activity of PSK2 itself can be regulated by apoptotic stimuli that reportedly activate JNK, non-transfected cells were incubated with a number of different agents. Endogenous PSK2 was immunoprecipitated from cell lysates, and its autophosphorylation was examined using in vitro kinase assays. Fig. 5 shows that the catalytic activity and autophosphorylation of PSK2 were increased 3 h after the addition of NaCl or staurosporine. Sorbitol also stimulated the catalytic activity of endogenous PSK2 at 1 h, but its effect was transient and declined thereafter (Fig. 5). In addition, NaCl and staurosporine each stimulated phosphorylation and activation of JNK at 3 h, whereas sorbitol caused a transient increase in phosphorylated JNK at 1 h (Fig. 5). In contrast, the microtubule-disrupting agent nocodazole failed to stimu-
late the activity of endogenous PSK2 or JNK within 3 h (data not shown). These results demonstrated that apoptotic stimuli can stimulate the catalytic activity of endogenous PSK2 and JNK in growing H1299 cells.

Caspase Activity Is Required for the Induction of Membrane Blebbing by PSK2—Caspase 3 exists as an inactive pro-enzyme (32 kDa) that is processed in cells undergoing apoptosis to produce large (17-kDa) and small (12-kDa) subunits that associate to form an active enzyme (27, 28). Treatment of H1299 cells with the apoptotic stimuli staurosporine and immunoblotting of cell lysates with anti-caspase 3 antibodies detected full-length endogenous caspase 3 (32 kDa) as well as two additional proteins at 17 and 12 kDa (Fig. 6). To determine whether PSK2 causes cleavage of endogenous caspase 3, lysates prepared from cells transfected with PSK2 were immunoblotted with this antibody and full-length caspase 3 (32 kDa), and the 17- and 12-kDa proteins were detected (Fig. 6). In contrast, the 17- and 12-kDa forms of caspase 3 were absent or much reduced in cells that were transfected with empty vector alone or PSK2 (K57A), respectively (Fig. 6).

Since PSK2 causes cleavage of caspase 3, we wanted to determine whether endogenous caspases were involved in the induction of apoptotic morphology by PSK2. Cultures were transfected with PSK2 and incubated with the cell-permeable broad spectrum caspase inhibitor Z-VAD-FMK, and the effects of PSK2 on cell morphology were examined. We found that Z-VAD-FMK prevented the blebbing response of cells to PSK2 but had no effect on their contraction when compared with transfected cells incubated in the absence of the caspase inhibitor (Fig. 7, A and B).

PSK2 Is a Substrate for Caspase 3—Several members of the GCK-like STE20 family are known to be substrates for caspase 3, and we set out to test whether this proteolytic enzyme could also cleave PSK2 (14–17, 25, 29). His-tagged PSK2 was translated in vitro in the presence of [35S]methionine, and labeled PSK2 immunoprecipitated with mouse anti-His monoclonal antibody and incubated with purified recombinant caspase 3. As shown in Fig. 8A, in vitro translated and precipitated PSK2 displayed a complex pattern of bands due to proteolysis and/or incomplete translation products during its synthesis in reticulocyte lysates. However, incubation of PSK2 with caspase 3 produced three additional bands at 80, 45, and 30 kDa that were absent when samples were incubated with the broad spectrum caspase inhibitor Z-VAD-FMK (Fig. 8A). Moreover, PSK2 (K57A) immunoprecipitated from transfected cell lysates was also cleaved by recombinant caspase 3 to produce a 45-kDa protein (Fig. 8B). These results demonstrate that PSK2 is cleaved by caspase 3 in vitro.

In addition, immunoblotting of cell lysates prepared from transfected cells detected full-length PSK2 at 155 kDa as well as two additional N-terminal fragments of PSK2 at 45 and 30 kDa (Fig. 8C). The 45- and 30-kDa protein fragments were not observed in cells transfected with kinase-defective PSK2 (K57A), demonstrating that the catalytic activity of PSK2 is required to stimulate its degradation (Fig. 8C). We also incubated PSK2-transfected cells with Z-VAD-FMK and found that this peptide abolished cleavage of PSK2 (Fig. 8C). However, mutation of a putative caspase recognition and cleavage site (373DVSD376) and expres-
sion of PSK2 (D376N) in cells failed to prevent production of the 45- or 30-kDa proteins (data not shown). PSK2 appears to be cleaved at non-consensus sites, and a similar observation has been reported for the related STE20 STE20-like kinase (25). These results suggest that exogenously expressed and catalytically active PSK2 is cleaved by caspases in vivo, and the caspase activation by PSK2 is needed to mediate its cleavage. Caspase activity was also required for the induction of membrane blebbing by PSK2.

Staurosporine CLEAVES Caspase 3 and Degrades Endogenous PSK2—Since staurosporine can activate caspase 3 and PSK2 is a substrate for this enzyme, we set out to determine whether endogenous PSK2 is cleaved during the apoptotic response to this agent. Non-transfected cells were treated with staurosporine, and the expression of endogenous PSK2 was examined by immunoblotting. Anti-PSK2 antibodies detected full-length endogenous PSK2 up to 8 h after the addition of staurosporine, but the protein was degraded thereafter (Fig. 9). Interestingly, pretreatment of cultures with Z-VAD-FMK abolished the degradation of endogenous PSK2 induced by staurosporine (Fig. 9). The anti-PSK2 antibody used to detect endogenous PSK2 was raised against amino acids 352–550 and was unable to detect the N-terminal fragments of PSK2 reported above. These results showed that the apoptotic agent staurosporine not only activates endogenous PSK2 but also causes cleavage and degradation of the protein in a caspase-dependent manner.

DISCUSSION
Northern blotting has shown that PSK2/TAO1 (also referred to as hKFC-B) is expressed in a wide variety of different tissues that include brain, heart, lung, testis, skeletal muscle, placenta, thymus, prostate, and spleen but may be absent in liver, kidney, and ovary (30, 31). Phylogenetic analysis of the mammalian STE20-like group of kinases has organized these proteins into 10 different subfamilies that include PAK (I-II) and GCK (I–VIII) (1). The GCK-VIII subfamily includes human PSK1 and PSK2 and their rat homologs, the thousand and one amino acid protein kinases (TAO2 and TAO1, respectively), as well as the human JNK-inhibitory protein (1, 24, 30, 32, 33). Previous studies have shown that some of these GCK-VIII proteins regulate the JNK and/or p38 MAPK signaling pathways. PSK1 activates JNK, and in addition, binds to and regulates microtubule organization and stability via its C terminus, and TAO1 and TAO2 stimulate p38 (23, 24, 30, 34). In contrast, JNK-inhibitory protein inhibits the basal level of JNK and has no effect on p38 (33). However, the downstream consequences of their effects on the MAPK signaling pathways have not been determined. Here we have shown that PSK2, which lacks the microtubule-binding domain found in PSK1, stimulates the JNK-signaling pathway and that the activity of JNK is required for PSK2 to induce apoptotic morphology since dominant negative JNK or a physiological inhibitor of JNK prevents its induction of cell contraction and membrane blebbing. Apoptotic stimuli that regulate JNK also increased the catalytic activity of endogenous PSK2.

A number of other GCK-like STE20s have also been associated with apoptosis and include MSTs 1–4, STE20-like kinase, and hematopoietic progenitor kinase 1 (HPK1) (14–17, 25, 29). Each of these kinases is a substrate for caspase 3 and their cleavage can enhance their catalytic activity (14–17, 25, 29). In the present study, we have shown that PSK2 is also a substrate for caspases in vitro and in vivo. However, apoptotic stimuli can increase the catalytic activity of full-length endogenous PSK2 (150 kDa) in the absence of cleavage. The broad spectrum caspase inhibitor Z-VAD-FMK prevents the stimulation of membrane blebbing by PSK2, demonstrating that this kinase requires caspase activity to induce this morphological response. Caspases must target other proteins such as ROCK-I to induce the full apoptotic response. Interestingly, staurosporine activates caspases, which mediate the cleavage and degradation of endogenous PSK2 during its induction of apoptosis. We have detected caspase-dependent N-terminal fragments of PSK2 in cells, and it is likely that such fragments could also contribute to the induction of apoptotic morphology. Indeed, we have found that PSK2 (1–416) is sufficient to activate JNK and induce apoptosis. We have shown that sorbitol stimulates PSK2 and JNK and causes cell rounding, but it is not possible to block this response using RNA interference for PSK2. PSK2 is one of at least 12 MAP3Ks that activate JNK, and others have shown that at least four MAP3Ks need to be down-regulated by small interfering RNA to prevent the activation of JNK by sorbitol (30, 35, 36). It is likely that other MAP3Ks can compensate for PSK2.
The importance of both JNK activation and caspases in the induction of apoptotic morphological changes by PSK2 may be explained by studies suggesting that JNK signaling causes mitochondrial cytochrome c release and subsequent activation of caspases (9). However, we found that Z-VAD-FMK does not prevent PSK2-induced cell contraction, and recent evidence suggests that some apoptotic morphological changes can be promoted via caspase-independent as well as caspase-dependent pathways (37). The GCK-like family member MST1 induces apoptotic changes via activation of the JNK-signaling pathway, and caspase inhibitors can prevent the stimulation of nucleosomal DNA fragmentation by MST1 (38). However, caspase inhibitors are unable to block the induction of chromatin condensation, cell contraction, or membrane blebbing by MST1, which occur independently of caspases (38). Taken together with our findings reported here, this suggests that caspases may not always be required for some members of the GCK subfamily to induce apoptotic morphology.

The role of caspase substrates in mediating particular apoptotic responses is poorly understood with a few exceptions that include MST-I-stimulated caspase cleavage of inhibitor of caspase-activated DNase, which results in the release and nuclear localization of CAD and its induction of DNA fragmentation (38). Caspases also cleave and activate PAK2 to induce the contraction and rounding of adherent cells, and cleavage of ROCK-I by caspases produces an activated fragment of ROCK-I that induces membrane blebbing (18–20). ROCK-I phosphor-cleavage of ROCK-I by caspases produces an activated fragment of PAK2 to induce the contraction and rounding of adherent cells, and the induction of chromatin condensation, cell contraction, or membrane blebbing by MST1, which occur independently of caspases (38). Taken together with our findings reported here, this suggests that caspases may not always be required for some members of the GCK subfamily to induce apoptotic morphology.

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