We have demonstrated previously that full-length prostate-derived sterile 20-like kinase 1-α (PSK1-α) binds to microtubules via its C terminus and regulates their organization and stability independently of its catalytic activity. Here we have shown that apoptotic and microtubule-disrupting agents promote catalytic activation, C-terminal cleavage, and nuclear translocation of endogenous phosphoserine 181 PSK1-α and activated N-terminal PSK1-α-induced apoptosis. PSK1-α, unlike its novel isoform PSK1-β, stimulated the c-Jun N-terminal kinase (JNK) pathway, and the nuclear localization of PSK1-α and its induction of cell contraction, membrane blebbing, and apoptotic body formation were dependent on JNK activity. PSK1-α was also a caspase substrate, and the broad spectrum caspase inhibitor benzylxoycarbonyl-VAD-fluoromethyl ketone or mutation of a putative caspase recognition motif (916DPGD919) blocked nuclear localization of PSK1-α and its induction of membrane blebs. Additional inhibition of caspase 9 was needed to prevent cell contraction. PSK1-α is therefore a bifunctional kinase that associates with microtubules, and JNK- and caspase-mediated removal of its C-terminal microtubule-binding domain permits nuclear translocation of the N-terminal region of PSK1-α and its induction of apoptosis.

Prostate-derived sterile 20-like kinase 1-α (PSK1-α)3 is a member of a family of more than 30 mammalian sterile 20 (STE20)-like kinases that regulate a diverse array of processes that include gene transcription, cell cycle progression, stress responses, cytoskeletal organization, and apoptosis (reviewed in Ref. 1). STE20s divide into two subfamilies according to their structure and regulation. The p21-activated kinases have a C-terminal catalytic domain and an N-terminal Cdc42/Rac-interacting domain, whereas the germinal center kinase (GCK)-like kinases possess an N-terminal catalytic domain and no Cdc42/Rac-interacting domain. p21-activated kinases interact with Rac or Cdc42 GTPases via the Cdc42/Rac-interacting domain and act as downstream effectors for these small GTP-binding proteins, but much less is known about the regulation of GCKs (2, 3). Most STE20s activate one or more of the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase, although their biological responses do not always require MAPKs (1). PSK1-α belongs to the GCK-like kinase class of STE20s and activates JNK via its N-terminal catalytic domain, and through its C-terminal regulatory domain, binds to microtubules and regulates their organization and stability (4, 5).

Several STE20 kinases and the JNK family of MAPKs have been implicated in apoptosis (1, 6). Apoptosis, or programmed cell death, is an important process in the development and homeostasis of multicellular organisms and is triggered by various stimuli, such as cytokines and DNA damage. Apoptosis is characterized by morphological changes that include cytoplasmic contraction, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (7, 8). These morphological alterations frequently involve the activation of caspases, a family of cysteine-dependent aspartate-directed proteases, either by Fas-associated death domain-mediated aggregation of death receptors or by release of cytochrome c from mitochondria (9, 10). Both stimuli trigger distinct initiator caspases to activate downstream effector caspases, which cleave and regulate various target proteins involved in signaling cell survival and proliferation, DNA synthesis and repair, as well as components of the cell cytoskeleton (10–17). In addition, pro-apoptotic proteins, such as the STE20 kinases mammalian STE20-like kinases 1–3 (MST1–3), STE20-like kinase, or hematopoietic progenitor kinase, are cleaved by caspases to produce constitutively activated fragments (18–23). However, the role of these target proteins in mediating particular apoptotic events is poorly understood, with a few exceptions that include caspase cleavage and the subsequent activation of the inhibitor of caspase-activated DNase leading to DNA fragmentation and Rho kinase-1 (ROCK-1), which plays an important role in membrane blebbing (24–28).
We have shown previously that PSK1-α specifically activates the JNK-signaling pathway via its N-terminal kinase domain, whereas the C terminus of PSK1-α binds to microtubules and regulates their organization and stability (4, 5). Here we show that apoptosis-inducing agents, such as paclitaxel and staurosporine, stimulated cleavage, activation, and nuclear localization of PSK1-α and that the N-terminal kinase domain of PSK1-α induced JNK- and caspase-dependent apoptotic morphological alterations. C-terminal cleavage and nuclear localization of catalytically active PSK1-α is mediated via JNK and caspase activity and is a necessary step for the induction of apoptosis by PSK1-α. In contrast, a novel splice variant PSK-1β, which has a different C-terminal sequence, does not activate JNK or induce apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—pRK5-Myc-PSK1-α and pRK5-Myc-PSK1-α (K57A) were made as described previously (4). pRK5-Myc-PSK1-α (1–745), pRK5-Myc-PSK1-α (1–745,K57A), and pRK5-Myc-PSK1-α (D919N) were prepared by PCR using appropriate oligonucleotides. pRK5-Myc-PSK1-β and pRK5-Myc-PSK1-β (K57A) were prepared from the human cDNA clone KIAA0881 obtained from the Kazusa DNA Research Institute (gift from Dr. T. Nagase). Colleagues donated the following plasmids: pCMV-FLAG-JNK1 (Dr. M. Karin, University of California), pGEX-c-Jun (Dr. J. Ham, Eisa London Research Laboratories), and dominant negative pCDNA3-FLAG-JNK1 (Dr. R. Davis, University of Massachusetts). The caspase inhibitors Z-VAD-fmk and Z-LEHD-fmk were obtained from Calbiochem. Paclitaxel 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, 2 × 10⁵ cells/2 ml of medium or 5 × 10⁶ cells/4 ml of medium were seeded onto 35- or 60-mm Petri dishes, respectively. After 16 h, the indicated plasmids were transfected into COS-1 cells or H1299 cells using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen).

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 Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris, pH 7.4). 100 μg of total protein were separated using 8–15% SDS-PAGE and transferred to nitrocellulose. Immunoblotting was carried out as described previously (4). Rabbit anti-phospho-MKK4 and anti-phospho-JNK antibodies were purchased from Cell Signaling Technology. Rabbit anti-phospho-c-Jun and mouse anti-α-tubulin antibodies were obtained from Abcam or Sigma, respectively. Mouse anti-Myc and anti-poly(ADP-ribose) polymerase (anti-PARP) antibodies were obtained from Santa Cruz Biotechnology or R & D Systems, respectively. Rabbit polyclonal antibody was raised against CPANS(P)FVGGTC coupled to keyhole limpet hemocyanin and affinity-purified to detect PSK1 phosphorylated on serine residue 181 (Eurogentec).

Nuclear and Cytoplasmic Fractionation—H1299 cells (2 × 10⁵) were seeded onto 100-mm Petri dishes in 8 ml of medium and treated as indicated. Cell cultures were scraped into ice-cold phosphate-buffered saline (4 ml), pelleted by centrifugation (600g, 5 min), and cytoplasmic or nuclear fractions isolated according to the manufacturer’s instructions (Bio-Vision Research Products). Total amounts of protein were determined using a protein assay kit (Pierce), and 100 μg of protein was separated using 10% SDS-PAGE and transferred to nitrocellulose for analysis by immunoblotting.

Immunoprecipitation, in Vitro Kinase Assays, Immunofluorescence, and Confocal Microscopy—These procedures were carried out as described previously (28). Briefly, for immunoprecipitation, 400 μg of protein in 1 ml of binding buffer (130 mM NaCl, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris-HCl, pH 7.6) was mixed with 3 μg of anti-FLAG mouse monoclonal antibody (Sigma) for 1 h at 4 °C and then 100 μl of 10% (v/v) protein G-Sepharose beads (Sigma) for 1 h at 4 °C. For in vitro kinase assays, beads were 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/mmol), and 1 μg of glutathione S-transferase-c-Jun protein or 1 μg of myelin basic protein and incubated for 45 min at 37 °C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting or analysis using a phosphorimaging device (Fuji). For immunofluorescence experiments, H1299 cells (2 × 10⁵) were transfected with the indicated plasmids at the times shown in the figure legends. Cultures were fixed in phosphate-buffered saline containing 3.7% formaldehyde (20 min), and cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (5 min). Myc-tagged PSKs, FLAG-tagged JNK, and actin were detected as described previously (28). Rabbit anti-PSK antibody PEP1 (5) was affinity-purified on a peptide column (GE Healthcare) and diluted 1:50 in blocking buffer for immunostaining followed by 1:400 Alexa 635-conjugated goat anti-rabbit IgG (Molecular Probes). In control experiments, immunoblotting and immunofluorescence signals were abolished in the presence of the appropriate peptide. Cells were imaged with a Bio-Rad MRC-1024 confocal laser-scanning microscope.

Caspase Cleavage Assays—In vitro caspase cleavage assays were performed as described previously (28).

RESULTS

PSK1-α (but Not Its Novel Isoform PSK1-β) Activated JNK—During the original isolation of PSK, we described an apparent isoform for this protein that is identical over the N-terminal kinase domain (amino acids 1–744) but contains a different C terminus (amino acids 745–1049) (4). Analysis of the human genome sequence has shown that a single gene located on chromosome 16 (locus 51677) encodes both isoforms of PSK. PSK1-α (previously referred to as PSK) and its novel isoform PSK1-β are generated from a primary transcript(s) containing the common exons 1–16 and either one (exon 17) or three additional exons (exons 18–20), respectively (Fig. 1A).

PSK1-α was shown previously to stimulate the JNK MAPK signaling pathway, and we set out to determine whether PSK1-β could also activate JNK (4). Myc-tagged PSK1-α, PSK1-β, or their identical N-terminal region PSK1-α (1–745)
were expressed in COS-1 cells and immune complexes for each protein prepared from cell lysates. In in vitro kinase assays, PSK1-α, PSK1-β, and PSK1-α-(1–745) each underwent autophosphorylation and phosphorylated myelin basic protein (MBP) (Fig. 1B), whereas their kinase-defective counterparts were unable to phosphorylate themselves or MBP (Fig. 1B).

PSK1-α, PSK1-β, or PSK1-α-(1–745) 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 PSK1-α or PSK1-α-(1–745) activated JNK and stimulated the phosphorylation of c-Jun, whereas PSK1-β was unable to increase the levels of phosphorylated c-Jun above those obtained using the kinase-defective mutants for each of these proteins (Fig. 1C). The failure of PSK1-β to activate JNK, when compared with its N-terminal fragment PSK1-α-(1–745), suggests that the C terminus of PSK1-β is inhibitory for this function.

PSK1-α-induced Cell Contraction, Membrane Blebbing, and Cleavage of PARP—Because 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 PSK1-α could stimulate changes in cell morphology that are associated with apoptosis (4, 6, 23, 29). In non-small cell lung carcinoma cells (H1299), PSK1-α and PSK1-α-(1–745) induced a marked reduction in cell spread area and caused cell rounding and substantial membrane blebbing as well as the formation of apoptotic bodies (Fig. 2, A–C). In contrast, kinase-defective PSK1-α (K57A) caused no significant cell contraction or membrane blebbing and localized to filamentous structures previously shown to be microtubules (Fig. 2, A–C) (5). PSK1-β localized to a different perinuclear site and had no detectable effect on either cell size or membrane morphology (Fig. 2, A–C). Kinase-defective PSK1-β (K57A), PSK1-α-(1–745), PSK1-α-(1–745,K57A), as well as green fluorescent protein (GFP) did not cause significant cell contraction or membrane blebbing (Fig. 2, B and C).

In addition to the effects of PSK1-α on cell morphology, we also tested whether PSK1-α induced cleavage of poly(ADP-ribose) polymerase (PARP), an event commonly associated with cells that are undergoing apoptosis (30). Expression of PSK1-α (but not PSK1-α (K57A), PSK1-β, or PSK1-β (K57A) caused cleavage of 116-kDa PARP to generate the 85-kDa fragment that is characteristic of apoptosis (Fig. 2D).

Paclitaxel-stimulated Activation and Phosphorylation of Endogenous PSK1—Analysis of the crystal structure of the kinase domain of PSK1-α (also referred to as TAO2) indicates that the phosphorylation of serine 181 in the activation loop of PSK1-α is involved in its conformational activation (31). To examine the activation and localization of endogenous kinases, an antibody was made against a peptide that mimicked PSK1-α phosphorylated on serine 181 (PSK1-pS181). To test whether the anti-PSK1-pS181 antibody could detect PSK1-α and PSK1-β, cells were transfected with Myc-tagged PSK1-α and PSK1-β, or PSK1-α-(1–745) were detected using a phosphorimaging device (Fuji). The expression of Myc-PSKs or FLAG-JNK was confirmed by immunoblotting total cell lysates.

FIGURE 1. Structure and activity of PSK1-α and PSK1-β, A, alternative splicing of a single gene product from chromosome 16 generates PSK1-α and PSK1-β. Exons 1–16 are common to both proteins and either one (exon 17) (black) or three additional exons (exons 18–20) (white) are required to produce PSK1-α (1235 amino acids) or PSK1-β (1049 amino acids), respectively. PSK1-α and PSK1-β are identical over their first 744 amino acids but have different C termini. PSK1-α and PSK1-β are functional kinases, but only PSK1-α activated JNK. B, and C, growing COS-1 cells were transfected with pRK5-Myc vector, pRK5-Myc-PSK1-α, pRK5-Myc-PSK1-β (K57A), pRK5-Myc-PSK1-β, pRK5-Myc-PSK1-β (K57A), pRK5-Myc-PSK1-(1–745), or pRK5-Myc-PSK1-(1–745,K57A) with (C) or without (B) CMV-FLAG-JNK. After 48 h, Myc-tagged PSKs (B) or FLAG-tagged JNK (C) were immunoprecipitated from cell lysates and taken for in vitro kinase assays using MBP (B) or c-Jun (C) as the substrate. MBP phosphorylated by PSKs or c-Jun phosphorylated by JNK were detected using a phosphorimaging device (Fuji). The expression of Myc-PSKs or FLAG-JNK was confirmed by immunoblotting total cell lysates.
kDa) after 2–6 h, suggesting that PSK1 could contribute to paclitaxel-stimulated JNK (Fig. 3B).

**PSK1-α Localized to the Nucleus as Well as Microtubules**—We have shown previously that PSK1-α/H9251 can localize to the cytoplasm and bind to microtubules (5). To examine the subcellular distribution of PSK1-α/H9251, cells expressing PSK1-α/H9251 or kinase-defective PSK1-α/K57A were immunostained with antibodies that recognize each end of the protein. 9E10 anti-Myc antibody was used to detect the N-terminal Myc epitope tag attached to PSK1-α/H9251 and demonstrated that PSK1-α/H9251 localized to microtubules in the cytoplasm; however, this antibody also showed that PSK1-α/H9251 was present in the nucleus (Fig. 4, A and B). In contrast, kinase-defective PSK1-α/K57A was only found on microtubules in the cytoplasm and was excluded from the nucleus, demonstrating that the catalytic activity of PSK1-α/H9251 was required for its nuclear localization (Fig. 4, A and B).

Cells were also co-stained with an antibody that was raised against the C terminus of PSK1-α/H9251 (amino acids 1216–1235) (named PEP1). This antibody recognized both PSK1-α/H9251 and PSK1-α/K57A on microtubules (Fig. 4A) but was unable to detect the nuclear form of PSK1-α/H9251 identified using the 9E10 antibody, demonstrating that nuclear PSK1-α lacked a recognizable C-terminal domain.
PSK1-α Regulates Apoptotic Morphology

FIGURE 4. PSK1-α localized to the cytoplasm and nucleus. A, growing H1299 cells were transfected with pRK5-Myc-PSK1-α or pRK5-Myc-PSK1-α (K57A), and after 24 h, the cultures were fixed and co-stained with anti-Myc 9E10 (first column), PEP1 (second column), or propidium iodide (third column). Composite panels show staining for PSK1-α using anti-Myc 9E10 (green), PSK1-α using PEP1 antibody (blue), or nuclei using propidium iodide to stain DNA (red) (fourth column). Scale bar, 10 μm. B, growing H1299 cells were transfected and Myc-PSKs expressed as indicated. The percentages of transfected cells that contained protein in the nucleus were determined by examining >100 cells in triplicate using confocal microscopy. Standard deviations are shown. C, growing H1299 cells were treated with or without paclitaxel (10 μM) as indicated, and at the times shown, cytoplasmic or nuclear fractions were prepared from cell lysates and immunoblotted with anti-PSK1-pS181 antibody (upper panel) or anti-Oct-1 antibody (lower panel). D, growing H1299 cells were pretreated with or without SP600125 (20 μM) for 16 h and paclitaxel (10 μM) added for 6 h where shown. Cytoplasmic or nuclear fractions were prepared from cell lysates and immunoblotted with anti-PSK1-pS181 antibody.

epitope (Fig. 4A). Taken together, these results suggest that full-length PSK1-α binds to microtubules in the cytoplasm, whereas a C-terminally truncated form of PSK1-α is present in the nucleus. We have shown previously that the C terminus of PSK1-α (amino acids 1064–1235) can bind to α- and β-tubulin, and it is plausible that the removal of these sequences permits the protein to relocate from the microtubules to the nucleus (5). Indeed, PSK1-α (1–745), which lacks the microtubule-binding domain, localized to the cell nucleus (Fig. 4B). Kinase-defective PSK1-α (1–745,K57A) also localized to the nucleus of transfected cells, unlike full-length PSK1-α (K57A), which remained in the cytoplasm (Fig. 4B). Removal of the C-terminal microtubule-binding domain was therefore sufficient for nuclear localization of PSK1-α. In contrast, PSK1-β and kinase-defective PSK1-β (K57A) were not detected in the nucleus (Fig. 4B).

These findings led us to examine the localization of phosphorylated endogenous PSK1-pS181 by immunoblotting. Cultures were treated with paclitaxel for 3 or 6 h and cytoplasmic or nuclear fractions prepared from cell lysates. Immunoblotting with anti-PSK1-pS181 antibody detected the presence of phosphorylated endogenous PSK1 (165 kDa) in both the cytoplasmic and nuclear fractions (Fig. 4C). In contrast, an additional 85-kDa N-terminal fragment of phosphorylated PSK1-pS181 was found solely in the nuclear fraction along with the nuclear marker protein Oct-1 (Fig. 4C). The paclitaxel-induced nuclear 85-kDa form of PSK1-pS181 was not observed when cultures were pretreated with the JNK inhibitor SP600125 (Fig. 4D). These results show that paclitaxel stimulated the production of an N-terminally phosphorylated form of endogenous PSK1-pS181 that was found exclusively in the nucleus.

JNK Activity Was Required for Nuclear Localization of PSK1-α and Its Induction of Cell Contraction and Membrane Blebbing—The results presented above demonstrate that the catalytic activity of PSK1-α and potentially its nuclear localization are important for the induction of cell contraction and membrane blebbing. To determine whether the activation of JNK by PSK1-α was involved in the stimulation of apoptosis, we tested the effects of dominant negative JNK and found that the expression of dominant negative JNK inhibited the induction of cell contraction and membrane blebbing by PSK1-α (Fig. 5, A and B). Dominant negative JNK expression resulted in the retention of PSK1-α on microtubules and its exclusion from the nucleus (Fig. 5, A and B). We also investigated the effects of the JNK inhibitor SP600125 on these processes and found that the inhibitor prevented nuclear localization of PSK1-α as well as its induction of cell contraction and membrane blebbing (Fig. 5, A and B). SP600125 did not inhibit the activity and autophosphorylation of transfected PSK1-α or subsequent phosphorylation of MKK4 or JNK but did prevent the phosphorylation of c-Jun (Fig. 5C). These results indicate that the activation of JNK by PSK1-α was required for its induction of membrane blebbing and cell contraction as well as nuclear localization.

Caspases Mediated Cleavage of PSK1-α—Several members of the GCK-like STE20 family are caspase 3 substrates, and we set out to determine whether this proteolytic enzyme could also cleave PSK1-α (18, 20, 23, 25, 32–34). His-tagged PSK1-α was translated in vitro in the presence of [35S]methionine, immunoprecipitated with mouse anti-His monoclonal antibody, and incubated with purified recombinant caspase 3. In vitro translated and precipitated PSK1-α displayed a complex pattern of bands due to proteolysis and/or incomplete translation products during its synthesis in reticulocyte lysates (Fig. 6A). However, incubation with caspase 3 produced two additional bands at 70 and 30 kDa that were absent when samples were incubated with the broad spectrum caspase inhibitor Z-VAD-fmk (Fig.
which declined after treatment with staurosporine (Fig. 6B). Interestingly, a novel 70-kDa form of PSK1-pS181 was detected 4–16 h after the addition of staurosporine, and this N-terminal fragment of PSK1 was smaller than the 85-kDa form observed previously with paclitaxel (Fig. 6B). Pretreatment of cultures with Z-VAD-fmk prevented the appearance of the 70 Da form of PSK1-pS181 but not the disappearance of the 165-kDa form of PSK1-pS181 (Fig. 6B). Caspase activity was therefore required to produce the 70-kDa N-terminal fragment of phosphorylated PSK1-pS181 during staurosporine-induced apoptosis.

**Caspase Activity Was Required for Nuclear Localization of PSK1-α and Its Induction of Apoptotic Morphology**—JNK activity was required for PSK1-α to localize to the nucleus and induce apoptosis, and others have shown the importance of JNK activation in stress-induced release of cytochrome c from the mitochondria and the subsequent stimulation of caspases and apoptosis (6, 35, 36). To investigate the involvement of caspases in the induction of apoptotic morphology by PSK1-α, cells transfected with PSK1-α or PSK1-α(D919N) were incubated with the caspase inhibitor Z-VAD-fmk. Z-VAD-fmk prevented PSK1-α-induced membrane blebbing, slightly reduced cell contraction, and decreased the nuclear localization of PSK1-α (Fig. 6, C and D). Z-VAD-fmk also prevented PSK1-α(D919N)-induced membrane blebbing and partially reduced cell contraction but had no inhibitory effect on the nuclear localization of PSK1-α(D919N) (Fig. 6, C and E).

Our finding that PSK1-α required JNK and caspase activity to induce membrane blebbing led us to examine whether the apoptotic morphological changes observed were a consequence of caspase activation via the mitochondrial pathway. H1299 cells were transfected with PSK1-α and cultures incubated in the presence of Z-LEHD-fmk, an inhibitor for caspase 9. Z-LEHD-fmk was considerably more potent than Z-VAD-fmk in inhibiting cell contraction in response to PSK1-α (Fig. 7). We also observed inhibition of membrane blebbing and nuclear localization of PSK1-α in transfected cells incubated with Z-LEHD-fmk (Fig. 7). Overall, the effects of the inhibitor for caspase 9 on PSK1-α were similar to that of dominant negative JNK. To examine whether the effects of PSK1-α on cell morphology could also be related to the death receptor-mediated caspase activation pathway, cells transfected with PSK1-α were incubated with both Z-LEHD-fmk and Z-VAD-fmk. No further reduction in cell contraction or membrane blebbing was observed in these cells, indicating that these morphological changes appear to be linked to the mitochondrial caspase 9–associated death pathway activated via JNK in cells that express PSK1-α (Fig. 7B). PSK1-α was catalytically active and phosphorylated itself, MBP, and activated JNK in the presence of both Z-LEHD-fmk and/or Z-VAD-fmk (data not shown). These results suggest that the induction of cell contraction and membrane blebbing by PSK1-α are linked to the mitochondrial death pathway.

**PSK1-α (D919N) Activated JNK, but Its Nuclear Localization and Effects on Membrane Blebbing Were Significantly Reduced**—Our results using the caspase inhibitors suggest that these proteolytic enzymes are involved in the cleavage, activation, and nuclear localization of PSK1-α and its induction of apoptotic morphology. PSK1-α has a putative caspase recognition and

---

**FIGURE 5. JNK activity was required for PSK1-α to induce apoptotic morphology.** A, growing H1299 cells were transfected with pRKS-Myc-PSK1-α with or without dominant negative (dn) pCDNA3-FLAG-JNK1 or the JNK inhibitor SP600125 (20 μM). After 48 h, cultures were fixed and co-stained with anti-Myc antibody and anti-rabbit FITC (PSK1-α), TRITC-phalloidin (actin), or M2 FLAG antibody and anti-mouse Alexa 633 (dn/JNK). Composite panels show staining for PSK1-α (green), actin (red), and dominant negative JNK (blue). Scale bar, 20 μm. B, cells were transfected with pRKS-Myc-PSK1-α with or without dominant negative pCDNA3-FLAG-JNK1 or the JNK inhibitor SP600125 (20 μM). The percentage of transfected cells undergoing cell contraction (black bars), membrane blebbing (gray bars), or containing PSK1-α in the nucleus (open bars) were determined by examining >100 cells in triplicate using confocal microscopy. Standard deviations are shown. C, growing H1299 cells were transfected with pRKS-Myc or pRKS-Myc-PSK1-α and treated with or without SP600125 (20 μM). After 24 h, cell lysates were immunoblotted with anti-PSK1-pS181 antibody, anti-phospho-MK4 antibody, anti-phospho-JNK antibody, anti-phospho-c-Jun antibody, or anti-α-tubulin antibody.

---

6A). These results demonstrate that PSK1-α was cleaved by caspase 3 in vitro.

To examine the involvement of caspases in the cleavage, activation, and phosphorylation of endogenous PSK1 in vivo, H1299 cells were incubated in the presence or absence of the cell-permeable broad spectrum caspase inhibitor Z-VAD-fmk and treated with staurosporine, which stimulates caspase 3 (28). Immunoblotting of cell lysates with anti-PSK1-pS181 antibody detected the 165-kDa form of the phosphoprotein,
cleavage motif (916DPGD919). To examine this site further, a point mutation was introduced into the caspase recognition motif to produce PSK1-α/D919N. Wild type PSK1-α or PSK1-α/D919N were co-transfected with FLAG-tagged JNK into H1299 cells and immunoprecipitates of JNK, PSK1-α, or PSK1-α/D919N prepared sequentially from cell lysates for in vitro kinase assays. Both forms of immunoprecipitated PSK1-α underwent autophosphorylation and phosphorylated MBP (Fig. 8A). In addition, PSK1-α and PSK1-α/D919N both activated JNK, demonstrating that the mutant PSK1-α/D919N was a fully functional kinase in H1299 cells (Fig. 8A).

Analysis of the effects of PSK1-α/D919N on cell morphology demonstrated that the mutation severely impaired the ability of the protein to induce membrane blebbing (Fig. 8, B and C). PSK1-α/D919N still caused some cells to contract, although more weakly than wild-type PSK1-α (Fig. 8, B and C). Interestingly, PSK1-α/D919N was predominantly retained in the cytoplasm of cells, indicating that the nuclear localization of PSK1-α was needed, as well as JNK activation, to induce the full apoptotic response (Fig. 8, B and C).

**DISCUSSION**

We have demonstrated that the STE20-like kinase PSK1-α induced apoptotic morphological changes that included cell contraction, membrane blebbing, and apoptotic body formation. PSK1-α also activated JNK, although a novel isoform of PSK1-α that we have identified here, PSK1-α/H9252, was unable to induce JNK activation or apoptosis (4). Both isoforms of PSK1 are encoded by a single gene on chromosome 16 and are identical over their N-terminal kinase domains but because of alternative splicing have different C termini that localize the proteins to distinct subcellular sites. In contrast, the N-terminal region that is common for both isoforms (amino acids 1–745) was distributed throughout the cytoplasm and did induce JNK signaling and apoptosis. Auto-inhibition of PSK1-α/H9252 by its C terminus appears unlikely to account for its inability to stimulate JNK, as full-length PSK1-α/H9252 was as catalytically active toward MBP as the N-terminal kinase domain alone. Because components of the JNK signaling cascade may be organized into defined signaling modules in vivo by their interaction with scaffold proteins such as the JNK-interacting proteins 1–4, it is plausible that the localization of PSK1-β and/or its conformation inhibits its activation.
ability to interact with components of the JNK pathway (37–40).

We have demonstrated previously that PSK1-α binds to microtubules and alters their organization and stability (5). Both the C-terminal region of PSK1-α (amino acids 745–1235) that lacks the kinase domain and kinase-defective PSK1-α (K57A) could bind to and stabilize microtubules, demonstrating that the catalytic activity of the protein is not required for these functions. In contrast, the ability of PSK1-α to induce apoptosis was dependent on its catalytic activity, which was also required for JNK activation, and PSK1-α-(1–745) was sufficient to stimulate both of these responses. Because dominant negative JNK or the caspase 9 inhibitor Z-LEHD-fmk blocked PSK1-α-induced apoptosis, it is plausible that the stimulation of JNK by PSK-1 α activates caspase 9. Indeed, JNK is known to act upstream of the mitochondrial death pathway causing cytochrome c release and activation of caspase 9 (36).

In contrast to p21-activated kinases, GCK-like STE20s such
PSK1-α Regulates Apoptotic Morphology

as PSK1-α lack a Cdc42/Rac-interacting domain, and much less is known about their activation and regulation. We have made an antibody that detects activated and phosphorylated forms of PSK1-pS181. The microtubule-stabilizing and apoptotic agent paclitaxel stimulated the activation of endogenous PSK1 at 165 and 85 kDa. The presence of both the 165- and 85-kDa forms of PSK1-pS181 in the nucleus suggests that these phosphoproteins are truncated and lack the microtubule-binding domains found in the C terminus of PSK1-α. The nuclear forms of PSK1-α did not possess a detectable C terminus in contrast to full-length PSK1-α, which was found on microtubules.

The nuclear localization of PSK1-α appeared to be important for its pro-apoptotic function. PSK1-α contains a putative caspase (912-DPGD) recognition motif, and although PSK1-α (D919N) stimulated JNK, its ability to localize to the nucleus and induce apoptosis was impaired, thereby correlating nuclear localization with apoptosis. PSK1-α contains two putative nuclear localization sequences at positions 605 and 633, and these sequences are present in the C-terminally truncated mutant PSK1-α-(1–745), which localizes to the nucleus. Our results suggest that removal of the C terminus of PSK1-α, containing the microtubule-binding region, results in its translocation to the nucleus, and this was dependent on its catalytic activity and JNK activation. Another GCK-like STE20, MST1, is excluded from the nucleus and localizes to the cytoplasm due to the presence of two functional nuclear export signals in its C terminus (25). Caspase-mediated cleavage of MST1, which is JNK-dependent, releases these sequences and produces an activated N-terminal kinase fragment that localizes to the nucleus (41). PSK1-α and MST1 therefore appear to be retained in the cytoplasm via different mechanisms, PSK1-α because of its C-terminal microtubule-binding region that tethers the protein to the cytoskeleton and MST1 because of its C-terminal nuclear export signals. Interestingly, the STE20-like kinase (SLK) induces apoptosis and contains a recognition motif that is cleaved by caspases to produce an N-terminal kinase domain (23). SLK can associate indirectly with microtubules, and it seems plausible that this protein might be regulated in a similar manner to PSK1-α (29).

Caspases cleave a number of GCK-like STE20s, including MST2, MST3, SLK, hematopoietic progenitor kinase 1, and the related kinase PSK2, and we found that PSK1-α was also a substrate for these proteolytic enzymes (18, 20, 21, 23, 28, 33). Stauroporine, a broad spectrum inhibitor of protein kinases, down-regulated the 165-kDa form of endogenous PSK1-pS181 but stimulated the appearance of a phosphorylated N-terminal fragment of PSK1 at 70 kDa that was blocked by Z-VAD-fmk. JNK has been shown to act upstream of the mitochondrial death pathway leading to the release of cytochrome c and caspase 9 activation, and we found that dominant negative JNK or the caspase 9 inhibitor Z-LEHD-fmk blocked nuclear localization of PSK1-α and its induction of apoptosis (36). PSK1-α could therefore stimulate JNK, leading to activation of caspase 9 and induction of apoptosis, whereby an N-terminally active PSK1 is required additionally in the nucleus. Recent studies have demonstrated that, upon JNK activation, MST1 is cleaved by caspase 3 and subsequently translocates to the nucleus where it phosphorylates histone H2B (42). Histone H2B has been shown to be essential for chromatin condensation, another hallmark for apoptosis (42, 43).

Little is known of how membrane blebbing is initiated during apoptosis, and it remains to be determined what potential nuclear substrate(s) PSK1-α may phosphorylate that lead to blebbing. However, we have found that PSK1-α-induced blebbing was mediated by ROCK-I, a serine/threonine kinase known to stimulate actomyosin-based contractility, which is important for bleb formation in apoptotic cells and is activated by caspase 3-dependent cleavage (data not shown) (26, 27, 44). Caspase substrates involved in cell contraction remain to be determined, although ROCK-I has also been implicated in this process in some cell types. We found that inhibition of ROCK-I activity using Y27632 had only a partial blocking effect on the induction of cell contraction by PSK1-α (data not shown), and taken together with the modest effect observed using Z-VAD-fmk, our results suggest that PSK1-α-induced cellular contraction may be initiated by caspase 9 activation.

In conclusion, further to our previous studies that identified a functional role for the N-terminal kinase domain of PSK1-α as a JNK activator (4), we have demonstrated that PSK1-α utilizes this pathway to induce apoptosis. The C-terminal microtubule-binding and regulatory domain of PSK1-α prevents its N-terminal kinase domain from entering the nucleus, and our results suggest that PSK1-α-induced JNK and caspase activation can lead to cleavage of PSK1-α. Subsequent translocation of C-terminally truncated forms of PSK1-α to the nucleus was required, as well as ROCK-I activation, for the blebbing process to occur. Cytoplasmic PSK1-α can still induce cell contraction, which is caspase-dependent. Almost 30 mammalian STE20s have been identified to date, and PSK1-α is the second member of the STE20-like superfamily that affects one of the hallmarks of apoptosis through nuclear localization. MST1 affects chromatin condensation, and we have now shown that PSK1-α induces membrane blebbing, raising the intriguing possibility that a subdivision of STE20-like kinases, in addition to caspase activation, may cooperate through nuclear activity to regulate apoptosis. Further understanding of the role of PSK1-α in the nucleus and potentially other members of the STE20-like kinases is likely to provide important insights into the mechanisms of apoptosis.

Acknowledgments—We thank Shaun Thomas, Ritu Garg, Mark Shipman, and Ron Senkus for many helpful discussions.

REFERENCES

1. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) Trends Cell Biol. 11, 220–230
2. Daniels, R. H., and Bokoch, G. M. (1999) Trends Biochem. Sci. 24, 350–355
3. Bagrodia, S., and Cerione, R. A. (1999) Trends Cell Biol. 9, 350–355
4. Moore, T. M., Garg, R., Johnson, C., Coptcoat, M. J., Ridley, A. J., and Morris, J. D. (2003) J. Biol. Chem. 278, 4311–4322
5. Mitsopoulos, C., Zihni, C., Garg, R., Ridley, A. J., and Morris, J. D. (2003) J. Biol. Chem. 278, 18085–18091
6. Davis, R. J. (2000) Cell 103, 239–252
7. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
8. Vaux, D. L., and Korsmeyer, S. J. (1999) Cell 96, 245–254
9. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424
10. Nunez, G., Benedict, M. A., Hu, Y., and Inohara, N. (1998) Oncogene 17, 3237–3245
11. Gervais, F. G., Thornberry, N. A., Ruffolo, S. C., Nicholson, D. W., and Roy, S. (1998) J. Biol. Chem. 273, 17102–17108
12. Levkau, B., Herren, B., Koyama, H., Ross, R., and Raines, E. W. (1998) J. Exp. Med. 187, 579–586
13. Wen, L. P., Fahrni, J. A., Troie, S., Guan, J. L., Orth, K., and Rosen, G. D. (1997) J. Biol. Chem. 272, 26056–26061
14. Widmann, C., Gibson, S., and Johnson, G. L. (1998) J. Biol. Chem. 273, 7141–7147
15. Boulares, A. H., Yakovlev, A. G., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S., and Smulson, M. (1999) J. Biol. Chem. 274, 22932–22940
16. Flygare, J., Armstrong, R. C., Wennborg, A., Orsan, S., and Hellgren, D. (1998) FEBS Lett. 427, 247–251
17. Schwab, B. L., Leist, M., Knippers, R., and Nicotera, P. (1998) J. Exp. Med. 187, 579–586
18. Chen, Z., Hutchison, M., and Cobb, M. H. (1999) J. Biol. Chem. 274, 28803–28807
19. Deng, Y., Pang, A., and Wang, J. H. (2003) J. Biol. Chem. 278, 11760–11767
20. Huang, C. Y., Wu, Y. M., Hsu, C. Y., Lee, W. S., Lai, M. D., Lu, T. J., Huang, C. L., Leu, T. H., Shih, H. M., Fang, H. I., Robinson, D. R., Kung, H. J., and Yuan, C. J. (2002) J. Biol. Chem. 277, 34367–34374
21. Lee, K. K., Ohyama, T., Yajima, N., Tsubuki, S., and Yonehara, S. (2001) J. Biol. Chem. 276, 19276–19285
22. Sabourin, L. A., and Rudnicki, M. A. (1999) Oncogene 18, 7566–7575
23. Sabourin, L. A., Tamai, K., Seale, P., Wagner, J., and Rudnicki, M. A. (2000) Mol. Cell. Biol. 20, 684–696
24. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) Nature 391, 43–50
25. Tsuruta, F., Sunayama, J., Mori, Y., Hattori, S., Shimizu, S., Tsujimoto, Y., Yoshioka, K., Masuyama, N., and Gotoh, Y. (2004) EMBO J. 23, 1889–1899
26. Cheung, W. L., Ajiro, K., Samejima, K., Kloc, M., Cheung, P., Mizzen, C. A., Beeser, A., Etkin, L. D., Chernoff, J., Earnshaw, W. C., and Allis, C. D. (2002) Nat. Cell Biol. 3, 339–345
27. Sebbagh, M., Renvoise, C., Hamelin, J., Riche, N., Bertoglio, J., and Breard, J. (2001) Nat. Cell Biol. 3, 546–552
28. Zihni, C., Mitsopoulos, C., Tavares, I. A., Ridley, A. J., and Morris, J. D. (2006) J. Biol. Chem. 281, 7317–7323
29. Wagner, S., Flood, T. A., O’Reilly, P., Hume, K., and Sabourin, L. A. (2002) J. Biol. Chem. 277, 37685–37692
30. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346–347
31. Zhou, T., Raman, M., Gao, Y., Earnest, S., Chen, Z., Machius, M., Cobb, M. H., and Goldsmith, E. J. (2004) Structure (Camb.) 12, 1891–1900
32. Sabourin, L. A., Tamai, K., Seale, P., Wagner, J., and Rudnicki, M. A. (2000) Mol. Cell. Biol. 20, 684–696
33. Lee, K. K., Murakawa, M., Nishida, E., Tsubuki, S., Kawashima, S., Sakamaki, K., and Yonehara, S. (1998) Oncogene 16, 3029–3037
34. Dan, I., Ong, S. E., Watanabe, N. M., Blagoev, B., Nielsen, M. M., Kajikawa, E., Kristiansen, T. Z., Mann, M., and Pandey, A. (2002) J. Biol. Chem. 277, 5929–5939
35. Tsuruta, F., Sunayama, J., Mori, Y., Hattori, S., Shimizu, S., Tsujimoto, Y., Yoshioka, K., Masuyama, N., and Gotoh, Y. (2004) EMBO J. 23, 1889–1899
36. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998) Science 288, 870–874
37. Whitmarsh, A. J., and Davis, R. J. (1998) Trends Biochem. Sci. 23, 481–485
38. Whitmarsh, A. J., Cavanagh, J., Yasuda, J., and Davis, R. J. (1999) Mol. Cell. Biol. 19, 7245–7254
39. Kelkar, N., Standen, C. L., and Davis, R. J. (2005) Mol. Cell. Biol. 25, 2733–2743
40. Lee, K. K., and Yonehara, S. (2002) J. Biol. Chem. 277, 12351–12358
41. Cheung, W. L., Ajiro, K., Samejima, K., Kloc, M., Cheung, P., Mizzen, C. A., Beeser, A., Etkin, L. D., Chernoff, J., Earnshaw, W. C., and Allis, C. D. (2003) Cell 113, 507–517
42. de la Barre, A. E., Angelov, D., Molla, A., and Dimitrov, S. (2001) EMBO J. 20, 6383–6393
43. Leverrier, Y., and Ridley, A. J. (2001) Nat. Cell Biol. 3, E91–93