Sterile 20 (STE20) protein kinases, which include germinal center kinases and p21-activated protein kinases, are known to activate mitogen-activated protein kinase pathways (c-Jun NH2-terminal kinase, p38, or extracellular signal-regulated kinase), leading to changes in gene transcription. Some STE20s can also regulate the cytoskeleton, and we have shown that the germinal center kinase-like kinase prostate-derived STE20-like kinase (PSK) affects actin cytoskeletal organization. Here, we demonstrate that PSK colocalizes with microtubules, and that this localization is disrupted by the microtubule depolymerizing agent nocodazole. The association of PSK with microtubules results in the production of stabilized perinuclear microtubule cables that are nocodazole-resistant and contain increased levels of acetylated α-tubulin. Kinase-defective PSK (K57A) or the C terminus of PSK (amino acids 745–1235) lacking the kinase domain are sufficient for microtubule binding and stabilization, demonstrating that the catalytic activity of the protein is not required. The localization of PSK to microtubules occurs via its C terminus, and PSK binds and phosphorylates α- and β-tubulin in vitro. The N terminus of PSK (1–940) is unable to bind or stabilize microtubules, demonstrating that PSK must associate with microtubules for their reorganization to occur. These results demonstrate that PSK interacts with microtubules and affects their organization and stability independently of PSK kinase activity.

The sterile 20 (STE20)1-like kinases are a large group of proteins whose catalytic domains have homology to the STE20 kinase of Saccharomyces cerevisiae involved in mitogen-activated protein kinase (MAPK) signaling of the pheromone response in yeast (1). More than 30 mammalian STE20-like kinases have been identified, and these proteins divide into two subfamilies according to their structure and regulation (reviewed in Ref. 2). The p21-activated kinases (PAKs) have a C-terminal catalytic domain and an N-terminal Cdc42/Rac interacting domain (CRIB), whereas the germinal center kinase (GCK)-like kinases possess an N-terminal catalytic domain and no CRIB. Most STE20s can act as MAPK kinase kinase kinases, upstream of the c-Jun N-terminal kinases (JNK), p38 and/or extracellular signal-regulated kinases (ERK) MAPK signaling pathways.

Recent work has demonstrated that some STE20s can also regulate the cell cytoskeleton. PAKs interact with Rac or Cdc42 GTPases via the CRIB domain and act as downstream effectors for these small GTP binding proteins to regulate the actin cytoskeleton (reviewed in Refs. 4, 5). Rac and Cdc42 stimulated morphological rearrangements are blocked by mutated PAK, and activated PAK down-regulates actin stress fibers and focal complexes (6–8). Some of the effects of PAK1 can be attributed to the phosphorylation of downstream kinases, such as myosin light chain kinase, which reduces its activity toward myosin light chain, and LIM kinase 1, which phosphorylates and inactivates the actin depolymerizing protein coflin to generate actin clusters (9, 10). X-PAK5 co-localizes to actin and microtubules (MTs) and produces stabilized MTs that are associated in bundles, and PAK1 accumulates at the MT-organizing center and along mitotic spindles during mitosis (11, 12).

Although GCK-like STE20s lack a CRIB domain, recent work has demonstrated that members of this subfamily of STE20s can also regulate the actin cytoskeleton. Proline- and alanine-rich STE20-related kinase associates with actin, and prostate-derived STE20-like kinase (PSK), Traf2- and Nek-interacting kinase, and STE20-like kinase (SLK) decrease actin stress fibers and focal adhesions and inhibit cell spreading (3, 13–15). SLK has recently been shown to associate with MTs at the cell periphery (16).

There is increasing evidence that actin filaments and MTs are coordinately regulated during the establishment and maintenance of cell polarity, division, and motility. The ability of MTs to undergo transitions between growth, shrinkage and pause are crucial for their function and the regulation of these processes. MT dynamics and stability are controlled by multiple factors, which include MT-associated proteins (MAPs), MT-affinity regulated kinases, severing factors (e.g. katanin), and catastrophe proteins (e.g. stathmin/OP18 and XKCM1) (reviewed in (17)). MAPs provide a focal point for MT regulation and act as structural proteins that lack enzymatic activity but promote the assembly of tubulin and MT stability. The affinity of MAPs for MTs is controlled by their phosphorylation, which causes the detachment of MAPs from MTs and results in MT

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The Prostate-derived Sterile 20-like Kinase (PSK) Regulates Microtubule Organization and Stability*
instability. A number of kinases can phosphorylate MAPs and thereby modulate MT stability (reviewed in Ref. 17). In contrast, phosphorylation of the MTdestabilizing protein stathmin inactivates the protein and stabilizes MTs (reviewed in Ref. 18). Several kinases, including PAK, modulate the phosphorylation of stathmin and may therefore regulate tubulin polymerization and MT stability (19). The signaling pathways and components that regulate MT dynamics remain to be determined, but small GTP binding proteins and their downstream targets, which include STE20-like kinases, seem to play crucial roles in controlling MT function.

Here we show that a recently identified member of the GCK-like family of STE20s, PSK (3), colocalizes with MTs and produces stabilized perinuclear MT cables that are nocodazole-resistant and contain increased levels of acetylated α-tubulin. These findings suggest that a major function for PSK is to transduce signals to the MT network.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pRK5PSK and pRK5PSK (K57A) were made previously (3). PSK (1–940) and PSK (745–1235) were prepared by PCR amplification using appropriate oligonucleotides and pRK5PSK as substrate, and DNA products were ligated into the pRK5 vector, which provides a 5′-MYC-epitope tag.

**Microinjection**—Cells were grown in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium. For microinjection, cells were seeded on glass coverslips and incubated for 2 days. Where appropriate, cells were serum-starved for 16 h before microinjection. Endotoxin-free plasmid DNA (100 ng/μl in phosphate-buffered saline; Qiagen) was microinjected into the nuclei of ~100 cells per coverslip. After 3–4 h, cultures were fixed for 20 min with 4% paraformaldehyde, 10% Me2SO, and 1% glucose in phosphate-buffered saline (37 °C) and permeabilized with 0.2% Triton X-100 for 5 min.

**Immunolocalization**—To detect MYC-tagged PSK, cells were incubated with 1:400 rabbit anti-MYC tag antibody (Santa Cruz Biotechnology) followed by 1:400 fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). To visualize tubulin, cells were incubated with either 1:100 mouse Cy3-conjugated

**FIG. 1.** PSK localizes to MTs. Growing Swiss 3T3 cells were microinjected with pRK5-MYC-PSK (wild type) and after 3.5 h, cells were treated with (C and D) or without (A and B) 5 μM nocodazole for 30 min and then fixed and costained to detect MYC-PSK (A and C) and MTs (B and D). Cells become vesiculated under these conditions. Scale bar, 20 μm.

**FIG. 2.** PSK constructs prepared for functional characterization. The kinase domain is shown in dark gray and the amino acids are indicated by numbers.
anti-β-tubulin antibody (TUB2.1; Sigma) or 1:100 mouse anti-α- tubulin antibody (DM1A; Sigma), followed by 1:400 TRITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories). To detect acetylated α-tubulin, cells were incubated with 1:100 mouse anti-acetylated-α-tubulin antibody (6–11B-1; Sigma) followed by 1:400 Alexa633-conjugated goat anti-mouse IgG (Molecular Probes). All antibodies were incubated in 20% fetal calf serum/phosphate-buffered saline. Cells were imaged with a confocal laser scanning microscope (Bio-Rad 1024). Image processing was performed using Adobe Photoshop 5.5.

Transfection—Human embryonic kidney 293 (1.3 × 10^5) or COS-1 (5 × 10^5) cells were seeded onto 80-mm Petri dishes and grown for 16 h before the indicated plasmids (3 μg) were transfected into cells with Lipofectin (Invitrogen). After 5 h, transfected cultures were returned to growth medium and incubated for 36 h.

Immunoblotting—Cells were lysed in lysis and binding 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 Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris, pH 7.4). 100 μg of each sample was analyzed by SDS-PAGE before proteins were transferred to nitrocellulose (Schleicher & Schuell). Samples were processed as described previously (3), except that rabbit anti-PSK serum was used as the primary antibody (raised against the amino acid sequences GTLAGRRSRTRQSSRALPPWR and EEEEAPIGTPRDPGDGC for PSK; Sigma Genosys).

Immunoprecipitation—Cells were lysed as described above, and 400 μg of protein were incubated with rabbit anti-PSK serum at 4 °C. Protein A-Sepharose beads (Sigma) were added to each sample, and after 1 h, beads were pelleted by centrifugation and washed three times in lysis and binding buffer.

In Vitro Kinase Assays—Beads were washed once in kinase buffer (20 mM Tris-HCl pH 7.4, 4 mM MgCl_2) and placed in 30 μl of kinase buffer containing 40 μM ATP, 5 μCi of γ-[^32P]ATP (Amersham Biosciences) and 1 μg of bovine α- and β-tubulin (Cytoskeleton), which predominantly form unpolymerized tubulin dimers under these conditions. Af-
and stained to detect MYC-PSK (Fig. 5). Swiss 3T3 cells were microinjected with pRK5-MYC-PSK (lanes 1 and 2), pRK5-MYC-PSK (K57A) (lanes 3, preblocked with 20% goat serum in phosphate-buffered saline overnight. Samples were incubated in 1 ml of kinase buffer (1 h, 4 °C), washed three times in kinase buffer, and separated using SDS-PAGE/6 M urea. α- and β-tubulin were detected by immunoblotting and enhanced chemiluminescence.

RESULTS

PSK Localizes to Microtubules—We have reported previously that PSK, a GCK-like kinase, activates the JNK MAPK pathway and regulates the actin cytoskeleton, and it showed a punctate or filamentous distribution when expressed in fibroblasts (3). To investigate the subcellular distribution of PSK in more detail, expression vectors encoding MYC-epitope tagged PSK were microinjected into the nuclei of Swiss 3T3 cells, and PSK was found to localize to a filamentous network in the cytoplasm of injected cells that closely resembled MTs (Fig. 1A). The localization of PSK to MTs was confirmed by costaining injected cells for PSK and the MT component β-tubulin (Fig. 1B). The immunofluorescence from PSK was more intense from MTs positioned around the nucleus than from MTs at the periphery of the cell, which appeared fragmented (Fig. 1, A and B). The staining patterns for both PSK and β-tubulin were disrupted by addition of the MT-depolymerizing agent nocodazole (5 µM), demonstrating that PSK and β-tubulin are closely associated (Fig. 1, C and D). Moreover, the fixation of cells in formaldehyde, not paraformaldehyde/Me2SO, resulted in the disruption of MTs and a tubulovesicular staining pattern for PSK (3). Costaining of injected cells for PSK and markers for other cytoskeletal structures (vimentin and actin) demonstrated that PSK localized specifically to the MT network (data not shown).

PSK Localizes to MTs via its C Terminus and Alters MT Organization—To determine whether the catalytic activity and/or specific sequences outside of the kinase domain of PSK are involved in localizing PSK to MTs, we prepared expression vectors encoding MYC-tagged full-length kinase-defective PSK (K57A) and the C terminus of PSK (amino acids 745–1235) for comparison with wild-type PSK and the N terminus of PSK (amino acids 1–940) containing the kinase domain (Fig. 2). PSK and kinase-defective PSK (K57A) both colocalized with β-tubulin (Fig. 3, A–D) showing that the catalytic activity of the protein is not required for its localization. PSK (745–1235), which lacks the entire kinase domain, also localized to MTs (Fig. 3, E and F), whereas PSK (1–940), containing the kinase domain, failed to localize to MTs and was cytoplasmic (Fig. 3, G and H). Taken together, these results demonstrate that the C terminus of PSK is both necessary and sufficient for PSK to localize to the MT network, and this process occurs independently of the protein’s catalytic activity.

The localization of PSK to MTs resulted in a significant change in MT organization. PSK generated MT cables around the nucleus that were absent in uninjected surrounding cells (Fig. 3, A and B). PSK (K57A) and PSK (745–1235), which both localize to MTs, also produced perinuclear MT cables (Fig. 3, C–F), and PSK was predominantly localized on these structures. Moreover, MTs appeared to be thicker in the presence of PSK, and co-staining for injected PSK and pericentrin, a marker for the MT-organizing center, demonstrated that the MT-organizing center was retained in cells expressing PSK (data not shown). PSK (1–940), which is catalytically active, failed to produce these MT structures, demonstrating that PSK must directly associate with MTs via its C terminus for their reorganization to occur (Fig. 3, G and H).

PSK Stabilizes MTs and Increases Acetylation of α-Tubulin—The ability of PSK to reorganize MTs into perinuclear cables raised the possibility that PSK might also alter the stability of MTs. To determine whether PSK could affect MT
stability, PSK was microinjected into cells, and nocodazole (0.5 μM) was added to the culture medium for 30 min. PSK stabilized MTs in the presence of nocodazole and prevented the loss of MT structures observed in surrounding uninjected cells (Fig. 4, A and B). The two other forms of PSK that localize to MTs, kinase-defective PSK (K57A) and PSK (745–1235), also produced nocodazole-resistant MTs, and PSK was found wherever MTs remained (Fig. 4, C–F). MT stabilization by PSK therefore requires protein localization but not catalytic activity, and this was again illustrated by the inability of PSK (1–940) to protect MTs from nocodazole (Fig. 4, G and H).

Tubulin undergoes several post-translational modifications, including the acetylation of α-tubulin on lysine residue 40; although this modification is a consequence, not a cause, of stabilization, the acetylation of α-tubulin is frequently used to distinguish between stable and dynamic forms of MTs (reviewed in Ref. 20). We therefore examined the effects of PSK on the levels of acetylated α-tubulin using anti-acetylated α-tubulin antibodies. The three forms of PSK that localize to MTs (PSK, kinase-defective PSK (K57A), and PSK (745–1235)) each increased levels of acetylated α-tubulin above those in neighboring uninjected cells (Fig. 5, A–F). The N-terminal kinase domain of PSK (1–940), which was unable to associate with MTs or generate nocodazole-resistant MTs, failed to stimulate the acetylation of tubulin (Fig. 5, G and H). Serum starvation of Swiss 3T3 cells seemed to result in a more cytoplasmic localization for full-length PSK, whereas C-terminal PSK (745–1235) remained tightly associated with the MTs under identical conditions (Fig. 5, A and E). Nocodazole-resistant and acetylated MTs are increased in Swiss 3T3 fibroblasts after serum stimulation (21, 22); however, the PSK effects observed here occurred in the absence of serum.

**PSK Binds Tubulin in Vitro**—Comparison of the C-terminal regulatory domain of PSK with other sequences present in the GenBank data base has shown that PSK does not share significant homology with any other proteins or with the MT-binding domains reported for MAPs, which contain a proline-rich sequence and three pseudorepeats (reviewed in Ref. 17). The ability of the C terminus of PSK (745–1235) to localize to MTs and produce stabilized perinuclear MT cables led us to investigate whether these sequences were able to interact with α- or β-tubulin in vitro. A C-terminal fragment of PSK fused to glutathione-S-transferase (GST) was prepared, and recombinant PSK (amino acids 1064–1235) was able to bind α- and β-tubulin in vitro, suggesting that PSK may interact directly with MTs (Fig. 6, lanes 1–3).

**Taxol Dissociates PSK from MTs**—The ability of PSK to bind and stabilize MTs led us to examine the effect of Taxol-stabilized MTs on PSK. Swiss 3T3 cells expressing PSK were incubated in the presence of Taxol for 30 min, and cultures were fixed and stained for PSK and MTs. Fig. 7 shows that the addition of Taxol causes PSK to dissociate from MTs and become cytoplasmic (Fig. 7, A and B), whereas the MTs formed a separate ring around the nucleus (Fig. 7, C and D). These results demonstrate that PSK can respond to Taxol-induced changes in MT dynamics and that PSK might dissociate from MTs that are sufficiently stable.

**PSK Phosphorylates Tubulin**—The localization of PSK to MTs brings the kinase into the local vicinity of a number of potential MT-associated substrates that are regulated by phosphorylation, and these proteins could lead to additional affects of PSK on MTs. Because PSK binds tubulin, we investigated the ability of PSK to phosphorylate tubulin. Plasmids encoding PSK or PSK (K57A) were transiently transfected into cell cultures, and immune complexes of each protein were assayed for in vitro kinase activity using α- and β-tubulin as substrates. The isoforms of tubulin were separated from each other using SDS-PAGE/6 M urea, and we found that PSK, but not kinase-defective PSK (K57A), phosphorylated α- and β-tubulin (Fig. 8A, lanes 2 and 3).

Interestingly, addition of the MT-stabilizing agent Taxol rapidly down-regulated the ability of PSK and, to a lesser extent...
PSK (1–940), to phosphorylate tubulin, demonstrating that the catalytic activity of PSK responds to Taxol-induced changes in MT stability (Fig. 8B, lanes 2, 3, 5, and 6). In addition, transfected PSK, which is normally expressed in cells as a protein doublet of 185 and 165 kDa (3), was converted to the smaller form of the protein in the presence of Taxol (Fig. 8B, lanes 2 and 5). Because 9E10 antibody detects the N-terminal MYC-epitope tag on both forms of PSK (3), it is likely that the 185-kDa form of the protein is either truncated at the C terminus to generate a protein of 165 kDa that potentially lacks MT localization sequences; alternatively, PSK might undergo a change in post-translational modification. In contrast, nocodazole had no effect on either the kinase activity or mobility of PSK (data not shown).

DISCUSSION

We have shown that the STE20-like kinase PSK localizes to MTs and produces stabilized perinuclear MT cables that are nocodazole-resistant and contain increased levels of acetylated α-tubulin. The N-terminal kinase domain of PSK (1–940) is unable to bind MTs or regulate their organization or stability, demonstrating that the association between PSK and MTs is required for these MT alterations to occur. The C terminus of PSK (745–1235) contains the MT localization and regulatory domain and recombinant PSK (1064–1235) binds α- and β-tubulin in vitro. The catalytic activity of PSK is not required for these effects on MTs because kinase-defective PSK (K57A) associates with MTs and regulates their organization and stability. The MT-stabilizing drug Taxol dissociates PSK from MTs and down-regulates the protein’s catalytic activity, suggesting that PSK is sensitive to Taxol-induced changes in MT stability.

The stabilized perinuclear MT cables generated by PSK are similar to the MT structures produced by another STE20, X-PAK5, which binds MTs and induces stabilized MTs that form whorls around the nucleus (11). X-PAK5, like PSK, binds to MTs via its non-catalytic regulatory domain, and neither the stabilization nor the reorganization of MTs require the protein’s catalytic activity (11). The Rho effector Rho kinase α and DCAMKL1 also generate MT clusters around the nucleus independently of their kinase activity, and another non-enzymatic protein, mDia, co-localizes and stabilizes MTs oriented toward the wound edge (23, 24). Interestingly, the ability of X-PAK5 to bind MTs is negatively regulated by its kinase activity because constitutively activated X-PAK5 is unable to bind MTs and relocates to the cytoplasm, where it causes dissolution of actin stress fibers and cell retraction (11). PSK also down-regulates actin stress fibers and induces cell rounding, and another STE20 SLK associates with MTs located at adhesion sites and reduces actin stress fibers (3, 16). Interestingly, the catalytic activity of PSK is required for its effects on actin, and it is plausible that the kinase activity of PSK might act in a manner similar to X-PAK5 and regulate its localization and function (3). How PSK kinase activity is regulated in vivo is not known, but we have been unable to detect clear differences between the localization of PSK and kinase-defective PSK (K57A) in injected cells. PSK does, however, become more cytoplasmic when cells are starved of serum, whereas the C terminus of PSK (745–1235) lacking the kinase domain, remains tightly bound to MTs. The identification of upstream regulators for PSK and its kinase activity will be required to explore these possibilities further.

Catalytic activity is not required by PSK to re-organize and stabilize MTs, but the localization of PSK to MTs would be expected to bring the kinase into the vicinity of a number of potential MT-associated substrates. PSK could therefore have additional regulatory affects on MTs that require its catalytic activity. PSK phosphorylates α- and β-tubulin, and others have shown that Syk and Src phosphorylate tubulin (25, 26). The physiological significance of this modification and the sites of phosphorylation are unknown, but it has been suggested that the phosphorylation of tubulin could alter its binding properties to signaling proteins via SH2 domain-mediated interactions and/or regulate the accessibility of kinases to their substrates (25, 26).

Although a number of kinases can regulate the activity and function of MT components, such as MAPs, by phosphorylation, only a few kinases have been shown to localize to MTs. Interestingly, MT-associated kinases include several components of MAPK signaling pathways, such as JNK and ERK (MAPKs), MAP kinase/ERK kinase (MEK), mixed-lineage kinase, X-PAK5 and SLK (MAPK kinase kinase kinases) (11, 16, 27–29). PSK can activate JNK (3), as can its rat homolog, Tao2 (30), and it is therefore possible that this occurs on MTs. Whether JNK is involved in regulating MT stability remains to be determined.
be determined, but JNK, ERK, p38, and glycogen synthase kinase-3β can each phosphorylate MAPs, such as tau, potentially regulating its affinity for MTs and their stability (31). Moreover, the MT-stabilizing agent Taxol activates JNK via mixed-lineage kinases such as MAP kinase/ERK kinase 1 and apoptosis-regulating kinase 3 (32, 33), but our finding that Taxol down-regulates the activity, expression, and localization of PSK indicates that PSK is unlikely to contribute to Taxol-induced JNK activation. Interestingly, ERK kinase activity has been shown to decrease the stability of MTs (34). In contrast, induced JNK activation. Interestingly, ERK kinase activity has been shown to decrease the stability of MTs (34). In contrast, induced JNK activation.

Moreover, the MT-stabilizing agent Taxol activates JNK via partially regulating its affinity for MTs and their stability (31). MT disassembly stimulates Rho and the formation of JNK-interacting proteins (37, 38).

In conclusion, MTs play important roles in the regulation of cell morphogenesis, division, migration, and vesicle trafficking, and the ability of MTs to undergo changes in their stability and dynamics are crucial for their function. Here we show for the first time that a member of the GCK family of protein kinases, which lack CRIB domains, can localize to MTs and regulate their stability and organization. These findings imply a functional role for PSK, and perhaps other GCKs, in the regulation of MT dynamics.

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