PSK, a Novel STE20-like Kinase Derived from Prostatic Carcinoma That Activates the c-Jun N-terminal Kinase Mitogen-activated Protein Kinase Pathway and Regulates Actin Cytoskeletal Organization*

(Received for publication, July 28, 1999, and in revised form, November 2, 1999)

Tanya M. Moore‡, Ritu Garg§, Caroline Johnson‡, Malcolm J. Coptcoat‡, Anne J. Ridley§, and Jonathan D. H. Morris¶

From the ‡Molecular Oncology Laboratory, Department of Academic Surgery, King’s College School of Medicine and Dentistry, Rayne Institute, 123 Coldharbour Lane, London SE5 9NU, the §Ludwig Institute for Cancer Research (University College London Branch), 91 Riding House Street, London W1P 8BT, and the ¶Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom

Degenerate polymerase chain reaction against conserved kinase catalytic subdomains identified 15 tyrosine and serine-threonine kinases expressed in surgically removed prostatic carcinoma tissues, including six receptor kinases (PDGFR, IGF1-R, VEGFR2, MET, RYK, and EPH-A1), six non-receptor kinases (ABL, JAK1, JAK2, TYK2, PLK-1, and EMK), and three novel kinases. Several of these kinases are oncogenic, and may function in the development of prostate cancer. One of the novel kinases is a new member of the sterile 20 (STE20) family of serine-threonine kinases which we have called prostate-derived STE20-like kinase (PSK) and characterized functionally. PSK encodes an open reading frame of 3765 nucleotides and contains an N-terminal kinase domain. Immunoprecipitated PSK phosphorylates myelin basic protein and transfected PSK stimulates MKK4 and MKK7 and activates the c-Jun N-terminal kinase mitogen-activated protein kinase pathway. Microinjection of PSK into cells results in localization of PSK to a vesicular compartment and causes a marked reduction in actin stress fibers. In contrast, C-terminally truncated PSK (1–349) did not localize to this compartment or induce a decrease in stress fibers demonstrating a requirement for the C terminus. Kinase-defective PSK (K57A) was unable to reduce stress fibers. PSK is the first member of the STE20 family lacking a Cdc42/Rac binding domain that has been shown to regulate both the c-Jun N-terminal kinase mitogen-activated protein kinase pathway and the actin cytoskeleton.

Cancer of the prostate is the most frequently diagnosed invasive cancer and the second most common cause of cancer-related death in males in North America (1, 2). Androgens play an important role in stimulating prostatic epithelial cell proliferation and initial treatment of patients with prostate cancer usually involves androgen ablation. Despite this, androgen-insensitive tumors often emerge after 2–3 years (3). The molecular basis for progression to cancer of the prostate is poorly understood, but there is increasing evidence that alterations in growth factor signaling pathways may be involved in the development of prostatic carcinomas. Epidermal growth factor (EGF),1 insulin-like growth factor 1 (IGF1), transforming growth factor α, interleukin-6 (IL-6), keratinocyte growth factor, and other fibroblast growth factors are all expressed in advanced prostate cancer providing potential autocrine growth factors for tumor cell growth (4–8). Furthermore, EGF, IGF1, and keratinocyte growth factor can transactivate the androgen receptor via nonsteroid transduction pathways, and alterations in expression of fibroblast growth factor receptor 11Hc, RET, Her2/neu, and ErbB1, -2, or -3 receptors have been detected in advanced prostate cancer (9–13). Activation of the mitogen-activated protein kinase (MAPK) pathways are also thought to play an important role in prostate cancer development with increased extracellular signal-regulated MAPK activation occurring with increased Gleason score and tumor stage (8, 14). The prostate cancer cell mitogen IL-6 also activates MAPK pathways and the androgen receptor (15).

The MAPK pathways play crucial roles in the regulation of a diverse array of responses including growth, differentiation, transformation, stress responses, and apoptosis, but their role in the development of prostate cancer remains to be determined (16–18). MAPK stimulation occurs via activation of a

1 The abbreviations used are: EGF, epidermal growth factor; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; TBS, Tris-buffered saline; IGF1, insulin-like growth factor 1; IGF1-R, insulin-like growth factor-1 receptor; PDGFR, platelet-derived growth factor B receptor; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, interleukin; MIP, myelin basic protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; CRIB, Cdc42/Rac binding domain; PSK, prostate-derived STE20-like kinase; RACE, rapid amplification of cDNA ends; MLC, myosin light chain; IL, inte...
FIG. 1. Identification of kinases expressed in three prostatic carcinoma tissues. The identity of each protein kinase is shown on the left of the aligned window of the deduced amino acid sequences encoded in PCR-amplified fragments. The number of individual isolates sequenced is shown on the right. Conserved amino acid sequences are shown in boldface and the conserved subdomains described by Hanks et al. (45) are indicated by Roman numerals (*).

MAPK kinase kinase (MAPKKK), which phosphorylates a dual specificity MAPK kinase (MAPKK), which in turn phosphorylates MAPK. The MAPKs currently consist of three related protein kinase cascades named after the final enzyme in each series; extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs)/stress-activated protein kinases, and p38s. The ERK cascade consists of ERK1 and -2 (MAPKs), MEK1 and -2 (MAPKKs), and A-RAF, B-RAF, and RAF-1 (MAPKKKs) and is predominantly involved in signaling cell growth, proliferation and differentiation. In contrast to ERK activation, pathways involved in the activation of JNK and p38 are less clear. The JNK cascade includes JNKs 1, 2, and 3 (MAPKs) and MKKs 4 and 7 (MAPKKs), while the p38 cascade contains p38α and β (MAPKs) and MKKs 3 and 6 (MAPKKs) (18–21). The JNK and p38 pathways are predominantly involved in the protective responses of cells to pro-inflammatory cytokines (e.g. tumor necrosis factor-α and IL-1) and cellular stresses (10, 16).

Recent attention has been focused on a family of serine-threonine kinases whose catalytic domain has homology to the sterile 20 (STE20) kinase of Saccharomyces cerevisiae involved in MAPK signaling of the pheromone response in yeast (26). Mammalian STE20-like kinases appear to act as MAPK kinase kinase kinases whose catalytic domain has homology to the sterile 20 (STE20) kinase family of protein kinases which we have called the STE20-like family of protein kinases (JNK and p38 pathways are predominantly involved in the signalling of the pheromone response in yeast (26). Mammalian STE20-like kinases appear to act as MAPK kinase kinase kinase (GCK), GCK-like kinase (GLK), hematopoietic progenitor kinase (HIPK), kinase homologous to SPS1/STE20 (KHS), lymphocyte-oriented kinase (LOK), STE20 oxidant stress response kinase (SOK), yeast SPS1/STE20-like kinase (YSK), and NCK interacting kinase (Nik) (27–41). MLK2 can also stimulate the ERK MAPK pathway, and PAks not only activate the JNK and p38 MAPK pathways but also regulate cytoskeletal organization stimulating disassembly of actin stress fibers and membrane ruffling (34, 42–44).

We set out to obtain a profile of kinases expressed in surgically removed prostatic carcinoma tissues (Gleason grades 6–8) using Rnazol B (Biogenesis). RNA (5 μg) was reverse-transcribed to cDNA using Superscript reverse transcriptase (Life Technologies, Inc.) primed with oligo(dT)17. PCR was performed using two degenerate primers 5'-CCTCGAGATCATC/T/GNGA/T/C/TG/T/C-3' and 5'-GAATTTGAATAA/GCGA/C/G/A/GA/T/C/TG-3' to target conserved sequences in subdomains VI and IX of the kinase catalytic domain (45, 46). PCR conditions were 93°C/90 s, 45°C/2 min, and 72°C/4 min for 30 cycles using 1 unit of Supertaq plus DNA polymerase (TaKaRa Bio) primed with oligo(dT)17. PCR was performed using two forward primers 5'-CTCGAGATCATC/T/GNGA/T/C/TG/T/C-3' and 5'-GAATTTGAATAA/GCGA/C/G/A/GA/T/C/TG-3' and a 534-bp product obtained and cloned into pGEM-T Easy vector (Promega). The 353-bp 3' RACE product was used to screen a Lambda Zap cDNA library prepared from T-47D carcinoma cells (Stratagene, kind gift from M. Crompton, ICR, London). Filters (Amersham Pharmacia Biotech) were hybridized at 65°C in a solution containing 5 μg/ml salmon testis DNA (Sigma) for 48 h. Stringency of the final wash was 1× SSPE, 0.1% (v/v) SDS at 65°C. CDNA clones were isolated from a screen of 1 × 10^8 phage clones and sequenced on both strands (MWG-Biotech). PCR to confirm the presence of PSK in human genomic DNA was carried out using a forward primer 5'-GACTACGTTGACATGACTTGGGCTGATC-3'. Nested PCR was carried out using two forward primers 5'-CTCGAGATCATC/T/GNGA/T/C/TG/T/C-3' and 5'-GAATTTGAATAA/GCGA/C/G/A/GA/T/C/TG-3' and a 534-bp product obtained and cloned into pGEM-T Easy vector (Promega). The 534-bp 3' RACE product was used to screen a Lambda Zap cDNA library prepared from T-47D carcinoma cells (Stratagene, kind gift from M. Crompton, ICR, London). Filters (Amersham Pharmacia Biotech, Hybond™-N+) were hybridized at 65°C in a solution containing 5 μg/ml salmon testis DNA (Sigma) for 48 h. Stringency of the final wash was 1× SSPE, 0.1% (v/v) SDS at 65°C. CDNA clones were isolated from a screen of 1 × 10^8 phage clones and sequenced on both strands (MWG-Biotech). PCR to confirm the presence of PSK in human genomic DNA was carried out using a forward primer 5'-CCTCGAGATCATGACTTGGGCTGATC-3'. Nested PCR was carried out using two forward primers 5'-CTCGAGATCATC/T/GNGA/T/C/TG/T/C-3' and 5'-GAATTTGAATAA/GCGA/C/G/A/GA/T/C/TG-3' and a 534-bp product obtained and cloned into pGEM-T Easy vector (Promega). The 353-bp 3' RACE product was used to screen a Lambda Zap cDNA library prepared from T-47D carcinoma cells (Stratagene, kind gift from M. Crompton, ICR, London). Filters (Amersham Pharmacia Biotech) were hybridized at 65°C in a solution containing 5 μg/ml salmon testis DNA (Sigma) for 48 h. Stringency of the final wash was 1× SSPE, 0.1% (v/v) SDS at 65°C. CDNA clones were isolated from a screen of 1 × 10^8 phage clones and sequenced on both strands (MWG-Biotech). PCR to confirm the presence of PSK in human genomic DNA was carried out using a forward primer 5'-GACTACGTTGACATGACTTGGGCTGATC-3'. Nested PCR was carried out using two forward primers 5'-CTCGAGATCATC/T/GNGA/T/C/TG/T/C-3' and 5'-GAATTTGAATAA/GCGA/C/G/A/GA/T/C/TG-3' and a 534-bp product obtained and cloned into pGEM-T Easy vector (Promega). The 353-bp 3' RACE product was used to screen a Lambda Zap cDNA library prepared from T-47D carcinoma cells (Stratagene, kind gift from M. Crompton, ICR, London). Filters (Amersham Pharmacia Biotech, Hybond™-N+) were hybridized at 65°C in a solution containing 5 μg/ml salmon testis DNA (Sigma) for 48 h. Stringency of the final wash was 1× SSPE, 0.1% (v/v) SDS at 65°C. CDNA clones were isolated from a screen of 1 × 10^8 phage clones and sequenced on both strands (MWG-Biotech).
frame was subcloned into a cytomegalovirus-based expression vector pRK5 containing a 5'-MYC-epitope tag. PSK (K57A) was prepared using the method of Kunkel et al. (47). Briefly, PSK (BamHI 23504 BamHI) was subcloned into pBluescript II SK and used to generate a mutated fragment using the oligonucleotide 5'-GAGGTGGTGGC- CATCGCGAAGATGATGTCCTACAG-3'. The resultant PSK (BamHI 233504 BamHI, K57A) fragment was ligated back into EfPlink-PSK (BamHI 3504–4492 XbaI).

PSK (amino acids 1–349) and PSK (1–349, K57A) were prepared by ligating the respective BamHI 21805 XhoI blunt-ended fragments of PSK into EfPlink MYC-tag cut with BamHI-EcoRI and containing an EcoRI blunt end.

**Northern Blotting**—A human multiple tissue Northern blot (MTN™, CLONTECH) was probed with an XhoI 1104–1800 XhoI-BamHI 3504–4492 XhoI fragment of PSK using the method described by the manufacturer.

**Transient Cell Expression, Immunoblotting, Immunoprecipitation, and Kinase Assays**—COS1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics (5%CO2, 37 °C). For transfection, 1.9 × 10^6 cells were seeded onto 100-mm Petri dishes and grown for 16 h before the indicated plasmids were co-transfected into COS1 cells for 5 h in the presence of 30 μl of Lipofectin/4 ml of Opti-MEM I. (Life Technologies, Inc.). Transfected cultures were transferred to 10 ml of growth medium and incubated for 48 h.

**Fig. 2.** Nucleotide and predicted amino acid sequences of PSK. Indicated are the Kozak sequence (broken line), start codon and stop codon (boldface), along with the kinase domain (underlined). The numbers to the left and right of the sequence refer to nucleotide and amino acid positions in the predicted protein, respectively.
For immunoblotting of whole cell lysates, cultures were lysed in 200 μl of gel sample buffer and 100 μg of each sample was separated by 10% or 15% SDS-PAGE before proteins were transferred to nitrocellulose (Schleicher & Schuell). MYC-tagged PSK or p38 proteins were detected using 9E10 anti-MYC tag antibody (Sigma), and FLAG-tagged JNK or ERK2 were detected using anti-FLAG tag M2 antibody (Sigma). Briefly, membranes were blocked with TBS (150 mM NaCl, 10 mM Tris, pH 7.4) containing 5% dried milk for 1 h at room temperature; incubated in TBS-Tween 20 (0.5%) containing either a 1/500 dilution of 9E10 or 5 μg/ml M2 for 1 h (room temperature); and finally incubated in TBS-Tween 20 (0.5%) containing 1/2000 anti-mouse horseradish peroxidase-conjugated antibody (Transduction Laboratories) and 1% dried milk for 1 h (room temperature). Membranes were processed using ECL according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

For JNK and p38 kinase assays, cells were lysed 48 h after transfection in 0.4 ml of lysis buffer A (1% (v/v) Triton X-100, 300 mM NaCl, 2.5 mM MgCl₂, 40 mM Na₄P₂O₇, 5 mM EGTA, pH 8.0, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, 0.1 mM Na₃VO₄, 3 mM phenylmethylsulfonyl fluoride, 25 mM Heps, pH 7.6). For ERK2 kinase assays or myelin basic protein (MBP) phosphorylation assays, cells were lysed in 0.4 ml of buffer B (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, 20 mM Tris, pH 8.0). Cell lysates were extracted for 10 min at 4 °C, and debris was removed by centrifugation (12,000 × g for 10 min at 4 °C).

For immunoprecipitation, 400 μg of protein were taken and made up to a final volume of 1 ml in binding buffer (50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, pH 8.0, 0.05% (v/v) Triton X-100, 40 mM Na₄P₂O₇, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 20 mM Heps, pH 7.6) for JNK or p38 kinase assays, or to 0.5 ml in buffer B for ERK2 and MBP phosphorylation assays. For JNK or ERK2 kinase assays, samples were incubated with 3 μg of anti-FLAG mouse monoclonal antibody for 1 h at 4 °C, and for MBP phosphorylation or p38 kinase assays, 3 μg of anti-MYC-tag

Fig. 3. A, alignment of the catalytic domain of PSK with those of other STE20 family members. The 11 catalytic subdomains are indicated above the alignment (I–XI). Identical amino acids are shown in black. B, diagram illustrating regions of homology between PSK and TAO1 or KIAA0881.
PSK, a Novel STE20-like Kinase That Regulates JNK/Actin

9E10 mouse monoclonal antibody was added for 1 h at 4 °C. 20 μl of protein G-Sepharose beads (Sigma; 1:1 in appropriate binding buffer) were added to each sample for incubation for another 1 h (4 °C), and beads were pelleted by centrifugation and washed two times in 0.5 ml of appropriate binding buffer.

For in vitro kinase assays, beads were washed in respective kinase buffers. JNK and p38 kinase assays were carried out in 30 μl of kinase buffer A (20 mM MgCl₂, 2 mM dithiothreitol, 20 mM p-nitrophenyl phosphate, 4 mM NaF, 0.1 mM Na₃VO₄, 20 mM Hepes, pH 7.6) containing 20 μM ATP, 5 μCi of [γ-³²P]ATP (Amersham Pharmacia Biotech, 3000 Ci/mmol) and either 3 μg of GST-c-Jun protein or 3 μg of GST-ATF2 protein, and incubated for 20 min or 30 min, respectively, at 30 °C. ERK2 or MBP kinase assays were carried out in 30 μl of kinase buffer B (20 mM MgCl₂, 2 mM MnCl₂, 30 mM Tris, pH 8.0) containing 0.25 mg/ml MBP, 10 μM ATP, and 1 μCi of [γ-³²P]ATP for 30 min at 30 °C. Kinase assays were terminated in gel sample buffer, heated to 100 °C for 5 min, and proteins separated by SDS-PAGE for transfer to nitrocellulose. Samples were processed by immunoblotting and analyzed by exposure to film or phosphorimaging (Fuji). Protein normalization and protein expression were confirmed for each sample by immunoblotting of cell lysates or immunoprecipitated material.

FLAG-tagged MKK3, MKK4, or MKK7 were transfected and immunoprecipitated as described for FLAG-tagged JNK except that recombinant GST-p38 (MKK3) or GST-JNK (MKK4 or -7) were used as substrate for in vitro kinase assays.

Microinjection, Immunolocalization, and Actin Detection—Swiss 3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FCS. For microinjection, cells were seeded at 3 × 10⁵/well (15 mm diameter) on glass coverslips (13 mm) and incubated for 2 days. Endotoxin-free plasmid DNA (100 ng/μl, Qiagen) was microinjected into the nuclei of approximately 100 cells (per coverslip), and, after 4 h, cultures were washed with PBS containing 0.9 mM CaCl₂ and 0.7 mM MgCl₂, fixed for 20 min with 3.7% formaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS.

To localize MYC-tagged PSK and actin filaments, cells were incubated with 1:500 anti-Myc tag antibody 9E10 (Santa Cruz Biotechnology) in PBS, 0.1% bovine serum albumin followed by 1:400 FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch) in PBS, 0.1% bovine serum albumin together with 0.1 μg/ml TRITC-conjugated phalloidin (Sigma). To localize PSK and vinculin, cells were incubated with 1:200 rabbit anti-Myc tag antibody (Santa Cruz Biotechnology) and 1:50 mouse anti-vinculin antibody (Sigma). Cells were imaged with a confocal laser scanning microscope (LSM 510, Zeiss). Image files were collected as a matrix of 1024 by 1024 pixels describing the average of 8 nuclei of approximately 100 cells (per coverslip), and, after 4 h, cultures were washed with PBS containing 0.9 mM CaCl₂ and 0.7 mM MgCl₂, fixed for 20 min with 3.7% formaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS.

Plasmids—The following plasmids were kindly provided by colleagues: pCMV-FLAG-JNK1 and pGEX-c-Jun (Dr. M. Karin, University of California and Dr. J. Ham, Eisai London Research Laboratories); pRK5-MYC-MLK2 (Dr. M. Terada, NCCRI, Tokyo); pCEF-HA-p38 (Dr. M. H. Cobb, University of Texas); and MKKs 3, 4, 7, GST-p38, and GST-JNK (Dr. R. Davis, Howard Hughes Medical Institute). pEXV-MYC-ERK2 was kindly provided by Dr. C. J. Marshall (Institute of Cancer Research, London), and ERK2 was subcloned into pCMV-FLAG (Sigma).

RESULTS

Kinase Expression in Human Prostatic Carcinoma Tissues—Total RNA was prepared from three surgically removed prostate tumors (Gleason grades 6–8) and used to obtain a profile of kinases expressed in prostatic carcinoma tissues. RNA was reverse transcribed to cDNA and amplified by PCR using degenerate oligonucleotides that correspond to the highly conserved amino acid sequences present in subdomains V1 (UI/VHRDL) and IX (DVWS/FLG) of the kinase catalytic domain (45, 46). This approach allowed us to amplify interconnecting sequences of approximately 200 bases between two subdomains that were kinase specific for each kinase. Sixty-six independent clones were sequenced. Based on this analysis, the deduced amino acid sequences (GenBank and EMBL data bases) identified 15 different tyrosine or serine-threonine kinases including six known receptor kinases (PDGFRB, IGFR1-B, VEGFR2, MET, RYK and EPY-A1), six non-receptor kinases (ABL, JAK1, JAK2, TYK2, PLK-1 and EMK), and three novel kinases (Fig. 1). Of the three tumors analyzed, one (Gleason grade 6) was more informative. All of the known kinases identified in this screen were represented at least once in this tumor, with IGF1-R being identified the most frequently (31% of clones sequenced for this tumor). Interestingly, 79% of clones sequenced from the other two tumors (Gleason grade 8) encoded PDGFB, while IGF1-R was sequenced twice and JAK1, ABL, and MET were each sequenced once. We also detected three novel kinases. GenBank database searches suggested that clone 1 was most related to the STE20 family of serine-threonine kinases with 52% homology to STE20 and clone 2 had most homology with the Caenorhabditis elegans predicted protein C46C2.1 (87%) and the Phycomyces blakesleeanus predicted protein PFPA (60%) (48, 49). Clone 3 now appears to be the human homolog of mouse PKL12 (50). We chose to focus on the novel kinase, which had significant homology to members of the STE20 family of serine-threonine kinases, because of the role that these proteins play in the regulation of cell growth and survival. This new kinase is analyzed in detail here.

Cloning of cDNAs Encoding a Novel Member of the STE20 Kinases—3′ RACE was carried out using a modification of the method of Frohman (51) to obtain more cDNA encoding the novel STE20-related kinase. This approach generated a PCR fragment of 534 bp, which was used to screen cDNA libraries leading to the isolation of two cDNA clones. One cDNA clone contained an insert of approximately 5 kb and was sequenced...
PSK, a Novel STE20-like Kinase That Regulates JNK/Actin

Fig. 5. Expression of PSK in COS1 cells. COS1 cells were transfected with either pRK5-MYC vector alone or various MYC-tagged PSK constructs and the expressed protein detected from the cell lysates by immunoblotting with anti-MYC 9E10 antibody.

A search of data bases (GenBank and EMBL) revealed that PSK had significant sequence homology over the kinase domain with members of the STE20 family of kinases including TAO1 (90%), MST1 (45%), NIK (44%), HPK1 (43%), GCK (42%), and STE20 (40%) (Fig. 2). The kinase domain was located at the N terminus of the protein and contains all 11 subdomains. The first kinase subdomain sequence (GHGSFG) is preceded by 34 amino acids and is followed by a crucial lysine residue at amino acid 57, which is required for ATP binding. The C-terminal domain contains several proline-rich regions representing predicted SH3 domain binding regions and a leucine-rich region. We have called this new protein PSK (prostate-derived STE20-like kinase).

PSK Is Homologous to STE20-related Kinases—A search of data bases (GenBank and EMBL) revealed that PSK had significant sequence homology over the kinase domain with members of the STE20 family of kinases including TAO1 (90%), MST1 (45%), NIK (44%), HPK1 (43%), GCK (42%), and STE20 (40%) (Fig. 3A). The presence of an N-terminal kinase domain and the stretch of 22 amino acids upstream of the first of the kinase catalytic subdomains is similar to other members of the STE20 family of kinases including MST1, NIK, GCK, HPK1, GLK, KHS, and HGK. Furthermore, PSK contains the amino acid sequence GTPY/FWMAPEV in subdomain VIII characteristic for the STE20 family and there is also no evident putative Cdc42/Rac binding domain (CRIB). The C-terminal domain of PSK has no significant homology with any STE20 family member.

The catalytic domain of PSK is most similar to the recently discovered rat TAO1 (thousand and one amino acids) protein kinase (53) with 90% identity over amino acids 22–278 (Fig. 3B). The C termini of PSK and TAO1 are 68% homologous between amino acids 278 and 744 and 23% homologous between amino acids 745 and 1001 (Fig. 3B). PSK contains an additional stretch of 15 glutamic acid residues (amino acids 378–392) that are not present in TAO1, and PSK also contains an additional 234 amino acids at the C terminus. A DNA sequence has recently been released encoding a predicted amino acid sequence for a human protein KIAA0881 (accession no. AB020688) (54) that also has homology to PSK and rat TAO1 (Fig. 3B). To confirm that PSK was distinct from TAO1 and KIAA0881, human genomic DNA was taken and subjected to PCR using a forward primer to nucleotides 2753–2774 of PSK, which are also present in KIAA0881, combined with a reverse primer that was specific for PSK (nucleotides 3180–3200). The PCR reaction generated a product of approximately 450 bp, which was cloned and sequenced and found to be PSK (data not shown). Two expressed sequence tags representing a single clone from human testis have recently been entered into the GenBank database (accession nos. AL048834 and AL048835) and encode 5’ and 3’ regions of PSK (903–1353 and 4220–4836 bp). Furthermore, an additional cDNA sequence encoding a potential rat homolog of PSK has been submitted to the GenBank database database during review of this manuscript (TAO2, accession no. AAD39480). This cDNA, however, encodes a much shorter protein of 993 amino acids. Taken together, this information indicates that PSK and TAO1 are distinct genes encoding proteins with closely related kinase domains.

Expression of PSK—Northern blotting analysis, using an XbaI 1105–1800 XhoI DNA fragment of PSK to probe mRNA from eight different tissues, detected a 5-kb transcript in prostate. PSK was ubiquitously expressed in all of the other tissues examined, and the strongest expression was found in testis (Fig. 4). We also used a BamHI 3504–4492 XhoI DNA fragment representing the 3’ end of PSK to detect the same 5-kb transcript in prostate and testis (data not shown).

PSK Is a Protein Kinase—MYC-tagged PSK was transiently transfected into COS1 cells and cell lysates were immunoblotted using antibody against the MYC-epitope tag. Two proteins of 165 and 185 kDa were specifically recognized in cells transfected with full-length PSK but absent from cells transfected with vector alone, suggesting possible post-translational processing or truncation of the protein (Fig. 5). Previous studies on other STE20-like kinases such as FLAG-tagged HGK have also revealed expression of a doublet (55).

To determine whether PSK had kinase activity, MYC-tagged PSK was transiently transfected into COS1 cells and an in vitro immune complex kinase assay was carried out on immunoprep-
PSK, a Novel STE20-like Kinase That Regulates JNK/Actin

PSK Activates the JNK MAPK Pathway—To determine whether PSK could activate MAPK pathways, MYC-tagged PSK was co-transfected together with FLAG-tagged JNK into COS1 cells and immune complexes of JNK were prepared from cell lysates for in vitro kinase assays using recombinant c-Jun as a substrate. Fig. 7A shows that transfected wild type PSK and also truncated PSK (1–349) stimulated phosphorylation of c-Jun 17.5- and 11.1-fold, respectively, when compared with cells transfected with JNK alone. In contrast, transfection of c-Jun 17.5- and 11.1-fold, respectively, with either full-length PSK or C-terminally truncated PSK (1–349, K57A) stimulated phosphorylation of MBP in cells transfected with vector alone (Fig. 6A). Additional phosphorylated proteins were also observed in the immune complex assay, at 185 and 45 kDa, and ran with the same mobility as full-length and truncated PSK, respectively, consistent with autophosphorylation of these protein kinases (Fig. 6B). This interpretation is supported by the absence of these phosphorylated proteins in cells transfected with kinase-defective PSK (K57A) or PSK (1–349, K57A) (Fig. 6B). These observations demonstrate that PSK and truncated PSK (1–349) act as functional kinases and that the kinase activity observed is attributable to PSK. Furthermore, addition of IL-1, transforming growth factor β, or serum to serum-starved cells expressing PSK failed to increase enzymatic activity of the immunoprecipitated protein toward MBP, suggesting that PSK is constitutively activated under these conditions (data not shown).

PSK Activates MKK4 and MKK7—To determine whether PSK could activate MAPK pathways, MYC-tagged PSK was co-transfected together with FLAG-tagged ERK2 or MYC-tagged p38 into COS1 cells. However, immune-complexes prepared from these cells failed to demnstrate any significant effect of PSK or PSK (1–349) on ERK2 activity toward MBP phosphorylation (Fig. 7B) or p38 activity toward ATP2 (Fig. 7C) compared with V12RAS (25.4-fold stimulation) or MLK2 (20.3-fold stimulation), respectively (Fig. 7, B and C). These findings demonstrate that PSK specifically activates the JNK MAPK pathway and has no detectable stimulatory effect on ERK or p38 MAPK pathways.

PSK Is Localized to a Vesicular Compartment—To determine the intracellular localization of PSK, expression vectors encoding PSK and various mutants were microinjected into the nuclei of Swiss 3T3 cells, and the localization of MYC epitope-tagged PSK determined 4 h after microinjection. Wild-type PSK localized predominantly to vesicles in the cytoplasm of the majority of injected cells (Fig. 8a), which in some cells had a tubulovesicular morphology (Fig. 8c). The localization of PSK was not detectably altered by serum starvation or serum re-stimulation (Fig. 8a and c), indicating that PSK constitutively associates with this vesicular compartment. The localization of PSK was clearly different from PAK1, which showed a uniform cytoplasmic distribution (Fig. 8e). Interestingly, the C-terminally truncated version of PSK (1–349) showed a diffuse cytoplasmic localization (ΔC, Fig. 8c), suggesting that the C-terminal region includes sequences that are required for targeting to the vesicular compartment. Kinase-defective PSK (K57A) showed a variable intracellular localization (KD; Fig. 8g). In approximately 50% of cells, it appeared diffusely cytoplasmic, whereas in the remainder of cells it showed a less homogeneous...
FIG. 7. PSK activates the JNK MAPK pathway. COS1 cells were transfected with either pRK5MYC vector alone or various MYC-tagged PSK constructs, control MYC-tagged V12RAS, or control MYC-tagged MLK2 along with FLAG-tagged JNK1 or FLAG-tagged ERK2 or MYC-tagged p38. A, FLAG-tagged JNK was immunoprecipitated with the M2 antibody and immunoprecipitates subjected to an in vitro kinase assay using GST-c-Jun as the substrate. Fold increases in c-Jun phosphorylation are shown when compared with cells transfected with JNK alone. B, FLAG-tagged ERK2 was immunoprecipitated with the M2 antibody and immunoprecipitates subjected to an in vitro kinase assay using MBP as the substrate. C, MYC-tagged p38 was immunoprecipitated with the 9E10 antibody and immunoprecipitates subjected to an in vitro kinase assay using GST-ATF2 as the substrate. Normalization of immunoprecipitated FLAG-JNK, FLAG-ERK2, or MYC-p38 and expression of MYC-tagged PSK proteins was confirmed by immunoblot analysis. PSK appears as a single band after 15% SDS-PAGE. D, COS1 cells were transfected with either pRK5MYC vector alone or various MYC-tagged PSK constructs along with FLAG-tagged MKK4 (a), MKK7 (b), or MKK3 (c). FLAG-tagged MKKs were immunoprecipitated and subjected to an in vitro kinase assay using GST-JNK or GST-p38 as substrate. MEKK3 and MLK2 were used as positive controls for MKK3 and MKK4 or MKK7, respectively.
distribution that nevertheless did not resemble that of wild-type PSK. This implies that autophosphorylation of PSK and/or phosphorylation of a downstream target are important for its localization. We have also analyzed localization of PSK in transfected COS1 cells and observed a similar requirement for the C terminus to localize PSK (data not shown).

PSK Induces Retraction and Actin Reorganization—We observed that cells expressing wild type PSK were clearly altered in morphology: they were more rounded, had fewer processes, and had a smaller spread area than surrounding uninjected cells or cells expressing kinase-defective PSK. To determine whether this cell shape change was reflected in altered actin cytoskeletal organization, we analyzed actin filament distribution in growing Swiss 3T3 cells following microinjection with expression vectors encoding wild-type PSK and various mutants. A marked change in the distribution of actin filaments was observed by 4 h after microinjection (Fig. 8b). Stress fibers were disorganized, and instead of being arranged in parallel bundles, were concentrated in shorter bundles at the periphery of the cell. A similar effect on actin stress fiber organization was observed both in growing Swiss 3T3 cells (Fig. 8b) and in starved cells acutely restimulated with FCS (Fig. 9b). In some cells, a significant decrease in the level of stress fibers was observed, and lamellipodia were not observed on any PSK-expressing cells (Figs. 8b and 9d, and data not shown). These changes were observed in multiple experiments, and with PSK in two different expression vectors, pRK5 and EPIlink. Staining for vinculin, a component of focal adhesions normally found at the ends of stress fibers (56), showed that vinculin-containing adhesions were re-organized in PSK-expressing cells, such that they were mostly present as small complexes at the periphery of the cells (Fig. 8d), consistent with the reorganization of the actin cytoskeleton. The PSK-mediated loss of processes was clear from the vinculin localization, as control cells had vinculin-containing focal adhesions at the end of processes.

The alteration in cell morphology and actin organization by PSK was dependent on kinase activity, since the kinase-defective mutant PSK (K57A) had no effect (Fig. 8h). In addition, the truncated form of PSK (1–349) did not effect actin organization (Fig. 8f). Taken together with the results on PSK localization, this suggests that correct localization of PSK is important for its effects on the actin cytoskeleton.

Expression of activated PAK kinases has been reported to induce loss of stress fibers in various cell types (42–44), so we compared the effect of PSK with that of an activated form of PAK1, PAK (L107F) (57). As previously reported in REF52 fibroblasts (42), PAK (L107F) did not induce detectable loss of stress fibers at early time points (4 h) after microinjection of the expression construct, although a decrease in stress fibers was observed at 24 h after injection (Fig. 8j). PAK1 (L107F)-expressing cells did not retract, indicating that the mechanism whereby PAK1 alters actin cytoskeletal organization is significantly different from that induced by PSK.

**DISCUSSION**

We have used reverse transcription-PCR to identify 15 tyrosine or serine-threonine kinases expressed in surgically removed prostate tumors. These include the receptor kinases IGF1-R, PDGFBR, VEGFR2, Eph-A1, MET, and RYK; the non-receptor kinases JAK1, JAK2, TYK2, ABL, EMK, and PLK; and three novel kinases. Many of these kinases have been shown to have oncogenic potential, and their presence in prostate tumors provides a basis for further analysis of signaling pathways involved in prostate cancer development.

PCR conditions were designed for kinase detection rather than quantitative analysis of kinase expression, but the most frequently sequenced clones using this approach were IGF1-R and PDGFBR. Others have shown that elevated levels of plasma IGF1 are associated with increased risk of prostate cancer development and that IGF1 is able to activate the ERK MAPK pathway in DU145 cells, and acts as a potent mitogen for prostate cancer cells (8, 58). Moreover, an IGF1-R-activated loop supports androgen-independent growth of DU145 prostate cancer cells and antisense to the IGF1-R suppresses growth and invasion of prostate cancer cells (6, 59). The presence of IGF1-R in all three of our screened prostatic carcinoma tissues supports the notion that IGF1-R may play a functional role in prostate cancer development. We also detected a number of other receptor kinases in our degenerate PCR screen of prostatic carcinoma tissues including PDGFRβ; PDGFR acts as a potent mitogen for prostate cells (60) and the oncogenic potential of PDGFRβ has been well characterized in other cancers. VEGFR2 and the HGF receptor c-MET also function to regulate cell growth, migration, and the actin cytoskeleton via autocrine and paracrine loops, and increases in expression of both vascular EGF and c-MET proteins have been reported in malignant prostate cells (61–63). The Eph-A1 receptor has not yet been investigated in prostate; however, overexpression of the Eph-A1 receptor has been reported in breast, lung, liver, and colon carcinoma tissues (64).
Some of the non-receptor kinases detected function as transduction molecules regulating MAPK pathways. c-ABL can regulate the ERK, JNK, and p38 MAPK pathways and the BCR-ABL oncogene induces transformation by activating the STE20-like GCKR (GCK-related) kinase and JNK MAPK pathway in CML cells (65–67). The Janus family of tyrosine kinases (JAK1–3, TYK2), which are able to induce rapid stimulation of interferon-responsive genes through latent cytoplasmic transcription factors, known as signal transducers and activators of transcription, have not yet been investigated in prostate but can regulate the ERK MAPK cascade in other cell types (68).

A profile of kinases expressed in a prostate carcinoma xenograft grown in mice has recently been published and detected some of the kinases reported here, including PDGFBR, EPH-A1, ABL, JAK1, and TYK2 (12). MAPK upstream regulators such as MKK3, MKK4, and MKK5 were also present in this malignant prostate xenograft. The different kinase profiles reported by us and by Robinson et al. (12) are most probably accounted for by the use of different degenerate primers and our use of surgically removed prostatic carcinoma tissues rather than a prostate cancer xenograft grown in mice. The number of kinases that we have detected is by no means complete and could probably be increased by using alternative degenerate primers and analyzing additional tumor tissues.

We were particularly interested in the three potentially novel kinases that were detected in our screen. Of these, one is a protein with 65% homology over its entire length to the C. elegans predicted protein C46C2.1 (49); however, none of the cDNAs that we isolated had an in-frame stop codon (data not shown) and although the predicted amino acid sequence appeared to contain all 11 kinase subdomains, a crucial lysine residue required for ATP binding was missing in the second catalytic domain (C190K). The protein could be expressed in COS1 cells, but immunoprecipitated protein was unable to phosphorylate MBP, even after a lysine residue had been introduced into the second catalytic subdomain (data not shown). The sequence of this kinase-defective protein (named KDP) has been submitted to the GenBank data base. (Another of the novel kinases turned out to encode the human homolog of the recently isolated murine protein kinase PKL12, a serine-threonine kinase, which is ubiquitously expressed but has unknown function (50).)

The third novel kinase encodes a new member of the STE20 kinase family, which we have named PSK. STE20 family members are known to be involved in regulating cell growth, transformation, differentiation, stress responses, cytoskeletal organization, cell motility, and apoptosis. They can be classified into two subfamilies according to their structure and regulation. The first group of enzymes includes the PAK and MLK proteins which have a C-terminal kinase domain and an N-terminal regulatory region containing a CRIB (69), while the second group of proteins have an N-terminal kinase domain and a C-terminal regulatory region that does not contain a CRIB. PSK is structurally related to the second group, which includes GCK, GLK, HGK, HPK1, KHS, MST1/2, NIK, and TAO1. Immune complexes of PSK phosphorylated MBP in vitro, demonstrating that it has kinase activity, and transfected PSK specifically stimulated the JNK MAPK pathway but not ERK MAPK or p38 MAPK in COS1 cells. This is similar to the response observed with GLK, HGK, HPK1, KHS, and NIK, which specifically stimulate the JNK pathway through MKK4, with GCK, GLK and NIK acting via MEKK1, HGK via TAK1, and HPK via MLK3 (28, 29, 37, 39, 41, 55, 70). In the case of HPK and NIK, this activity has been attributed in part to direct interaction with MLK3 and MEKK1, respectively, through homologous regions in their non-catalytic C-terminal domains (39). PSK has no homology in its C-terminal region with these kinases, but we have shown that PSK activates MKK4 and MKK7, which regulate the JNK pathway. The C-terminal region of PSK also contains proline-rich motifs, which could potentially bind to SH3 domains, and some of these sites could therefore be important in protein-protein interactions.

Among the STE20 family, PSK shows the closest homology to TAO1, with 90% amino acid identity within the kinase domain (53). Outside the kinase domain, however, homology is substantially reduced. Despite the similarity of their catalytic domains, the specificities of PSK and TAO1 for MAPK pathways appear to differ in cells. Transfected TAO1 interacts with MKK3, apparently through its C-terminal non-catalytic do-

![Fig. 9. Effects of PSK on serum-starved and serum-stimulated cells. Swiss 3T3 cells were starved for 16 h and then microinjected with pRK5-MYC-PSK (act). Cells were incubated for 4 h and then fixed (a and b) or stimulated with 10% FCS for 10 min and then fixed. Cells were co-stained with mouse anti-MYC epitope antibodies, followed by FITC-labeled anti-mouse IgG to show PSK (a and c) and with TRITC-phalloidin to show actin filaments (b and d). Bar represents 20 μm.](image)
main, and stimulates MKK3 but not MKK4 activity, which would normally be expected to activate p38 but not JNK. C-terminally truncated TAO1 (1–416) can, however, stimulate MKK4 activity in vitro (53), suggesting that specificity of MAPK pathway activation in cells is mediated by selective interactions of the C-terminal domain. Indeed, we observe that the C-terminal region is essential for localization of PSK to a vesicular cytoplasmic compartment, and this may be important for its function. Our finding that transfected PSK, unlike TAO1, specifically activates JNK does not, however, appear to be solely due to the different C termini of PSK and TAO1, since truncated PSK (1–349) containing the N-terminal kinase domain was also able to activate JNK, albeit less potently than full-length PSK. The inability of Hutchison et al. (53) to co-express TAO1 with JNK or p38 in 293 cells makes further comparisons with PSK difficult at the present time.

Of the STE20-like kinases, only PAKs have so far been shown to induce changes in actin organization in mammalian cells. PAKs interact with the GTPases Cdc42 and Rac, which regulate the formation of filopodia and lamellipodia, respectively (56). Various mutated forms of PAKs can induce loss of actin stress fibers and focal complexes, stimulate formation of filopodia and lamellipodia, and/or block Cdc42 and Rac effects on the cytoskeleton (42–44, 71). In contrast, MLK2 and MLK3 also interact with Cdc42 and Rac but do not regulate the actin cytoskeleton (34, 40). The cell retraction, loss of processes, and actin reorganization observed with PSK is significantly different from the loss of stress fibers induced by activated PAK1. Stress fibers consist of actin and non-muscle myosin II filaments, and increased MLC phosphorylation normally correlates with an increase in the number and/or contractility of stress fibers (72). Both MLC and MLC kinase have been reported to be targets for PAKs (73–75). Expression of activated PAK1, however, can lead to either an increase or decrease in MLC phosphorylation depending on the system (44, 74). We have found that immunoprecipitated PSK does not phosphorylate purified myosin light chain kinase in vitro nor does transfected PSK alter phosphorylation of MLC on serine 19 following activation by immunoblotting of COS1 cell lysates, whereas expression of activated RhaA does induce increased MLC phosphorylation, as previously reported (76) (data not shown). An alternative possibility for how PSK acts is suggested by its localization to cytoplasmic vesicles. As its ability to induce reorganization of stress fibers correlates with this localization, it may play a role in trafficking of proteins involved in actin organization or focal adhesion formation. Indeed, there is good evidence that some focal adhesion components, including paxillin, are normally associated with intracellular vesicles and need to be transported to the plasma membrane in order for focal adhesions and associated stress fibers to form efficiently (77).

In conclusion, we have demonstrated for the first time that a STE20-like kinase which lacks a CRIB, PSK, can not only selectively activate the JNK MAPK pathway but also affects actin cytoskeletal organization. By coordinating these two responses, PSK may play a role in regulating cell migration.

Acknowledgments—We thank Drs. Mark Crompton and Alan Mackay for helpful discussions and Fumio Matsumura for myosin light chain kinase and anti-phospho-MLC antibodies.

REFERENCES

1. National Cancer Institute of Canada (1997) Canadian Statistics, p. 15. National Cancer Institute of Canada, Toronto, Canada
2. Parker, S. L., Tong, T., Bolden, S., and Wing, P. A. (1996) Cancer Stat. Cancer J. Clin. 46, 5–27
3. Rechcigl, C. G. (1991) in Mechanisms of Progression to Hormone-independent Growth of Breast and Prostatic Cancer (Barnes, P. M. M. J., Romijn, J. C., and Schroder, F. H., eds) pp. 97–122, Parthenon Publishing Group, New York
PSK, a Novel STE20-like Kinase That Regulates JNK/Actin