Identification and Characterization of a Novel Ste20/Germinal Center Kinase-related Kinase, Polyploidy-associated Protein Kinase*

Kazu Nishigaki‡§, Delores Thompson‡, Takashi Yugawa‡, Karen Rulli‡, Charlotte Hanson‡, Joan Cmarik‡, J. Silvio Gutkind†, Hiromi Teramoto†, and Sandra Ruscetti‡¶

From the Basic Research Laboratory, NCI, National Institutes of Health, Frederick, Maryland and Oral and Pharyngeal Cancer Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892

A novel protein kinase, polyploidy-associated protein kinase (PAPK), was isolated using a subtraction cDNA library approach from a mouse erythroleukemia cell line that had been induced to polyploidy after serum withdrawal. PAPK shares homology with members of the Ste20/germinal center kinase family of protein kinases and is ubiquitously expressed as two spliced forms, PAPK-A and PAPK-B, that encode for proteins of 418 and 189 amino acids, respectively. The expression of endogenous PAPK-A protein increased after growth factor withdrawal in murine hematopoietic and fibroblast cells. When tested in an in vitro kinase assay, PAPK-A was activated in response to the stress-inducing agent hydrogen peroxide and slightly by fetal calf serum. Biochemical characterization of the PAPK-A-initiated pathway revealed that this novel kinase does not affect MAP kinase activity but can stimulate both c-Jun N-terminal kinase 1 (JNK1) and ERK6/p38. The kinase activity of PAPK appears to be required for the activation of ERK6/p38 but not JNK1. When an inducible construct of PAPK-A was expressed in stably transfected NIH3T3 cells, the cells exhibited distinct cytoskeletal changes and became resistant to apoptotic cell death induced by serum withdrawal, effects of PAPK that require its kinase activity. These data suggest that PAPK is a new member of the Ste20/germinal center kinase family that modulates cytoskeletal organization and cell survival.

The sterile-20 (Ste20) family of serine-threonine kinases was first discovered, and extensively studied, as an essential component for the pheromone-response pathway in Saccharomyces cerevisiae (1, 2). It has also been recognized that this family of kinases plays a key role in several other known yeast signaling pathways. They include the control of morphological changes, cytokinesis, response to nutrient starvation, and localizing cell growth with respect to the division plate (3–5).

Over the past few years, there has been a tremendous increase in the number of mammalian homologues of Ste20 kinases identified. Based on their structure, these mammalian Ste20-like kinases can be divided into two subfamilies (6). The first class, p21-activated kinases (PAKs), is activated upon binding to the guanine triphosphatases Cdc42 and/or Rac1. Upon binding to Cdc42/Rac1-GTP, PAKs undergo a conformational change, which enables autophosphorylation and subsequent activation of the kinase to occur (7). PAKs have been shown to be involved in changes in cell motility, morphology (8), apoptosis (9–12), and transformation (13, 14). PAKs have also been implicated in activating MAP kinases, including ERK, JNK/SAPK, and p38 (8).

The second class of Ste20-related kinases is the germinal center kinase (GCK), which contains an N-terminal catalytic domain and lacks the p21-binding domain. A large number of serine/threonine kinases belonging to the GCK family have been implicated as upstream regulators of MAP kinase signaling pathways (15, 16). The GCK family kinases include GCK (17, 18), hematopoietic progenitor kinase-1 (HPK1) (19, 20), NCK-interacting kinase (NIK; also referred to as HGK) (21, 22), GCK-like kinase-23, kinase homologous to SPS1/STE20 (KHS1; also referred to as GCKR) (24, 25), STE20/oxidant stress-response kinase-1 (SOK-1; also referred to as YSK1, stk25) (26, 27), mammalian STE20-like kinase-1 (MST1; also referred to as Krs-2) (28, 29), mammalian STE20-like kinase-2 (MST2; also referred to as Krs-1) (28, 29), mammalian STE20-like kinase-3 (MST3) (30, 31), MST4 (also referred to as MASK) (32–34), misshapen/NIK-related kinase (35), Traf2 and NCK interacting kinase (TNIK) (36), NIK-related kinase (also referred to as NESK) (37, 38), lymphocyte-oriented kinase (39), SLK (40, 41), PASK/SPAK (42, 43), TAO1 (44), PSKI/TAO2 (45, 46), and JNK/SAPK-inhibitory kinase (JIK; also referred to as DPK) (47, 48). All GCKs contain N-terminal Ste20-like kinase domains and long C-terminal regulatory domains. Several members of the GCK group have been shown to be potent and selective activators of SAPK/JNK or p38.

In the present study, using a subtractive screen for polyploidy induction, we cloned and characterized a widely expressed molecule termed polyploidy-associated protein kinase (PAP kinase; PAPK), a novel member of the mammalian Ste20/GCK kinase family. We demonstrate in this report that PAPK, like many other GCK family members, is able to specifically activate the JNK and ERK6/p38 pathway when over-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence may be addressed: Basic Research Laboratory, Bldg. 469, Rm. 205, NCI-Frederick, Frederick, MD 21702-1201. Tel.: 301-846-5740; Fax: 301-846-6164; E-mail: nishigaki@ncifcrf.gov.

§ To whom correspondence may be addressed: Basic Research Laboratory, Bldg. 469, Rm. 205, NCI-Frederick, Frederick, MD 21702-1201. Tel.: 301-846-5740; Fax: 301-846-6164; E-mail: ruscetti@ncifcrf.gov.
expressed in 293T cells. In addition, overexpression of PAPK in NIH3T3 cells resulted in resistance to cell death and led to distinct changes in cell morphology. Our results suggest that PAPK represents a novel molecule regulating cytoskeleton structures and cell survival.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Expression vectors of pCDNAII-ERA2K, pCDNAII-HA-JNK1, pCDNA-HA-p38, pCEFL-HA-p38γ, pCEFL-HA-ERK6/ p38γ, and pCEFL-HA-ERK5 were described previously (49, 50). The expression plasmids for GST-SEK1 (pEGB-SEK1) and GST-SEK1-RR (pEGB-SEK1-RR) encoding a dominant-negative SEK1 (51) were a generous gift of John M. Kyriakis (Massachusetts General Hospital). The expression plasmids MKK7 and MKK7-K66E encoding a dominant-negative MKK7 (52) were a generous gift of Tse-Hua Tan (Baylor College of Medicine). pFC-MEKK1, pFC-MEKK3, and pFC-MKK3 were purchased from Stratagene.

**Cell Culture and Transfection—**29T, NIH3T3, HeLa, ELA, and Friend erythroleukemia (C9, DS19 (53)) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS). HCD-57, an erythroidopietin (Epo)-dependent erythroleukemia cell line, were maintained as described previously (54). Interleukin 3-dependent BaF3, FDCP2, and 32D cells were maintained in RPMI 1640 medium with 10% FCS and 10% WEHI-conditioned medium (as an IL-3 source). NIH3T3 cells were stably expressing the regulatory GAL4-DDBh/PR-LBD/p65 AD fusion protein from the pSwitch plasmid (GeneSwitch-3T3) were obtained from Invitrogen. To obtain clones with mifepristone-inducible expression of PAPK-A and PAPK-A(K89M), GeneSwitch-3T3 cells were transfected with 2 μg of the pG /V5-His plasmid (Invitrogen) carrying the coding region of Myc-tagged PAPK-A, PAPK-A(K89M), and cDNA, and empty vector by using LipofectAMINE 2000 (Invitrogen) and subjected to selection for stable transfectants with 0.4 μg of Zeocin per ml. Two to 3 weeks later, colonies were picked, expanded, and tested for induction of the gene by mifepristone (1 × 10–8 M) (Invitrogen). 293T and HeLa cells in 60-mm culture dishes (2 × 105) were transfected with various expression plasmids by using LipofectAMINE 2000 in accordance with the manufacturer’s instructions.

**cDNA Subtraction—**cDNA subtraction was performed using a PCR-selected cDNA Subtraction kit (Clontech). The mRNA from C9 cells cultured for 24 h in serum-free medium was used as the “driver.” The mRNA from polyploid C9 cells cultured for 5 days in serum-free medium was used as the “tester.” The subtractive screening was performed according to the manufacturer’s instructions. A partial fragment of PAPK was obtained as a candidate-positive cDNA clone.

**Cloning of PAPK and DNA Construct—**5′ and 3′ RACE analyses for the cDNA fragment obtained were performed using C9 erythroleukemia cDNA as a template with Marathon cDNA amplification kit (Clontech) in accordance with the manufacturer’s instructions. The internal primers used were 5′-CAGTCTCAGGCGCACTACCTACGAGCAAC-3′ (sense) for 3′RACE) and 5′-CAGTCTTGCGAGCTCGTGGTTATTTCTCC-3′ (antisense for 5′). Each RACE product was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced. Full-length mouse PAPK-A and PAPK-B were obtained by PCR using cDNA from C9 cells as a template with the Marathon cDNA amplification kit (Clontech). EcoRI-SalI fragments of full-length PAPK-A and PAPK-B were cloned into pCMV-Myc (Clontech). A kinase-inactive form of PAPK-A (PAPK-A-K89M) was constructed by substituting lysine 89 with a methionine using a QuickChange site-directed mutagenesis kit (Stratagen) with the mutagenic primers 5′-GACAACGTGTTGGCATGTTGCTAGACGAAAG-3′ (sense) and 5′-CTTCCAGCTGGTTATCAAGCTACGCTGGTTATTTCTCC-3′ (antisense) (the mutated bases are underlined) by PCR and cloned into pCMV-myc.

**Generation of Polyclonal Antibodies against PAPK—**An antiserum against PAPK was raised in rabbits by immunization with a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to the C-terminal 20 amino acids (amino acids 399–418, SPWSLEFQFYRPPDDKDPWVF). Anti-PAPK antibodies were typically used at a 1:1000 dilution.

**RNA and Protein Analysis—**Poly(A+) RNA was prepared with the FastTrac Kit (Invitrogen). Mouse Multiple Tissue Northern blots were purchased from Origene. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and aprotinin and leupeptin at 1 μg/ml each). Cells were lysed on ice for 20 min, and supernatant was collected at 14,000 rpm at 4°C.

**RESULTS**

**cDNA Cloning and Identification of PAPK, a Novel Ste20/GCK-like Kinase—**In the course of studying erythroleukemia (MEL) cell lines derived from mice infected with the Friend virus, cDNA subtraction was performed using a cDNA probe (PAPK-A) from a gene that was shown by subtractive cDNA screening to be differentially expressed under the two conditions. The subtractive screening was performed using a cDNA probe (PAPK-A) from a gene that was shown by subtractive cDNA subtraction to be significantly differentially expressed under the two conditions.

**FIG. 1. Serum withdrawal from mouse erythroleukemia cells induces polyplody and up-regulation of a 2.4-kb transcript.** A, the Friend MEL cell line C9 was cultured in DMEM containing 10% FCS (left panel) (steady state) or grown for 5 days in serum-free medium (right panel), which induces polyplody. The cells were stained with HEMA 3 stain (Fisher) and photographed at ×40 magnification. B, poly(A) RNA was prepared from C9 cells grown in 10% FCS (left lane) (steady state) or for 5 days in serum-free medium (right lane) (induced to polyplody). Northern blotting was then carried out using a cDNA probe (PAPK-A) from a gene that was shown by subtractive cDNA screening to be differentially expressed under the two conditions. Lysates were subjected to immunoblotting, as described previously (54), using mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals), mouse monoclonal anti-Myc antibody (7E10, Clontech), mouse monoclonal anti-FLAG antibody (M2, Stratagene), mouse monoclonal anti-GST antibody (Cell Signaling), rabbit anti-JNK antibody (Cell Signaling), and rabbit anti-phospho-JNK antibody (Cell Signaling). For the PAPK and MAP kinase assays, protein was immunoprecipitated with anti-Myc monoclonal antibody (7E10) or anti-HA monoclonal antibody (12CA5) at 4°C for 5 h or overnight, respectively. Immunocomplexes were recovered with protein G-agarose (Upstate Biotechnology, Inc.) for 1 h. Beads were washed twice with lysis buffer and twice with kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol, 0.1 mM orthovanadate for the MAP kinase assay or 20 mM HEPES, pH 7.6, 10 mM MgCl2, 2 mM dithiothreitol, 0.1 mM orthovanadate for the PAPK assay). Samples were then resuspended in 50 μl of kinase reaction buffer containing 5 or 10 μg of (1 μg/particle) ATP and 500 μM cold ATP. After 30 min at 30°C, the reactions were terminated by addition of 10 μl of 4× Laemmli buffer. In vitro kinase assays were performed using as substrates myelin basic protein (MBP, Upstate Biotechnology, Inc.) for ERK6/p38, ERK5, and PAPK; GST-Erk1 (residues 307–428, Cell Signaling) for ERK2; or GST-ATF2 (residues 19–96, Cell Signaling) for JNK, p38, and p38γ. Samples were separated by SDS-14% PAGE, transferred to nitrocellulose filters, and subjected to autoradiography.

**Cell Death Assay—**In order to induce apoptosis in NIH3T3 cells stably expressing vectors, cells were plated at 1 × 105 cells per well in 12-well plates for 1 day and then washed in DMEM. Cells were cultured with serum-free DMEM in the presence or absence of mifepristone (1 × 10–7 M). At the indicated times, cells were stained with Hoechst 33342 (Molecular Probes) and visualized by using a fluorescence microscope. Abnormal nuclei were scored for apoptosis in a blind fashion. To analyze a marker for cells undergoing apoptosis, we carried out Western blot analysis by using a mouse monoclonal antibody to cleaved PARP (7C9; Cell Signaling).

**Morphological Analyses—**1 × 105 cells grown in 60-mm dishes were cultured for 1 day in serum containing medium and then transferred to DMEM with 10% FCS in the presence mifepristone (1 × 10–7 M). At the indicated times, the cultures were examined by phase contrast microscopy, and the percentages of cells undergoing morphological change were counted. Three separate experiments were carried out, and for each experiment, a total of 200 cells per culture were examined for each time point. The percentage of cells exhibiting extensions that were longer than the length of the cell body was determined.
spleen focus-forming virus, we observed that growing these cells in serum-free medium induced a dramatic change in their morphology. Within 3–5 days, the cells changed from erythroblastic to giant polyploid cells (Fig. 1A). Although the altered cells resemble megakaryocytes, they do not express typical megakaryocyte markers such as acetylcholinesterase and CD41 (data not shown). In order to isolate genes in MEL cells whose expression is altered after induction of this morphological change, we performed a subtractive cDNA screening using mRNAs from the MEL cell line C19 (53) before and after induction. Among the isolated cDNA clones, we found a cDNA fragment corresponding to a gene that is up-regulated during induction of polyploidy. Northern blot analysis using this cDNA revealed that the corresponding mRNA for this gene is normally present in C19 cells but is dramatically up-regulated 5 days after induction of the cells to polyploidy (Fig. 1B).

3'- and 5'-RACE analyses showed that at least two mRNA variants with different 3'-sequences were isolated, and their cDNAs were sequenced (Fig. 2A). Sequence analysis revealed that the larger of the two should encode a polypeptide of 418 amino acids that contains a kinase domain (amino acids 58–369) in the center of the coding region. The smaller message appears to be encoded by a gene containing a stop codon within the kinase-encoding domain and should encode for a 189-amino acid polypeptide lacking 172 amino acids from the C terminus. We named these proteins polyploidy-associated protein kinase (PAPK)-A and PAPK-B. The calculated molecular mass of PAPK-A is 46.8 kDa, whereas that of PAPK-B is 21.4 kDa.

Sequence analysis indicates that PAPK-A is a novel protein.

**Fig. 2.** Primary structure of PAPK and homologies with known MAP kinases. A, nucleotide and deduced protein sequence of PAPK-A cDNA and its spliced variant PAPK-B. The GenBank™ accession numbers for PAPK-A and PAPK-B are AB057666 and AB057667. B, sequence alignment of the kinase domain of PAPK-A with other GCK family kinases. C, phylogenetic tree of GCK family kinases. Generation of the phylogenetic tree was carried out using the Phylip program.
kinase belonging to the Ste20/GCK family of serine/threonine kinases. When the kinase domain of PAPK-A is compared with those of other protein kinases in this family (Fig. 2B), it shows the highest homology to that of NY-Br-96 (52% homology) and SPAK (30% homology). The phylogenetic tree for the PAPK-A kinase domain is shown in Fig. 2C. Within the sequence of PAPK-A there are two consensus Src homology 3-binding sites (PXxxP) (55) at amino acids 382 and 397, which may be involved in protein-protein interactions, and a consensus AKT phosphorylation site (RXRXXS) (56) at amino acid position 16 (residues 11–16). When the sequence for PAPK-A was BLASTed against the assembled mouse genomic sequence in the NCBI data base, it was found on mouse chromosome 1.

Expression of PAPK—The expression pattern of PAPK was examined using a mouse multitissue Northern blot (Fig. 3). When PAPK-A cDNA was used as a probe, a 2.4-kb transcript was shown to be expressed ubiquitously, with higher expression levels in brain, heart, kidney, liver, and testis. A similar pattern was observed using a probe corresponding to nucleotides 692–863 of PAPK-A, which are missing in PAPK-B.

To identify and characterize the PAPK-A protein, we produced anti-PAPK-A antibody by immunizing rabbits with a peptide corresponding to the C terminus of the predicted protein. The specificity of the anti-PAPK antibody was confirmed using 293T cells transfected with a Myc-tagged PAPK-A cDNA expression plasmid. Both the anti-PAPK antibody and the anti-Myc antibody recognized the same protein (data not shown). Because the antibody is directed against the C terminus of PAPK-A, which is missing in PAPK-B, it recognizes only PAPK-A and detects a 48-kDa protein in the C19 and DS19 (53, 54) MEL cell lines (Fig. 4A). When C19 and DS19 cells are induced to polyploidy by growth in serum-free medium, the level of PAPK-A protein is significantly increased (Fig. 4A). However, when serum-grown MEL cells are induced to differentiate with the chemical hexamethylenebisacetamide, PAPK-A levels decrease (Fig. 4B). In addition to MEL cells, a large amount of PAPK-A protein can also be detected in the mouse T-cell line EL4 grown in serum but not in other mouse cell lines examined, including the Epo-dependent erythroleukemia cell line HCD-57, the IL-3-dependent myeloid cell lines FDCP2 and 32D, the IL-3-dependent proB cell line BaF3, and the embryonic mouse fibroblast cell line NIH3T3 (Fig. 4A). Of interest, however, is the observation that the levels of PAPK-A can be increased by withdrawing Epo from HCD-57 cells (Fig. 4C) or removing IL-3 from FDCP2, 32D, or BaF3 cells (data not shown), each of which results in apoptosis of the cells. These data suggest that PAPK-A is up-regulated during apoptosis. Furthermore, when NIH3T3 cells were starved for 24 h in DMEM with 0.5% bovine serum albumin and then cultured in DMEM with 10% FCS, lysesates were examined for expression of PAPK-A by Western blotting. D, NIH3T3 cells were starved for 24 h in DMEM with 0.5% bovine serum albumin, and then cultured in DMEM with 10% FCS. Lysesates were then examined for expression of PAPK-A by Western blotting.

PAPK Functions as a Protein Kinase—To determine whether PAPK has protein kinase activity, we transfected 293T cells with Myc-tagged PAPK-A, isolated the protein from the cell lysate by immunoprecipitation with an anti-myc monoclonal antibody, and carried out an immune complex kinase assay using myelin basic protein (MBP) as an exogenous substrate. Cells transfected with empty vector or with vector expressing Myc-tagged PAPK-B, which is missing the majority of the kinase domain of PAPK-A and is not expected to have any kinase
activity, were used as controls. Although the immunoprecipitates from cell lysates prepared from 293T cells transfected with vector alone or with PAPK-B phosphorylated MBP to a limited extent, the kinase activity was markedly increased in immunoprecipitates from cells transfected with PAPK-A (Fig. 5). These data indicate that PAPK-A, but not PAPK-B, is a functional protein kinase. To eliminate the possibility that an associated kinase might be co-precipitating with PAPK-A, we generated a kinase-inactive form of PAPK-A, designated K89M-PAPK-A, by replacing lysine 89 in the ATP-binding domain with a methionine. When tested in the same immune complex kinase assay, the level of phosphorylation of MBP by kinase-inactive mutant was less when compared with that of wild-type PAPK-A (Fig. 5). Thus, in addition to its own kinase activity, PAPK-A may also function as a scaffold for other protein kinases as this kinase-inactive mutant of PAPK-A still resulted in some MBP phosphorylation.

**Activation of PAPK by Cellular Stimuli**—To determine whether PAPK-A is activated by any of the cellular stimuli that are known to activate MAP kinases, we transfected HeLa cells with Myc-tagged PAPK-A and carried out the PAPK-A kinase assay after exposing the cells to various cellular stresses or cytokines (Fig. 6). PAPK-A kinase activity markedly increased (greater than 5-fold) after treatment of the cells with H$_2$O$_2$ and slightly (almost 2-fold) after exposure to fetal calf serum. Treatment with 12-O-tetradecanoylphorbol-13-acetate, tumor necrosis factor-$\alpha$, anisomycin, NaCl, or IL-1 (Fig. 6), as well as with tunicamycin, thapsigargin, or sorbitol (data not shown), did not increase PAPK-A activity. The level of overexpressed PAPK-A was verified by immunoblotting with anti-Myc antibody (bottom panel). Similar data were obtained using 293T cells (data not shown). Thus, PAPK-A may be stimulated by a limited number of extracellular stimuli.

**PAPK Activates MAP Kinase Pathways**—To determine whether PAPK is a component of any of the known MAP kinase pathways, we tested the effect of overexpression of PAPK-A and PAPK-B on the activation of various MAP kinases (Fig. 7). 293T cells were transiently transfected with HA-tagged ERK2, JNK1/SAPK, p38$\alpha$, p38$\beta$, ERK5, or ERK6/p38$\gamma$ with or without mammalian expression vectors encoding Myc-tagged PAPK-A or PAPK-B. The HA-tagged kinases were then immunoprecipitated from the cell lysates and analyzed for kinase activity using the appropriate substrate. As shown in Fig. 7, co-expression of PAPK-A with ERK2 (A), p38$\alpha$ (C), p38$\beta$ (D), or ERK5 (F) failed to activate their kinase activity even though each kinase could be activated by previously described upstream activating kinases. In contrast, both JNK1 (B) and ERK6/p38$\gamma$ (E) were markedly activated (2.45- and 2.57-fold, respectively) by over-expression of PAPK-A. Interestingly, JNK1, but not ERK6/p38$\gamma$, was also slightly activated (1.5-fold) by overexpression of PAPK-B. By using anti-phospho-JNK antibodies, we were also able to detect phosphorylated JNK1 in cells overexpressing PAPK-B (data not shown). This suggests that although the kinase activity of PAPK is required for maximal activation of JNK1, PAPK might also have a function as a scaffold or adapter protein.

**JNK Activation by PAPK—MKK7 is one of the upstream activators of JNK1.** To determine whether PAPK-A activates JNK1 through MKK7, 293T cells were transiently co-transfected with HA-tagged JNK1 with or without Myc-tagged PAPK-A, FLAG-tagged MKK7, or a combination. After transfection, HA-tagged JNK1 was immunoprecipitated from cell lysates, and a kinase assay was carried out using the ATP2 fusion protein as a substrate. Although MKK7 activates JNK1 in 293T cells, the kinase activity is enhanced when MKK7 is co-expressed with PAPK-A (Fig. 8A, left panel). When 293T cells expressing HA-tagged JNK1 are co-transfected with a kinase-inactive mutant of PAPK-A (K89M-PAPK-A), JNK1 is activated, and the kinase activity is slightly enhanced when the mutant is co-expressed with MKK7 (Fig. 8A, right panel). When 293T cells expressing HA-tagged JNK1 were transiently co-transfected with Myc-tagged PAPK-A and a FLAG-tagged dominant-negative MKK7 and starved of serum for 12 h, PAPK-A-induced JNK1 activation was inhibited (Fig. 8B, left panel). However, K89M-PAPK-A induced JNK1 activation was not inhibited by the dominant-negative MKK7 (Fig. 8B, right panel). These results suggest that although PAPK-A functions upstream of MKK7, different mechanisms for JNK1 activation by PAPK-A might exist.

**Overexpression of PAPK-A in NIH3T3 Cells Induces Morphological Changes**—To determine whether overexpression of PAPK-A had any effect on cell morphology or viability, we
generated inducible NIH3T3 cell lines that expressed either wild-type PAPK-A or kinase-inactive K89M-PAPK-A under the control of a mifepristone-responsive element. In this system, addition of mifepristone induced expression of the recombinant proteins (Fig. 9A). Cells expressing an empty vector were used as a control.

Before mifepristone treatment, all of the cell lines looked morphologically similar when they were examined by phase contrast microscopy (Fig. 9B). However, when mifepristone was added to the cultures, only those expressing PAPK-A showed a large increase in the number of cells exhibiting a distinct stretching-like morphology associated with extensions in a stellate shape (Fig. 9B). To quantitate the number of cells exhibiting this distinct morphology, we counted 300 cells from each uninduced or induced cell line in triplicate, and we determined the frequency of cells that exhibited extensions that were longer than the body length. By using these criteria, <10% of the cells in each culture exhibited this morphology before mifepristone treatment (Fig. 9C), and when the control line or the line expressing the kinase-inactive mutant of PAPK-A (K89M) was treated with the drug, the frequency remained at <10% 24 or 48 h later. However, 24 h after mifepristone treatment of the line expressing PAPK-A, 20% of the cells exhibited this distinct morphology, and the frequency rose to 29% by 48 h (Fig. 9C). These data indicate that PAPK-A induces morphological changes in NIH3T3 cells and that this function requires its kinase activity.

Overexpression of PAPK-A in NIH3T3 Cells Blocks Cell Death—The fact that withdrawal of cytokines or FCS consistently results in increased expression of PAPK-A suggested that PAPK might have a function that promotes or inhibits apoptosis. When PAPK-A is overexpressed in NIH3T3 cells growing in DMEM with 10% FCS, the cells did not undergo apoptosis, indicating that PAPK-A is not pro-apoptotic. Because serum withdrawal induces apoptosis in NIH3T3 cells, we tested whether expression of PAPK in these cells made them resistant or more sensitive to apoptosis. Our results indicate that NIH3T3 cells overexpressing PAPK-A are resistant to cell death induced by serum withdrawal. Fig. 10A shows a typical picture of uninduced and induced cells after growth in seru-
free medium for 30 h. All of the uninduced cells show condensed nuclei typical of apoptotic cells. After induction with mifepristone, NIH3T3 cells stably expressing empty vector or the kinase-inactive K89M-PAPK-A mutant are apoptotic, whereas those expressing PAPK-A appear healthy. To determine the kinetics for apoptotic cell death, we stained nuclei with Hoechst 33342 at various times after serum withdrawal (Fig. 10B). Although 26.7% ± 1.6 of NIH3T3 cells expressing empty vector (closed squares) and 40.8% ± 3.2 of NIH3T3 cells expressing kinase-inactive PAPK-A (closed triangles) show apoptosis 48 h after serum withdrawal, only 10.9% ± 2.4 of those expressing PAPK-A show apoptosis (closed diamonds). Interestingly, NIH3T3 cells overexpressing the kinase-inactive K89M-PAPK-A mutant (closed triangles) are even more sensitive than uninduced or vector-induced NIH3T3 cells for cell death at 24, 48, and 72 h after serum withdrawal, suggesting that this kinase-inactive mutant of PAPK-A may be acting as a dominant-negative mutant for endogenous PAPK-A. Furthermore, to monitor a marker of cells undergoing apoptosis, we examined cells for expression of cleaved PARP, one of the main targets of caspase 3. As shown in Fig. 10C, whereas cleaved PARP could be detected 24 and 48 h after serum withdrawal in control cells or cells expressing the kinase-inactive mutant of PAPK-A, cells expressing wild-type of PAPK-A did not show significant expression of cleaved PARP. To determine whether PAPK-A-induced protection against cell death involved JNK1 activation, we carried out Western blot analysis using anti-phospho-JNK antibody. As shown in Fig. 10D, JNK phosphorylation was detected in cells expressing wild-type PAPK-A after serum withdrawal, but we failed to detect JNK phosphorylation in control cells (vector). Interestingly, even higher levels of JNK phosphorylation were detected after induction of the kinase-inactive mutant of PAPK-A, which does not protect the cells from apoptosis induced by serum withdrawal. These
data suggest that PAPK-A kinase activity is not required for phosphorylation of JNK and that JNK activation by PAPK-A is not sufficient to protect cells from death under these conditions. Unfortunately, it is not technically possible to detect phosphorylation of endogenous p38/H9253 in NIH3T3 cells, so we were unable to determine whether PAPK-A activation of this kinase plays a role in protecting the cells from apoptosis induced by serum withdrawal.

**DISCUSSION**

We have cloned and characterized a murine protein kinase, PAPK, which when overexpressed promotes cell survival as well as cytoskeletal changes. From our phylogenetic tree analysis of the kinase domain, we determined that PAPK is a Ste20-like kinase that is most closely related to the GCK subfamily of these serine/threonine kinases. Unlike GCK kinase family members that have an N-terminal kinase domain and a C-terminal regulatory domain (15, 16), PAPK has a kinase domain in the center of the protein and lacks the long C-terminal regulatory domain. Among known GCK kinase family members, PAPK is most highly related to NY-BR-96 (GenBank™ accession number AF308302), with 52% identity within the kinase domain. While this work was in progress, we also identified the human orthologue of PAPK-A, which is similar to ALS2CR2 (GenBank™ accession number, AB038950), an uncharacterized gene cloned in a search for genes involved in juvenile amyotrophic lateral sclerosis on chromosome 2q33-q34 (57). We also isolated an inactive mutant of PAPK, termed PAPK-B. Analysis of the genomic sequence of PAPK-B indicated that it is an alternatively spliced isoform of PAPK. PAPK-B is missing a 172-bp region of putative exon 9, causing a frameshift within the kinase domain and premature termination. PAPK-B does not exhibit kinase activity toward MBP using exogenous substrate.

In this study, we have focused on elucidating the function of
Like other GCK family members, PAPK-A exhibits kinase activity toward MBP. In addition, PAPK-A resembles other GCK family kinases in that overexpression activates JNK. When a putative kinase-inactive form of PAPK-A, K89M-PAPK-A, was generated by replacing lysine 89 in the ATP-binding site with a methionine and tested in an in vitro kinase assay, phosphorylation of MBP was reduced but not to background levels.Interestingly, we observed that PAPK-B and the kinase-inactive K89M-PAPK-A also activated JNK as determined by the in vitro kinase assay and by Western blot analysis using anti-phospho-JNK antibodies. These data suggest that PAPK-A, in addition to harboring protein kinase activity, may also bind other signal transducing molecules, including kinases that can phosphorylate MBP and/or activate JNK. Other GCK kinases have been shown to activate the JNK pathway in the absence of kinase activity (18). For example, a mutant of TNIK that lacks a kinase domain was shown to activate JNK by binding to TRAP2 (36, 58), which potently activates JNK (59). The nature of the PAPK-associated proteins is still unknown and under current investigation.

M KK7 is an upstream activator of JNK (71). We found that PAP kinase-mediated JNK activation was inhibited by dominant-negative MKK7 and enhanced by expression of MKK7. These observations suggest that PAP-K is upstream of MKK7. Unlike PAP-K-induced activation of JNK, activation of JNK by the kinase-inactive mutant of PAP-K is not inhibited by dominant-negative MKK7, suggesting that different mechanisms for JNK activation by PAP kinase exist involving the aforementioned putative PAPK-associated proteins. Because SEK1/MKK4 is also an upstream activator of JNK1 (71), PAP kinase may also activate JNK1 through SEK1/MKK4. Our preliminary data demonstrate that PAP kinase-mediated JNK activation is enhanced by expression of SEK1/MKK4 (data not shown). However, we are unable to conclude that the SEK1/MKK4 pathway plays a role in PAP-K-induced activation of JNK1 because dominant-negative SEK1/MKK4 directly inhibits the basal activity of JNK. Further detailed analysis will be required to determine whether PAP-K-induced activation of JNK involves SEK1/MKK4.

Although we did not detect ERK2, p38α, p38β, or ERK5 activation by PAP kinase, PAP-K, but not PAP-B, preferentially activated ERK6/p38γ. This suggests that unlike JNK activation by PAP kinase, activation of ERK6/p38γ requires PAPK-A kinase activity. It is unknown whether other GCKs activate ERK6/p38γ. It was shown previously (49, 60) that ERK6/p38γ is activated by MMK4, MKK3, and MKK6. ERK6/p38γ is preferentially expressed in heart, skeletal muscle, lung, thymus, and testes (61–63). Because PAPK-A expression is elevated in some of these tissues, it will be interesting to explore the possibility that PAPK-A, via the ERK6/p38γ pathway, plays a role in the physiology of these tissues.

We found that PAPK-A is activated to a greater extent by oxidative stress induced by H2O2 than by other cellular stimuli such as cytokines, growth factors, and other stressors, suggesting that PAP kinase may represent a unique redox-sensitive kinase. Oxidative stress has been linked to both cell death (apoptosis) and cell survival (64). Thus, PAPK may be involved in redox-sensitive signal transduction pathways for cell survival. Although PAPK is ubiquitously expressed in adult tissues, PAPK-A expression levels increase when cells undergo apoptosis.

We isolated PAP kinase from an MEL cell line that was derived from a mouse infected with the Friend spleen focus-forming virus. The erythroid progenitors that serve as targets for spleen focus-forming virus were recently shown to be bipotent (erythroid and megakaryocytic) (65), suggesting that MEL cells may also be bipotent cells. When MEL cells are grown in serum-free medium, PAPK levels are greatly increased and the cells become polyploid, resembling megakaryocytes. When the cells are grown in serum and hexamethylenebisacetamide, which induces their differentiation into red blood cells, PAPK levels decrease. These observations suggest that PAPK may regulate the erythroid/megakaryocyte commitment pathway. We are currently carrying out studies to test this possibility.

Unfortunately, we were unable to make stable lines of either MEL cells or fibroblasts constitutively expressing PAPK-A. Thus, in this study, we used an inducible system for expression of PAPK in NIH3T3 cells. Cells overexpressing PAPK-A exhib-
it a stretching morphology associated with membrane extensions in a stellate shape, as described previously for H-Ras- and RIT-transformed NIH3T3 cells (49). Our preliminary data using immunofluorescence and confocal microscopy to identify filamentous actin-containing structures suggest that actin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A may induce actin rearrangement. Such morphological changes might be associated with cell motility. The morphological changes induced by PAP kinase-A appear to require its kinase activity. Ste20 family members such as PAKs (66) and GCK family members such as SLK (67), PASK (68), PSK (46), and TNIK (36) have all been implicated in the regulation of cytoskeletal reorganization. It has been reported that the low molecular weight GTPase Rho regulates the formation of actin stress fibers and the assembly of focal adhesions and that the inhibition of Rho function by C3 toxin blocks stress fiber formation (69, 70). It is possible that PAPK-A modulates the activity of Rho family proteins. Further experiments are necessary to determine this possibility.

Our studies demonstrate that PAPK-A expression in NIH3T3 cells confers resistance to cell death induced by serum withdrawal, a function that requires kinase activity. Activation of the JNK pathway has been shown to play a role in pro-apoptotic pathways (71). However, it is unlikely that activation of JNK by PAPK-A has an anti-apoptotic function, due to the fact that the kinase-dead form of this protein is unable to protect cells from death despite its ability to activate JNK. Activation of p38 may also be required for the anti-apoptotic function of PAPK-A. Alternatively, the quality or quantity of JNK activation by PAPK-A may contribute to cell survival. Of interest, among Ste20 kinase family members, only PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members such as Bax or Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members. The morphological changes induced by PAP kinase-A indicate that the inhibition of Rho function by C3 toxin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A modulates the activity of Rho family proteins. For example, PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members. The morphological changes induced by PAP kinase-A indicate that the inhibition of Rho function by C3 toxin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A modulates the activity of Rho family proteins. For example, PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members. The morphological changes induced by PAP kinase-A indicate that the inhibition of Rho function by C3 toxin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A modulates the activity of Rho family proteins. For example, PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members. The morphological changes induced by PAP kinase-A indicate that the inhibition of Rho function by C3 toxin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A modulates the activity of Rho family proteins. For example, PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members. The morphological changes induced by PAP kinase-A indicate that the inhibition of Rho function by C3 toxin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A modulates the activity of Rho family proteins. For example, PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members. The morphological changes induced by PAP kinase-A indicate that the inhibition of Rho function by C3 toxin stress fibers have disappeared in cells overexpressing PAPK-A, suggesting that PAPK-A modulates the activity of Rho family proteins. For example, PAKs have been shown to induce BAD phosphorylation, resulting in a reduction in the interaction between BAD and Bcl-2 or Bcl-XL and an increase in the association of BAD with pro-apoptotic Bcl-2 family members.
59. Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., and Levrero, M. (1997) *Science* **275**, 200–203

60. Cuenda, A., Cohen, P., Buce-Scherrer, V., and Goedert, M. (1997) *EMBO J.* **16**, 295–305

61. Lechner, C., Zahalka, M. A., Giot, J. P., Moller, N. P., and Ullrich, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4355–4359

62. Mertens, S., Craxton, M., and Goedert, M. (1996) *FEBS Lett.* **383**, 273–276

63. Court, N. W., dos Remedios, C. G., Cordell, J., and Bogoyevitch, M. A. (2002) *J. Mol. Cell. Cardiol.* **34**, 413–426

64. Kamata, H., and Hirata, H. (1999) *Cell. Signal.* **11**, 1–14

65. Vanzuccchi, A. M., Paoletti, F., Linari, S., Cellai, C., Caporale, R., Ferrini, P. R., Sanchez, M., Migliaccio, G., and Migliaccio, A. R. (2000) *Blood* **95**, 2559–2568

66. Hall, A. (1996) *Science* **275**, 509–514

67. Sabourin, L. A., Tami, K., Seale, P., Wagner, J., and Rudnicki, M. A. (2000) *Mol. Cell. Biol.* **20**, 684–696

68. Tsutsumi, T., Ushiro, H., Kosaka, T., Kayahara, T., and Nakano, K. (2000) *J. Biol. Chem.* **275**, 9157–9162

69. Ridley, A. J., and Hall, A. (1992) *Cell* **70**, 389–399

70. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) *J. Biol. Chem.* **251**, 1001–1007

71. Davis, R. J. (2000) *Cell* **103**, 239–252

72. Wolf, D., Witte, V., Laffert, B., Blume, K., Stromer, E., Trapp, S., d’Aloja, P., Schurmann, A., and Baur, A. S. (2001) *Nat. Med.* **7**, 1217–1224

73. Jakobs, R., Moertl, E., and Koeppe, M. A. (2001) *J. Biol. Chem.* **276**, 16624–16634

74. Tang, Y., Zhou, H., Chen, A., Pittman, R. N., and Field, J. (2000) *J. Biol. Chem.* **275**, 9106–9109
Identification and Characterization of a Novel Ste20/Germinal Center Kinase-related Kinase, Polyploidy-associated Protein Kinase
Kazuo Nishigaki, Delores Thompson, Takashi Yugawa, Karen Rulli, Charlotte Hanson, Joan Cmarik, J. Silvio Gutkind, Hidemi Teramoto and Sandra Ruscetti

J. Biol. Chem. 2003, 278:13520-13530.
doi: 10.1074/jbc.M208601200 originally published online February 6, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M208601200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 74 references, 42 of which can be accessed free at http://www.jbc.org/content/278/15/13520.full.html#ref-list-1