Identification and Characterization of PS-GAP as a Novel Regulator of Caspase-activated PAK-2*

Mark A. Koeppel§§, Corine C. McCarthy‡, Erin Moertl‡, and Rolf Jakobi‡¶¶

From the §Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the ¶Department of Biochemistry, Kansas City University of Medicine and Biosciences, Kansas City, Missouri 64106

Received for publication, September 13, 2004, and in revised form, October 4, 2004

Published, JBC Papers in Press, October 7, 2004, DOI 10.1074/jbc.M410530200

p21-activated protein kinase (PAK)-2 is a member of the PAK family of serine/threonine kinases. PAKs are activated by the p21 G-proteins Rac and Cdc42 in response to a variety of extracellular signals and act in pathways controlling cell growth, shape, motility, survival, and death. PAK-2 is unique among the PAK family members because it is also activated through proteolytic cleavage by caspase-3 or similar proteases to generate the constitutively active PAK-2p34 fragment. Activation of full-length PAK-2 by Rac or Cdc42 stimulates cell survival and protects cells from cell death, whereas caspase-activated PAK-2p34 induces a cell death response. Caspase-activated PAK-2p34 is rapidly degraded by the 26 S proteasome, but full-length PAK-2 is not. Stabilization of PAK-2p34 by preventing its polyubiquitination and degradation results in a dramatic stimulation of cell death. Although many proteins have been shown to interact with and regulate full-length PAK-2, little is known about the regulation of caspase-activated PAK-2p34.

Here, we identify PS-GAP as a regulator of caspase-activated PAK-2p34. PS-GAP is a GTPase-activating protein for Cdc42 and RhoA that was originally identified by its interaction with the tyrosine kinase PYK-2. PS-GAP interacts specifically with caspase-activated PAK-2p34, but not active or inactive full-length PAK-2, through a region between the GAP and SH3 domains. The interaction with PS-GAP inhibits the protein kinase activity of PAK-2p34 and changes the localization of PAK-2p34 from the nucleus to the perinuclear region. Furthermore, PS-GAP decreases the stimulation of cell death induced by stabilization of PAK-2p34.

In multicellular organisms, cell metabolism needs to be tightly regulated by extracellular signals and intracellular signaling pathways. Because of the variety of signals cells receive, the precise balance and modulation of various signals are critical for normal function. Dysregulation of cell signaling pathways can result in cell death or malignant transformation. Protein kinases play a critical role in modulation of a wide variety of signals, and many protein kinases have been identified as oncogenes or tumor suppressor genes, demonstrating their critical role in cell signaling (1, 2).

The p21-activated protein kinases (PAKs) are a family of cellular serine/threonine kinases. The PAK family includes PAK-1 (α-PAK), PAK-2 (γ-PAK), and PAK-3 (β-PAK) as well as PAK-4, PAK-5, and PAK-6, a less closely related second group of PAK family proteins (3–9). PAKs are named for their activation by the monomeric p21 G-proteins Cdc42 and Rac. Active Cdc42 and Rac bind to a region within the regulatory domain of PAKs. This binding region overlaps with an autoinhibitory region within PAK-1, PAK-2, and PAK-3, and p21 binding induces conformational changes that lead to PAK activation (10–12). PAK-4, PAK-5, and PAK-6 appear to lack this autoinhibitory region, although they are still activated by binding of active Cdc42 and Rac (7, 13).

PAKs have been implicated in a variety of cellular functions, including regulation of cell shape and motility through effects on the actin cytoskeleton and integrin signaling pathways and regulation of cell survival and death (14–19). PAKs appear to accomplish these different functions by interaction with a variety of other signaling molecules. The best known PAK interaction partners are the p21 monomeric G-proteins Cdc42 and Rac. Indeed, the PAK family was first identified in an overlay screen for proteins that interact with activated Rac (3). Binding of Cdc42 and Rac to the so-called PBD (p21-binding domain) or CRIB (Cdc42/Rac-interactive binding) domain results in activation of PAKs. Interestingly, sphingolipids also interact with the same region and activate PAKs (20). Additionally, the adaptor protein Nck binds to a proline-rich motif within the regulatory domain of PAK (residues 12–16 of PAK-1) and has been implicated in recruiting PAKs to activated growth factor receptor complexes (21–23). Pix/COL proteins, which are guanine nucleotide exchange factors, have also been shown to modulate PAK activity through binding to a third, atypical proline-rich region within the regulatory domain of PAK (residues 187–196 of PAK-1) (24–26). More recently, PAK-3 has been shown to interact with paxillin, which acts as a scaffolding adaptor protein in integrin signaling, through a region within the PAK regulatory domain that may include the Nck-binding site (27). Paxillin can compete with Nck for PAK binding; surprisingly, it also appears to compete with Pix, even though they are believed to interact with distinct regions within the regulatory domain of PAK.

PAK-1, PAK-2, and PAK-4 have been shown to suppress cell death and to promote cell survival through phosphorylation of...
PS-GAP, a Novel Regulator of Caspase-activated PAK-2

the pro-apoptotic protein Bad (28–31). Constitutive activation of PAKs appears to be involved in malignant transformation, cancer development, and cancer cell invasion. Expression of constitutively active PAK-4 results in anchorage-independent growth (32, 33). Elevated protein or activity levels of PAK-1, PAK-2, and PAK-4 have been detected in various cancer cell lines, and elevated PAK activity has been shown to be required for proliferation of MDA-MB435 breast cancer cells (33–35). Dominant-negative PAK constructs reduce invasion of MDA-MB435 breast cancer cells (35).

PAK-2 is unique among the PAKs because of the existence of a cleavage site for caspase-3 or a caspase-3-like protease within the regulatory domain. Proteolytic cleavage C-terminal of Asp1212 removes most of the regulatory domain, generating a constitutively active PAK-2p34 catalytic fragment (36, 37). Caspase-mediated generation of PAK-2p34 has been observed in response to a variety of apoptotic stimulants (31, 36, 38, 39). Additionally, ectopic expression of PAK-2p34 stimulates cell death (40–42). Therefore, PAK-2 appears to have dual and opposing functions in the regulation of cell survival and death. Activated full-length PAK-2 stimulates cell survival and suppresses cell death, whereas proteolytically activated PAK-2p34 induces a cell death response. We have shown recently that localization and protein levels of PAK-2p34 are tightly regulated (42). Subcellular localization of PAK-2 is regulated by nuclear export and nuclear localization signals. In full-length PAK-2, the nuclear export signal dominates over the nuclear localization signal, resulting in cytoplasmic localization. Caspase cleavage disrupts the nuclear export signal and results in nuclear accumulation of PAK-2p34. Protein levels of PAK-2p34 are regulated by ubiquitination and degradation by the 26 S proteasome. Caspase-activated PAK-2p34 is rapidly degraded by the 26 S proteasome, but full-length PAK-2 is not. Expression of epitruncated-ubiquitinated PAK-2p34 prevents its polyubiquitination and degradation, and stabilization of PAK-2p34 results in dramatic stimulation of programmed cell death (42).

Interestingly, cleavage of PAK-2 to the PAK-2p34 fragment removes interaction sites for Nck, Pix/COOL proteins, paxillin, and Cdc42/Rac/spingolipids, thereby freeing the PAK-2p34 fragment from these known regulators of PAK signaling. Here, we report the identification of a novel PAK-2 regulator, PS-GAP. PS-GAP is a GTPase-activating protein (GAP) for Cdc42 and RhoA previously identified by interaction with the tyrosine kinase PYK-2 (43). PS-GAP interacts selectively with caspase-activated PAK-2p34 both in vitro and in vivo, but does not interact with full-length PAK-2. The interaction with PS-GAP regulates the activity and subcellular localization of caspase-activated PAK-2p34. PS-GAP inhibits the protein kinase activity of PAK-2p34 in vitro and changes the localization of PAK-2p34 from the nucleus to the perinuclear region. Furthermore, PS-GAP appears to regulate the ability of caspase-activated PAK-2p34 to induce programmed cell death. Expression of PS-GAP reduces levels of cell death induced by stabilization of PAK-2p34. PS-GAP is the first identified protein that specifically regulates pro-apoptotic caspase-activated PAK-2p34, but not anti-apoptotic full-length PAK-2, a critical step in elucidating the pro-apoptotic PAK-2p34 signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—Yeast strain PJ69-4a was a generous gift from Dr. P. James (University of Wisconsin, Madison, WI (44). Yeast strain Y190, two-hybrid vectors pAS2-1 and pACT2, the Advantage 2 PCR kit, mouse heart Marathon Ready cDNA, the monoclonal anti-green fluorescent protein (EGFP) Living Colors antibody, and expression vector pRevTRE were obtained from Clontech. The Frozen-EZ Yeast Transformation II kit was from Zymo Research. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs Inc. The QiAprep spin miniprep kit and plasmid midi kit and the pDRIVE PCR cloning kit were purchased from QIAGEN Inc. The Geneclean III kit was from Biotek, Inc. BioMax MR and BioMax M3 autoradiography films were from Eastman Kodak. The Thermoscript RT-PCR kit and Platinum Taq polymerase, plasmid pcDNA3.1, Dulbecco’s modified Eagle’s medium, Dulbecco’s phosphate-buffered saline (DBS-PBS), trypsin/EDTA, Express Five SFM medium, Cellfectin, anti-Myc monoclonal antibody, and customized oligonucleotide primers were obtained from Invitrogen. Fetal bovine serum was from HyClone Laboratories. The baculovirus expression vector pAcG2T and Baculogold baculovirus helper DNA were from Pharmingen. Genejamber G3 PORC baculovirus transformation reagent and Escherichia coli XL2-Blue were from Stratagene. TransIT-TLT1 transfection reagent was from Mirus. Tris-buffered saline and SuperSignal chemiluminescent reagent were from Pierce. Bacterial expression vector pGEX2-T and reduced glutathione-Sepharose were obtained from Amersham Biosciences. Immuno-Fluoro mounting medium and [γ-32P]GTP were from ICN Biomedicals, Inc. [γ-32P]ATP was obtained from PerkinElmer Life Sciences. Mouse RNA from various tissues was a gift from Dr. S. Duncan (Medical College of Wisconsin). Rabbit anti-PS-GAP polyclonal antibody was a gift from Dr. W.-C. Xiong (University of Alabama at Birmingham, Birmingham, AL). Anti-FLAG monoclonal antibody, aga rose-conjugated anti-FLAG monoclonal antibody, and anti-hemagglutinin (HA) monoclonal antibody were obtained from Sigma. Agarose-conjugated anti-Myc antibody, anti-HA polyclonal antibody was obtained from Santa Cruz Biotechnology. Mammalian expression vectors pExpress/HA and pRetroIRE5/GFP were generated previously (42, 45). Mammalian expression clones for FLAG-tagged PAK-2p34 and PAK-2p34-K278R in pRETROIRES/GFP were generated previously (42, 45). mammalian expression clones for FLAG-tagged PAK-2, PAK-2L1106F, and PAK-2-K278R in pRevTRE were generated previously. Plasmid pMT107 encoding His-tagged ubiquitin (46) was provided by Dr. D. Bohmann (University of Rochester, Rochester, NY). The T7 coupled reticulocyte lysate system for in vitro transcription/translation was from Promega.

Yeast Two-hybrid Library Screening and Analysis—The kinase-deficient PAK-2p34-K278R mutant was subcloned into the Gal4 DNA-binding domain vector pAS2-1 in-frame with the Gal4 DNA-binding domain. The PAK-2p34-K278R bait was used to screen a mouse embryonic fibroblast cDNA library in vector pACT2 (47). Yeast PJ69-4a cells transformed with these clones were grown in YPD medium plus 0.4 M NaCl on adenine-deficient medium. Adenine-deficient medium allows for the growth of yeast cells that are capable of transcribing reporter gene, which is under the control of the GAL4 promoter in strain PJ69-4a (44). All colonies capable of growth on adenine-deficient medium were considered potential positive clones. Plasmid DNA was isolated from these clones by digestion with lyticase using Orca’s instructions. PCR products were purified by agarose gel electrophoresis and labeled with [35S]methionine by a coupled in vitro transcription/translation reaction. An aliquot of 10 μg of GST fusion protein was mixed with 100 μl of pull-down buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, and 1% Triton X-100) plus 0.8% bovine serum albumin and incubated on ice for 1 h. An aliquot of 20 μl of glutathione-Sepharose beads was added, and the mixture was incubated for 1 h at 4 °C. Beads were washed with pull-down buffer and analyzed by SDS-PAGE and autoradiography.

Molecular Cloning—To obtain full-length PS-GAP cDNA, mouse heart cDNA was amplified into pDON3.1, and protein was synthesized and labeled with [35S]methionine by a coupled in vitro transcription/translation reaction. An aliquot of 10 μg of GST fusion protein was mixed with 10% of the in vitro transcription/translation reaction in 200 μl of pull-down buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, and 1% Triton X-100) plus 0.8% bovine serum albumin and incubated on ice for 1 h. An aliquot of 20 μl of glutathione-Sepharose beads was added, and the mixture was incubated for 1 h at 4 °C. Beads were washed with pull-down buffer and analyzed by SDS-PAGE and autoradiography.

Molecular Cloning—To obtain full-length PS-GAP cDNA, mouse heart cDNA was amplified by PCR (49) using the forward primer PS-GAP-5 (5′-TTTTGTTCTATATGCGCCCTGACCCCTGAGGGTTATA), corresponding to the start codon region of PS-GAP (43), and the reverse primer PS-GAP-3′ (5′-ACATCTTACAGCTAGCAGTACATATTCTTGGAGA), corresponding to the stop codon region of clone pBK18 and elongated using the Advantage 2 PCR kit according to the manufacturer’s instructions. PCR products were purified by agarose gel electro-
Phosophoresis and extraction using the GeneClean III kit and ligated into the pDRIVE vector. Clones were identified through plasmid isolation using QIAprep spin miniprep kits, followed by restriction digestion and agarose gel electrophoresis. Selected clones were analyzed by DNA sequencing. Sequences were assembled and analyzed using Vector NTI Suite 7.1 (Informax). BLAST (NCBI Protein Database) was used for homology searches. To facilitate subcloning of PS-GAP, an internal BamHI site in PS-GAP-a was disrupted by site-directed mutagenesis according to the megagaper PCR method (50, 51) without changing the amino acid sequence. The megamper was amplified with PS-GAP-5’ and 3’-BamHI-3’ (5’-TCTCTCTGAGCGTTCTTCCCTGCGCATCTCCTCTCCCCGAGACCATGTCGCTAGATC; and P5, 5’-CACTTGTGAAATTTCTCCCGAAGCCTTTCGATTGAT) and primer pair P3/P4 (P3, 5’-TAACGCTATGAAAGTTTTTTGCAACTTGCGCTG; and P4, 5’-CTGATGATCTTTGCGCCCTGCTTGCATTTGAT) were used to amplify an aliquot of the RT reactions by PCR for 40 cycles with Platinum Taq polymerase. Products were separated by gel electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Cell Culture and Transfection—Human embryonic kidney 293T cells (American Tissue Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin and grown at 37 °C in a humidified atmosphere of 5% CO2. For transfection, cells were seeded at densities that will allow them to reach 50% confluency within 16–24 h. Plasmid DNAs for epitope-tagged PS-GAP-a and PAK-2 constructs were transfected into cells using Genejamer or TransIT-LT1 transfection reagent. At 48 h after transfection, cells were harvested and lysed. To stabilize recombinant PS-GAP-a, cells were cotransfected with pMT107 encoding Histagged ubiquitin (45).

Immunoprecipitation and Western blotting—293T cells transfected with epitope-tagged PS-GAP-a and PAK-2 constructs were lysed in modified radioimmunoprecipitation assay buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholate, 1.5 mM MgCl2, and 0.2 mM sodium orthovanadate). Protein concentrations were determined by the Bradford assay using bovine γ-globulin as a protein standard. 500 μg of lysate protein was diluted with PBS to a final concentration of ~5 μg/μl and incubated overnight with 20 μl of agarose-conjugated anti-FLAG or anti-HA antibody at 4 °C. Immunocomplexes were washed with pull-down buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, and 1% Triton X-100) and analyzed by Western blotting. Western blotting was performed using cells lysates (30 μg of protein) or immunoprecipitates (from 150–250 μg of cell lysate) by SDS-PAGE, followed by semidry transfer to polyvinylidene membranes. Chimeriluminescence detection was performed using SuperSignal reagent and horseradish peroxidase-conjugated secondary antibodies.

Rho-GAP Assays—RhoA, Rac1, and Cdc42 were expressed in E. coli XL2-Blue as GST fusion proteins using pGEX2-T, whereas PS-GAP-a was expressed as a GST fusion protein in TN-5B1-4 cells using pAcG2T. Recombinant GST fusion proteins were absorbed to glutathione-Sepharose as described above and eluted with 10 mM reduced glutathione. Purified GST-RhoA, GST-Rac1, and GST-Cdc42 were dialyzed overnight at 4 °C in 40 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% β-mercaptoethanol, and 5% glycerol. For Rho-GAP assays, GST fusion proteins were preloaded at 30 °C for 5 min using 50 mM Tris-HCl (pH 7.6), 2 mM EDTA, 100 mM NH4Cl, 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol, and 0.1 mM [γ-32P]GTP (800 cpm/pmol) and placed on ice. MgCl2 and GTP were added to 12 and 2 mM, respectively. GTPase activity was monitored by incubating preloaded Rho GTPase for 5 min at room temperature in the absence or presence of PS-GAP and spotting on Whatman 3MM filters. Filters were washed with 10% v/v glycerol (50 mM Tris-HCl (pH 7.6), 100 mM NH4Cl, 1 mM MgCl2, and 7 mM β-mercaptoethanol) and dried, and the remaining [γ-32P]GTP was analyzed by scintillation counting. The effects of PAK-2p34 on GTPase activity were measured by preincubation of PS-GAP-a with 10 mM MgCl2 and 200 μM ATP for 15 min at 30 °C in the presence or absence of GST-PK-2p34 prior to performing the GAP assay as outlined above.

Kinase Assays—Autophosphorylation and kinase activity of purified recombinant GST-PK-2p34 (0.1–0.2 μg) and GST-PK-2-T402E (0.1–0.2 μg) were determined in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 2 mM dithiothreitol, and 200 μM [γ-32P]ATP (1000 cpm/pmol) for 30 min at 30 °C. 1 μg of myelin basic protein or histone H4 was used as substrate. Kinase assays were performed in the presence and absence of purified GST fusion proteins of PS-GAP containing the region between the SH3 domains, the SH3 domain, or the region between the GAP and SH3 domains plus the SH3 domain. Autophosphorylation of PAK-2p34 was analyzed by SDS-PAGE on 12% gels and autoradiography. 32P incorporation into myelin basic protein or histone H4 was analyzed by SDS-PAGE on 15% gels, respectively, followed by scintillation counting of excised protein bands.

Immunofluorescence—293T cells plated on polylysine-coated chamber slides were transfected with pRevTRE-HA-PS-GAP-a and pRevTRE-EGFP-PAK-2p34. At 48 h after transfection, cells were washed twice with D-PBS and fixed with 4% paraformaldehyde in D-PBS for 15 min at room temperature. Cells were washed twice with PBS and permeabilized using D-PBS containing 0.2% Triton X-100 for 10 min. Cells were washed for 5 min with D-PBS containing 0.1% Tween 20 and blocked in D-PBS containing 10% goat serum, 100 mM ethanolamine, and 0.1% Tween 20. Cells were washed with D-PBS plus 0.1% Tween 20 and incubated overnight at 4 °C in D-PBS containing 5% goat serum, 0.1% Tween 20, and 10 μg/ml Alexa Fluor 595-conjugated anti-HA antibody. Cells were washed three times with D-PBS containing 0.2% Tween 20 and once with D-PBS. Nuclei were stained by incubation in 0.05 μg/ml 4′,6-diamidino-2-phenylindole in D-PBS for 5 min. Cells were then washed once with D-PBS, and slides were mounted using Immuno-Fluo mounting medium. Immunofluorescence was analyzed by fluorescence microscopy.

Analysis of Programmed Cell Death—293T cells were cotransfected with reporter constructs for EGFP-PAK-2p34, His-tagged ubiquitin (His-Ub), and/or Myc-PS-GAP-a. Cells were stained with 10 μg/ml Hoechst 33342 for 10 min and analyzed by fluorescence microscopy. To determine the levels of programmed cell death, 500 cells expressing EGFP-PAK-2p34 were counted and analyzed for apoptotic chromatin condensation.

RESULTS

Identification of a Novel PAK-2-Interacting Protein—To identify novel proteins that interact with PAK-2, we performed a yeast two-hybrid screen in a mouse embryonic fibroblast cDNA library. So far, no substrates or regulators have been identified for caspase-activated PAK-2p34. Therefore, we used a construct containing residues 213–524, which correspond to the caspase-cleaved PAK-2p34 fragment, as a bait. Transformation of yeast with PAK-2p34 results in an ~10-fold lower number of yeast colonies and delayed growth compared with the kinase-deficient PAK-2p34-K278R mutant. PAK-2p34 is also toxic in E. coli and induces cell death in mammalian cells (40–42). Therefore, we used the kinase-deficient PAK-2p34-K278R mutant for the yeast two-hybrid screening. As a result of the library screening, we isolated 10 clones that selectively induced the reporter genes when cotransfected with PAK-2p34-K278R, but not with an empty vector control. Partial DNA sequencing revealed that clones p/K1 and p/K6 and clones p/K4, p/K11, p/K18, and p/K17 were duplicates, whereas clones p/K5, p/K15, p/K16, and p/K18 were unique. Sequence analysis by a BLAST search of the NCBI Database revealed that clone p/K18, an ~600-bp cDNA, has homology to the C terminus of Rho-GAP proteins such as Graf and oligophrenin (52–54). A subsequent BLAST search several months later revealed that clone p/K18 encodes a 191-amino acid C-terminal fragment of PS-GAP, a Rho-GAP recently identified in a yeast two-hybrid screen using the tyrosine kinase PYK-2 as a bait (43). The interaction between p/K18 and PAK-2 was further examined by yeast two-hybrid analyses and GST pull-down assays (Fig. 1). Clone p/K18 was cotransfected into yeast with PAK-2p34-K278R, wild-type PAK-2, kinase-deficient PAK-2-K278R, constitutively active PAK-2-T402E, the regulatory domain of PAK-2, or an empty vector as a negative control. Interaction was examined by filter-lift assay for β-galactosidase activity (Fig. 1A). Cotransformation of clone p/K18 with PAK-2p34-K278R resulted in strong induction of β-galactosidase activity.
yeast cells were assayed for which expresses the catalytically inactive proteolytic fragment PAK-clone p/K18 as a fusion with the Gal4 activation domain, and pAS2-1, assay. p/K18 was examined by yeast two-hybrid analysis and GST pull-down B as a negative control showed no induction of nase activity, whereas cotransformation with the empty vector induction of domain fragment of PAK-2 with clone p/K18 did not result in nase-deficient, or constitutively active PAK-2 or the regulatory domain variant PS-GAP-s, which lacks the 103 N-terminal residues and starts at Met^104, was described previously (43).

PS-GAP-a, PS-GAP-b, and PS-GAP-c differ from the previously described PS-GAP-m and PS-GAP-s in a stretch of 4 consecutive amino acids within the region between the PH and GAP domains. In addition, there are several single nucleotide polymorphisms, some of which result in amino acid polymorphisms. These polymorphisms were observed in several clones that were amplified by independent PCRs, suggesting that they correspond to genetic diversity between different animals. The cDNA used in the PCR amplifications was from a pool of 200 mice. Seven amino acid polymorphisms exist between PS-GAP-a, PS-GAP-b, and PS-GAP-c. More exist between these variants and the previously described PS-GAP-m and PS-GAP-s variants.

Expression of PS-GAP Variants—The mouse brain cDNA used to amplify full-length PS-GAP was derived from a pool of 200 mice. Therefore, differences between the three isolated PS-GAP variants could be due to differences between individual animals. To determine whether the sequence gaps observed in PS-GAP-b and PS-GAP-c correspond to polymorphisms in different animals or represent splice variants within the same animal, we performed RT-PCR using mRNA from brain, heart, kidney, lung, and testes of a single mouse as well as mRNA from BALB/3T3 mouse fibroblasts (Fig. 3). Primer sets flanking the gaps observed in PS-GAP-b and PS-GAP-c were used in RT-PCR experiments to amplify PS-GAP cDNA, PS-GAP-a, and PS-GAP-c cDNA clones were amplified as positive controls and markers. Amplification of PS-GAP-a and PS-GAP-c with primers flanking the gap at the start of the GAP domain (P1/P2) produced an ~400-bp PCR product, whereas amplification of PS-GAP-b, which lacks a sequence encoding 22 amino acids within this region, produced a PCR product of ~340 bp. Amplification of PS-GAP-a and PS-GAP-b using primers flanking the gap in the region between the GAP and SH3 domains (P3/P4) produced an ~480-bp PCR product, whereas amplification of PS-GAP-c, which lacks a sequence encoding 51 amino acids within this region, produced a PCR product of ~330 bp.

When primer pair P1/P2 was used to amplify transcripts from mouse tissues and BALB/3T3 fibroblasts, PCR products of 400 and 340 bp corresponding to the isolated variants were generated. Both PCR products were generated in all tissues tested, but relative amounts varied between tissues. For example, the 340-bp PCR product was present at the lowest levels in testes and BALB/3T3 fibroblasts. In addition, products >400 bp and <340 bp were also generated, suggesting that there may be additional variants within this region. When primer pair P3/P4 was used to amplify cDNA from mouse tissues and BALB/3T3 fibroblasts, PCR products of 480 and 330 bp corre-
sponding to the isolated variants were generated. Both products were generated in all tissues tested, but relative levels varied between tissues. For example, the 480-bp product was present at the lowest levels in testes, whereas the 330-bp product was detected at the lowest levels in brain and heart. The data indicate that the frame-preserving sequence gaps observed within PS-GAP-b and PS-GAP-c represent splice vari-

ts of PS-GAP-a.

PS-GAP protein levels were examined in various mouse tissues as well as in BALB/3T3 mouse fibroblasts and human embryonic kidney 293T cells. Tissue or cell lysates were analyzed by Western blotting using the anti-PS-GAP antibody (Fig. 4). Immunoreactive bands were present at the lowest levels in testes, whereas the 330-bp product was detected at the lowest levels in brain and heart. The data indicate that the frame-preserving sequence gaps observed within PS-GAP-b and PS-GAP-c represent splice vari-

ts of PS-GAP-a.

PS-GAP protein levels were examined in various mouse tissues as well as in BALB/3T3 mouse fibroblasts and human embryonic kidney 293T cells. Tissue or cell lysates were analyzed by Western blotting using the anti-PS-GAP antibody (Fig. 4). Immunoreactive bands were present at the lowest levels in testes, whereas the 330-bp product was detected at the lowest levels in brain and heart. The data indicate that the frame-preserving sequence gaps observed within PS-GAP-b and PS-GAP-c represent splice vari-

ts of PS-GAP-a.

PS-GAP protein levels were examined in various mouse tissues as well as in BALB/3T3 mouse fibroblasts and human embryonic kidney 293T cells. Tissue or cell lysates were analyzed by Western blotting using the anti-PS-GAP antibody (Fig. 4). Immunoreactive bands were present at the lowest levels in testes, whereas the 330-bp product was detected at the lowest levels in brain and heart. The data indicate that the frame-preserving sequence gaps observed within PS-GAP-b and PS-GAP-c represent splice vari-

ts of PS-GAP-a.

PS-GAP Interacts with PAK-2 in Mammalian Cells—

We have identified PS-GAP as a novel PAK-2-interacting protein. In yeast two-hybrid analyses and GST pull-down assays, clone p/K18, the 191-amino acid C-terminal fragment of PS-GAP-a, interacts specifically with PAK-2p34-K278R, but not with full-length PAK-2. However, the interaction with full-length PAK-2 could require more N-terminal amino acid sequences of PS-GAP-a, which are not present within clone p/K18. Therefore, we used full-length wild-type PAK-2, kinase-deficient PAK-2-K278R, and constitutively active PAK-2-L106F in addition to PAK-2p34 and PAK-2p34-K278R to analyze the interaction with full-length PS-GAP-a. 293T cells were cotransfected
with PS-GAP-a containing an N-terminal HA tag (HA-PS-GAP-a) and PAK-2 constructs containing an N-terminal FLAG tag (FLAG-PAK-2, FLAG-PAK-2-K278R, FLAG-PAK-2-L106F, FLAG-PAK-2p34, and FLAG-PAK-2p34-K278R). PAK-2p34 and kinase-deficient PAK-2p34-K278R are rapidly degraded by 26 S proteasome; however, degradation of PAK-2p34 and PAK-2p34-K278R can be inhibited by coexpression of epitope-tagged ubiquitin (42). Therefore, cells were cotransfected with a construct encoding His-Ub to stabilize FLAG-PAK-2p34 and FLAG-PAK-2p34-K278R. Cell lysates were used in reciprocal co-immunoprecipitation experiments with agarose gel electrophoresis and visualized by ethidium bromide staining. Approximate sizes of the markers are indicated in base pairs on the left. The positions and orientations of the primers within PS-GAP are shown at the bottom.

To verify that the interaction between PS-GAP-a and PAK-2p34 is specific, 293T cells were cotransfected with PS-GAP-a containing an N-terminal Myc tag (Myc-PS-GAP-a) and FLAG-PAK-2p34, FLAG-PAK-2p34-K278R, or FLAG-PAK-2-L106F. 293T cells transfected with Myc-PS-GAP-a alone were used as negative controls. Cell lysates were used in co-immunoprecipitation experiments with agarose-conjugated anti-FLAG antibody. Anti-FLAG immunoprecipitates and cell lysates were analyzed by Western blotting with anti-Myc and anti-FLAG antibodies (Fig. 5C). The anti-Myc antibody detected Myc-PS-GAP-a in all cell lysates. Myc-PS-GAP-a co-immunoprecipitated with FLAG-PAK-2p34 and FLAG-PAK-2p34-K278R, whereas with FLAG-PAK-2-L106F, the levels of Myc-PS-GAP-a were low and indistinguishable compared with the negative control. Western blotting with the anti-FLAG antibody was performed to verify immunoprecipitation of FLAG-tagged proteins. As the levels of immunoprecipitated FLAG-PAK-2 constructs were approximately equal, it appears that Myc-PS-GAP-a precipitates with FLAG-tagged constructs of PAK-2p34 and kinase-deficient PAK-2p34-K278R, but not constitutively active PAK-2-L106F. The results of the immunoprecipitation experiments indicate that PS-GAP-a specifically interacts with caspase-activated PAK-2p34, but not with active or inactive forms of full-length PAK-2, in mammalian cells.

PAK-2p34 Does Not Affect the GAP Activity of PS-GAP—To examine the physiological significance of PAK-2p34/PS-GAP-a interactions, we examined the activity of a GST fusion protein of full-length PS-GAP as a GAP for the p21 monomeric G-proteins RhoA, Rac1, and Cdc42 in the absence and presence of PAK-2p34 (Fig. 6). Purified recombinant RhoA, Rac1, and Cdc42 were preloaded with [γ-32P]GTP, and GTPase activity was analyzed in the absence and presence of purified full-length PS-GAP-a. Full-length PS-GAP stimulated the intrinsic GTPase activity of Cdc42 and, to a much lower extent, RhoA in a dose-dependent manner, but had no significant effect on the GTPase activity of Rac1 (Fig. 6A). To examine whether the interaction with PAK-2p34 affects the GAP activity of PS-GAP, we determined the stimulation of the intrinsic GTPase activity of RhoA, Rac1, and Cdc42 in the presence of an equimolar amount or a 5-fold excess of purified PAK-2p34. Because PS-GAP contains a putative PAK-2 phosphorylation site (KRAS<sup>264</sup>) directly N-terminal to the PH domain, the GAP activity of PS-GAP could be modified through phosphorylation of PS-GAP by PAK-2p34. Therefore, PS-GAP-a was preincubated with GST-PAK-2p34 in the presence of ATP and MgCl<sub>2</sub> prior to GAP assays. As a control, PS-GAP-a was preincubated with ATP and MgCl<sub>2</sub> alone. To compensate for different levels of GAP activity, we used 20 nM PS-GAP-a in experiments with RhoA and Rac1 and 5 nM PS-GAP-a in experiments with Cdc42. Preincubation in the presence of PAK-2p34 had no significant effect on the GAP activity of PS-GAP-a for RhoA, Rac1, or Cdc42 (Fig. 6B). Similar results were obtained when PAK-2p34 was added directly to the GAP assay without a preincubation period with ATP and MgCl<sub>2</sub> (data not shown). The data indicate that the interaction with PAK-2p34 does not affect the GAP
activity of PS-GAP by phosphorylation or allosteric interaction.

Effects of PS-GAP on the Protein Kinase Activity of PAK-2p34—To examine whether PS-GAP is phosphorylated by PAK-2p34, we performed kinase assays using purified PAK-2p34 and PS-GAP. GST-PAK-2p34 was incubated in the presence of [γ-32P]ATP and purified recombinant GST-PS-GAP-a or GST fusion proteins of fragments of PS-GAP-a, and phosphorylation was analyzed by SDS-PAGE and autoradiography. No
FIG. 6. The GAP activity of PS-GAP-a is not affected by the interaction with PAK-2p34. Recombinant full-length PS-GAP-a was analyzed in RhoGAP assays with 5 nM [γ-32P]GTP-loaded RhoA, Rac1, and Cdc42. The remaining [γ-32P]GTP was analyzed by filter assay and scintillation counting. A, intrinsic GTPase activity (Intr.) and GTPase activity in the presence of 5, 10, and 20 nM purified PS-GAP-a were determined over 5 min. Values are shown as the percentage of initial [γ-32P]GTP loading of RhoA, Rac1, and Cdc42. Results are shown as the means ± S.D. (n = 6). B, intrinsic GTPase activity of p21 GTPases (Intr.) and GTPase activity in the presence of PS-GAP-a (control (con)) and PS-GAP-a preincubated with a 1-fold (1×) or 5-fold (5×) molar ratio of PAK-2p34 over PS-GAP-a were determined over 5 min. Assays with RhoA and Rac1 were performed with 20 nM PS-GAP-a, whereas assays with Cdc42 were performed with 5 nM PS-GAP-a. Results are shown as the means ± S.D. (n = 4).

FIG. 7. Effects of PS-GAP on PAK-2p34 kinase activity. Autophosphorylation and protein kinase activity of purified PAK-2p34 and PAK-2-T402E for myelin basic protein and histone H4 were examined in the absence and presence of recombinant PS-GAP. A, autophosphorylation of GST-PAK-2p34 (p34) was determined in the absence and presence of a 20-fold molar excess of the PAK-2p34-binding domain (BD) or the SH3 domain of PS-GAP-a. Assays were performed in quadruplicates, and autophosphorylation was analyzed by SDS-PAGE, followed by autoradiography. Results shown are representative of three independent experiments. B, the protein kinase activity of GST-PAK-2p34 for myelin basic protein was determined in the absence and presence of a 20-fold molar excess of full-length PS-GAP-a, the PAK-2p34-binding domain plus the SH3 domain (BD-SH3), the PAK-2p34-binding domain, or the SH3 domain of PS-GAP-a. The protein kinase activity of PAK-2-T402E was determined in the absence and presence of a 20-fold molar excess of the PAK-2p34-binding domain plus the SH3 domain of PS-GAP-a. Results are shown as the means ± S.D. (n = 4) of a representative of three independent experiments. C, the protein kinase activity of GST-PAK-2p34 for histone H4 was determined in the absence and presence of a 20-fold molar excess of the PAK-2p34-binding domain plus the SH3 domain of PS-GAP-a. Results are shown as the means ± S.D. (n = 4) of a representative of three independent experiments. D, the dose-response effect of PS-GAP on the protein kinase activity of GST-PAK-2p34 was determined with myelin basic protein and increasing amounts of the PAK-2p34-binding domain plus the SH3 domain of PS-GAP-a. Results are shown as the means ± S.D. (n = 4) of a representative of four independent experiments. Protein kinase activities in B–D were analyzed by SDS-PAGE and scintillation counting of excised bands. The levels of phosphorylation were normalized to 1 μg of PAK-2p34 or PAK-2-T402E.
significant phosphorylation of PS-GAP-a was observed, suggesting that PS-GAP-a is not a substrate for PAK-2p34 (data not shown). However, a fragment of PS-GAP that contains the region between the GAP and SH3 domains inhibited autophosphorylation of PAK-2p34 (Fig. 7A). To examine whether PS-GAP binding inhibits PAK-2p34 kinase activity, we performed kinase assays using myelin basic protein as a PAK substrate in the presence of purified GST-PS-GAP-a constructs (Fig. 7B). GST fusion proteins of full-length PS-GAP-a, the region between the GAP and SH3 domains, and the region between the GAP and SH3 domains plus the SH3 domain resulted in decreased PAK-2p34 activity for myelin basic protein. A GST fusion protein of the SH3 domain of PS-GAP showed no inhibition of PAK-2p34 activity for myelin basic protein. Therefore, the region between the GAP and SH3 domains appears to be the PAK-2p34-binding domain of PS-GAP-a. The GST fusion protein of the PAK-2p34-binding domain plus the SH3 domain had no effect on the activity of full-length PAK-2-T402E, which is consistent with the results showing that full-length PAK-2 does not interact with PS-GAP.

To verify that inhibition of PAK-2p34 kinase activity by PS-GAP-a is not limited to phosphorylation of myelin basic protein, kinase assays were also performed using histone H4 as a substrate (Fig. 7C). The GST fusion protein of the PS-GAP-a fragment containing the PAK-2p34-binding domain plus the SH3 domain also inhibited PAK-2p34 kinase activity for histone H4, indicating that inhibition of PAK-2p34 kinase activity by binding to PS-GAP is a general phenomenon and is not limited to phosphorylation of myelin basic protein. To characterize the inhibition of PAK-2p34 activity by binding of PS-GAP, we performed a dose-response curve using different ratios of the GST fusion protein of the PS-GAP-a fragment containing the PAK-2p34-binding domain plus the SH3 domain (Fig. 7D). Maximal inhibition was observed at an ~20-fold molar excess of the PS-GAP-a fragment. These data suggest that PS-GAP down-regulates PAK-2p34 kinase activity and that this effect is mediated through the direct interaction with the PAK-2p34-binding domain of PS-GAP.

**PS-GAP Prevents Nuclear Accumulation of PAK-2p34**—To examine the effects of PS-GAP on PAK-2p34 in vivo, we used transient expression of recombinant PS-GAP-a and PAK-2p34 in 293T cells. Expression of EGFP fusion constructs was used to study the localization of PS-GAP-a and PAK-2p34 in live cells (Fig. 8A). EGFP-PS-GAP-a was detected in the cytoplasm, but not in cell nuclei. EGFP-PAK-2p34 was detected primarily in nuclei. This is consistent with previous results showing that caspase-activated PAK-2p34 is targeted to the nucleus, whereas full-length PAK-2 is localized in the cytoplasm (42). Surprisingly, coexpression of EGFP-PAK-2p34 and HA-PS-GAP-a changed the localization of EGFP-PAK-2p34 from the nucleus to the cytoplasm.

To further characterize the change in PAK-2p34 localization by PS-GAP-a, we performed a combination of EGFP fluorescence and direct immunofluorescence experiments using recombinant expression of EGFP-PAK-2p34 and HA-PS-GAP-a (Fig. 8B). EGFP-PAK-2p34 and HA-PS-GAP-a were expressed in 293T cells and detected in paraformaldehyde-fixed cells using EGFP fluorescence and direct immunofluorescence with Alexa Fluor 594-conjugated anti-HA antibody. In the absence of HA-PS-GAP-a, EGFP-PAK-2p34 was detected primarily in the nucleus. HA-PS-GAP-a was detected in the cytoplasm at the perinuclear region and the cell periphery. However, coexpression of both EGFP-PAK-2p34 and HA-PS-GAP-a resulted in co-localization of both proteins to the perinuclear region within the cytoplasm. Similar results were obtained when FLAG-PAK-2p34 was used and detected by direct immunofluorescence with a fluorescein isothiocyanate-conjugated anti-FLAG antibody. Therefore, the interaction with PS-GAP-a appears to prevent nuclear accumulation of PAK-2p34.

**PS-GAP Reduces the Ability of PAK-2p34 to Induce Cell Death**—To examine whether the interaction with PS-GAP pro-
FIG. 9. Effects of PS-GAP on degradation of PAK-2p34. 293T cells were transfected with a construct for EGFP-PAK-2p34 or cotransfected with constructs for EGFP-PAK-2p34, His-Ub, and Myc-PS-GAP-a as indicated. Lysates (30 μg of protein) were analyzed by Western blotting using anti-EGFP (upper panel) and anti-Myc (lower panel) antibodies. The molecular masses of molecular mass standards are shown in kilodaltons on the left; the positions of recombinant EGFP-antibodies. The molecular masses of molecular mass standards are indicated by red or yellow arrows, respectively. The levels of cell death were determined by apoptotic chromatin condensation of transfected cells 48 h after transfection. Expression and localization of EGFP-PAK-2p34 were detected by EGFP fluorescence, and apoptotic chromatin condensation was analyzed by staining with Hoechst 33342. EGFP-PAK-2p34-expressing cells with condensed chromatin or normal chromatin are indicated by red or yellow arrows, respectively. The levels of cell death were quantified by analyzing 500 cells expressing EGFP-PAK-2p34 for apoptotic chromatin condensation. The levels of apoptotic cells are shown as percentages of cells that express EGFP-PAK-2p34. Cells coexpressing EGFP-PAK-2p34 and Myc-PS-GAP-a exhibited perinuclear localization of EGFP-PAK-2p34, whereas cells expressing EGFP-PAK-2p34 alone exhibited primarily nuclear localization of EGFP-PAK-2p34. As indicated by this difference in subcellular localization ~70% of the cells that were transfected with EGFP-PAK-2p34, His-Ub, and Myc-PS-GAP-a expressed both EGFP-PAK-2p34 and Myc-PS-GAP-a. This number is in agreement with our immunofluorescence studies of cells cotransfected with EGFP-PAK-2p34 and HA-PS-GAP-a.

FIG. 10. Coexpression of PS-GAP prevents PAK-2p34-induced cell death. 293T cells were cotransfected with constructs for EGFP-PAK-2p34, His-Ub, and Myc-PS-GAP-a as indicated. Cell death was determined by apoptotic chromatin condensation of transfected cells 48 h after transfection. Expression and localization of EGFP-PAK-2p34 were detected by EGFP fluorescence, and apoptotic chromatin condensation was analyzed by staining with Hoechst 33342. EGFP-PAK-2p34-expressing cells with condensed chromatin or normal chromatin are indicated by red or yellow arrows, respectively. The levels of cell death were quantified by analyzing 500 cells expressing EGFP-PAK-2p34 for apoptotic chromatin condensation. The levels of apoptotic cells are shown as percentages of cells that express EGFP-PAK-2p34. Cells coexpressing EGFP-PAK-2p34 and Myc-PS-GAP-a exhibited perinuclear localization of EGFP-PAK-2p34, whereas cells expressing EGFP-PAK-2p34 alone exhibited primarily nuclear localization of EGFP-PAK-2p34. As indicated by this difference in subcellular localization ~70% of the cells that were transfected with EGFP-PAK-2p34, His-Ub, and Myc-PS-GAP-a expressed both EGFP-PAK-2p34 and Myc-PS-GAP-a. This number is in agreement with our immunofluorescence studies of cells cotransfected with EGFP-PAK-2p34 and HA-PS-GAP-a.

PAK-2 appears to be a bifunctional modulator of both cell survival and cell death pathways (42, 55). Full-length PAK-2 stimulates cell survival, whereas caspase-activated PAK-2p34 stimulates programmed cell death. Therefore, it is critical to understand the regulation of anti-apoptotic PAK-2 and pro-apoptotic PAK-2p34 signaling pathways. Although full-length PAK-2 is known to be regulated through interactions with a number of other signaling molecules, little is known about the regulation of PAK-2p34 signaling. In this study, we have identified PS-GAP as a novel regulator of caspase-activated PAK-2p34. PS-GAP interacts specifically with PAK-2p34, but not with full-length PAK-2. This is surprising since PAK-2p34 is a proteolytic product of PAK-2, which is identical to residues 213–524 of PAK-2. Therefore, the interaction site with PS-GAP appears to be masked in full-length PAK-2. The crystal structure of PAK-1 predicts that inactive PAKs exist as antiparallel dimers that dissociate or unfold upon activation by p21 G-proteins (12). Therefore, the interaction site with PS-GAP could be masked by interactions between the PAK monomers. However, this does not explain why PS-GAP does not interact with PAK-2-T402E and PAK-2-L106F, two constitutively active mutants of PAK-2 that should be in an open conformation. Therefore, the interaction site with PS-GAP appears to be masked by intramolecular interactions within full-length

DISCUSSION

PAK-2 appears to be a bifunctional modulator of both cell survival and cell death pathways (42, 55). Full-length PAK-2 stimulates cell survival, whereas caspase-activated PAK-2p34 stimulates programmed cell death. Therefore, it is critical to understand the regulation of anti-apoptotic PAK-2 and pro-apoptotic PAK-2p34 signaling pathways. Although full-length PAK-2 is known to be regulated through interactions with a number of other signaling molecules, little is known about the regulation of PAK-2p34 signaling. In this study, we have identified PS-GAP as a novel regulator of caspase-activated PAK-2p34. PS-GAP interacts specifically with PAK-2p34, but not with full-length PAK-2. This is surprising since PAK-2p34 is a proteolytic product of PAK-2, which is identical to residues 213–524 of PAK-2. Therefore, the interaction site with PS-GAP appears to be masked in full-length PAK-2. The crystal structure of PAK-1 predicts that inactive PAKs exist as antiparallel dimers that dissociate or unfold upon activation by p21 G-proteins (12). Therefore, the interaction site with PS-GAP could be masked by interactions between the PAK monomers. However, this does not explain why PS-GAP does not interact with PAK-2-T402E and PAK-2-L106F, two constitutively active mutants of PAK-2 that should be in an open conformation. Therefore, the interaction site with PS-GAP appears to be masked by intramolecular interactions within full-length
PKA2 that are released by removing the N-terminal 212 residues through proteolytic cleavage.

PS-GAP was originally detected in a yeast two-hybrid screen using a fragment of PYK-2 as a bait protein, and the SH3 domain of PS-GAP was shown to interact with PYK-2 (43). The region between the GAP and SH3 domains of PS-GAP-a was sufficient to inhibit the protein kinase activity of PKA2p34 and was named the PKA2p34-binding domain. Interestingly, the splice variant PS-GAP-c lacks 51 amino acids within this region immediately N-terminal to the SH3 domain, and interactions between PKA2p34 and PS-GAP could be modulated by alternative splicing. Splice variant PS-GAP-b differs from PS-GAP-a in the region between the PH and GAP domains, including the N terminus of the GAP domain. Because PS-GAP-b lacks the N-terminal 12 amino acids of the GAP domain, it is unclear if it can function as a Rho-GAP. Two additional PS-GAP variants, PS-GAP-m and PS-GAP-s, were described previously (43). PS-GAP-m could correspond to PS-GAP-a, but there are some differences in the sequence, viz. in 4 consecutive amino acids within the region between the PH and GAP domains. Because this appears to be a region where splicing occurs, these differences could also be due to alternative splicing. The results of our RT-PCR with primers flanking the sequence gap observed in PS-GAP-b suggest that two more splice variants exist within this region. Therefore, PS-GAP appears to be regulated by multiple alternative splicing events, and the various splice variants may serve different functions within the cell.

To analyze the physiological function of the interaction between PS-GAP and PKA2p34, we measured the GAP activity of full-length PS-GAP-a for RhoA, Rac1, and Cdc42, and examined whether PKA2p34 affects the GAP activity of PS-GAP. Full-length PS-GAP-a stimulated the GTPase activity of Cdc42 and, to a lesser extent, RhoA, but not Rac1. In previous studies, the isolated GAP domain of PS-GAP was shown to stimulate the GTPase activity of RhoA and, to a lesser degree, Cdc42, but not Rac1 or Ran; however, recombinant expression of full-length PS-GAP in 293T cells decreased the levels of active GTP-bound Cdc42 more than those of RhoA (43). Therefore, it appears that full-length PS-GAP and the isolated GAP domain differ in their affinities for Cdc42 and RhoA and that Cdc42 is the preferred target of full-length PS-GAP. The interaction between PS-GAP and PKA2p34 could affect the Rho-GAP activity of PS-GAP, and this could involve phosphorylation of PS-GAP by PKA2p34. PS-GAP has a consensus site for phosphorylation by PKA2 (RKR(S/T)) at Ser264. However, PKA2p34 (or full-length PKA2) did not phosphorylate PS-GAP-a to a significant level. Therefore, it appears that PS-GAP-a is not a substrate for PKA2p34. In addition, PKA2p34 did not affect the GAP activity of PS-GAP for Cdc42, RhoA, or Rac1. Therefore, the interaction with PKA2p34 does not regulate the GAP activity of PS-GAP by phosphorylation or allosteric interaction.

Although PS-GAP is not a substrate or target of PKA2p34, it appears to be a specific regulator of PKA2p34. In protein kinase assays, full-length PS-GAP-a and fragments containing the PKA2p34-binding domain inhibited PKA2p34 kinase activity toward itself and toward exogenous substrates such as myelin basic protein and histone H4. Although inhibition of PKA2p34 kinase activity was pronounced, PS-GAP did not decrease the kinase activity of the constitutively active full-length PKA2-T402E mutant. Therefore, the interaction with PKA2p34 specifically inhibits the protein kinase activity of caspase-activated PKA2p34, but not full-length PKA2. Maximal inhibition of PKA2p34 activity required a 20-fold molar excess of PS-GAP, which is quite high for a physiologically relevant event. However, physiological inhibition of PKA2p34 by PS-GAP could require a post-translational modification or a cofactor of PS-GAP that was not present in the in vitro protein kinase assays.

In addition to inhibition of PKA2p34 activity, PS-GAP affects the subcellular localization of PKA2p34. Coexpression of PS-GAP changes the localization of recombinant PKA2p34 from the nucleus to the cytoplasm. We have shown recently that full-length PKA2 is localized in the cytoplasm, whereas caspase-activated PKA2p34 accumulates in the nucleus (42). PS-GAP is a cytoplasmic protein that localizes to the perinuclear region and the cell periphery (43). Because coexpressed PKA2p34 and PS-GAP co-localize to the perinuclear region, it appears that PS-GAP prevents nuclear accumulation of caspase-activated PKA2p34.

Caspase-activated PKA2p34 is rapidly degraded by the 26 S proteasome, but full-length PKA2 is not. Expression of epitope-tagged ubiquitin stabilizes PKA2p34 by inhibiting its polyubiquitination and degradation. Stabilization of PKA2p34 results in a dramatic stimulation of cell death (42). Although expression of PS-GAP slightly increases PKA2p34 levels, it does not result in significant stabilization of PKA2p34 or in stimulation of cell death. However, expression of PS-GAP with PKA2p34 and epitope-tagged ubiquitin reduces the levels of cell death, indicating that PS-GAP counteracts cell death induced by caspase-activated PKA2p34. Therefore, PS-GAP appears to protect cells from cell death by inhibiting the kinase activity and/or by preventing nuclear accumulation of proapoptotic caspase-activated PKA2p34.

Acknowledgments—We thank Dr. P. Jarno for yeast strain PJ69-4a and Dr. D. Bohmann for providing plasmid pM1107 encoding His-tagged ubiquitin. We also thank Dr. W.-C. Xiong for providing the anti-PS-GAP antibody and Dr. S. Duncan for the mouse tissue mRNA samples. We thank Jerry W. Marlin (Kansas City University of Medicine and Biosciences) for technical assistance.

REFERENCES

1. Krebs, E. G. (1994) Trends Biochem. Sci. 19, 439
2. Cohen, P. (1995) Trends Biochem. Sci. 17, 408–413
3. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) Nature 367, 40–46
4. Manser, E., Chong, C., Zhao, Z. S., Leung, T., Michael, G., Hall, C., and Lim, L. (1995) J. Biol. Chem. 270, 25070–25078
5. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. (1995) J. Biol. Chem. 270, 22731–22737
6. Abo, A., Qu, J., Cammarano, M. S., Dan, C., Fritsch, A., Baud, V., Beilsle, B., and Minden, A. (1998) EMBO J. 17, 6537–6540
7. Yang, F., Li, X., Sharma, M., Zarnegar, M., Lim, B., and Sun, Z. (2001) J. Biol. Chem. 276, 15345–15353
8. Dan, C., Nath, N., Liberto, M., and Minden, A. (2002) Mol. Cell. Biol. 22, 567–577
9. Zhao, Z., Manser, E., Chen, X. Q., Chong, C., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 2153–2163
10. Tu, H., and Wiegler, M. (1999) Mol. Cell. Biol. 19, 602–611
11. Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000) Cell 102, 387–397
12. Jaffer, Z. M., and Chernoff, J. (2002) Int. J. Biochem. Cell Biol. 34, 713–717
13. Lim, L., Manser, E., Leung, T., and Hall, C. (1996) Eur. J. Biochem. 242, 171–185
14. Sells, M. A., and Chernoff, J. (1997) Trends Cell Biol. 7, 162–167
15. Krain, L. G., and Bokoch, G. M. (1998) Int. J. Biochem. Cell Biol. 30, 857–862
16. Bagrodia, S., and Cerione, R. (1999) Trends Cell Biol. 9, 350–355
17. Bochk, G. M. (2000) Annu. Rev. Biochem. 71, 743–781
18. Bochk, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S., and Knaus, U. G. (1996) J. Biol. Chem. 271, 8137–8144
19. Bochk, G. M., Wang, Y., Bohl, R. P., Sells, M. A., Quilliam, L. A., and Knaus, U. G. (1996) J. Biol. Chem. 271, 6574–6579
20. Galisteo, M. L., Chernoff, J., Su, Y. C., Skolnik, E. Y., and Schlessinger, J. (1999) J. Biol. Chem. 274, 20997–21000
21. Zhao, Z., Manser, E., and Lim, L. (1998) Mol. Cell. Biol. 20, 6947–6950
22. Bokoch, G. M., Wang, Y., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998) Mol. Cell 1, 183–192
23. Hashimoto, S., Tsubouchi, A., Mazaki, Y., and Sabe, H. (2001) J. Biol. Chem. 276, 183–192
PS-GAP, a Novel Regulator of Caspase-activated PAK-2

53664
276, 6037–6045
28. Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C., and Bokoch, G. M. (2000) Mol. Cell. Biol. 20, 453–461
29. Tang, Y., Zhou, H., Chen, A., Pittman, R. N., and Field, J. (2000) J. Biol. Chem. 275, 9106–9109
30. Gesoutta, N., Qu, J., and Minden, A. (2001) J. Biol. Chem. 276, 14414–14419
31. Jakobi, R., Moerli, E., and Koeppel, M. A. (2001) J. Biol. Chem. 276, 16624–16634
32. Qu, J., Cammarano, M. S., Shi, Q., Ha, K. C., de Lanerolle, P., and Minden, A. (2001) Mol. Cell. Biol. 21, 3523–3533
33. Callow, M. G., Clairvoyant, F., Zhu, S., Schryver, B., Whyte, D. B., Bischoff, J. R., Jallal, B., and Smeal, T. (2002) J. Biol. Chem. 277, 550–558
34. Vadlamudi, R. K., Adam, L., Wang, R.-A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M.-C., and Kumar, R. (2000) J. Biol. Chem. 275, 36238–36244
35. Mira, J. P., Benard, V., Groffen, J., Sanders, L. C., and Knauß, U. G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 185–189
36. Rudel, T., and Bokoch, G. M. (1997) Science 276, 1571–1574
37. Walter, B. N., Huang, Z., Jakobi, R., Tuazon, P. T., Alnemri, E. S., Litwack, G., and Traugh, J. A. (1998) J. Biol. Chem. 273, 28733–28739
38. Tang, T. K., Chang, W. C., Chan, W. H., Yang, S. D., Ni, M. H., and Yu, J. S. (1998) J. Cell. Biochem. 70, 442–454
39. Chan, W. H., Yu, J. S., and Yang, S. D. (1998) J. Protein Chem. 17, 485–494
40. Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., and Williams, L. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13642–13647
41. Rudel, T., Zenke, F. T., Chuang, T. H., and Bokoch, G. M. (1998) J. Immunol. 160, 7–11
42. Jakobi, R., McCarthy, C. C., Koeppel, M. A., and Stringer, D. K. (2003) J. Biol. Chem. 278, 38675–38685
43. Ren, X. R., Du, Q. S., Huang, Y. Z., Ao, S. Z., Mei, L., and Xiong, W.-C. (2001) J. Cell Biol. 152, 971–984
44. James, P., Halladay, J., and Craig, E. A. (1996) Genetics 144, 1425–1436
45. Jakobi, R., McCarthy, C. C., and Koeppel, M. A. (2002) BioTechniques 33, 1218–1222
46. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) Cell 78, 787–798
47. Fields, S., and Song, O. (1989) Nature 340, 245–247
48. Jakobi, R., Huang, Z., Walter, B. N., Tuazon, P. T., and Traugh, J. A. (2000) Eur. J. Biochem. 267, 4414–4421
49. Mullis, K. B., and Faloona, F. A. (1987) Methods Enzymol. 155, 335–350
50. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 404–407
51. Jakobi, R., and Traugh, J. A. (1992) J. Biol. Chem. 267, 23894–23902
52. Billuart, P., Bienvenu, T., Ronce, N., des Portes, V., Vinet, M. C., Zenni, R., Roest Crollius, H., Carrie, A., Fauchereau, F., Cherry, M., Briault, S., Hamel, B., Fryns, J. P., Belôjord, C., Kahn, A., Moraine, C., and Chelly, J. (1998) Nature 392, 923–926
53. Hall, C., Monfries, C., Smith, P., Lim, H. H., Kozma, R., Ahmed, S., Vanniasingham, V., Leung, T., and Lim, L. (1990) J. Mol. Biol. 211, 11–16
54. Hildebrand, J. D., Taylor, J. M., and Parsons, J. T. (1996) Mol. Cell. Biol. 16, 3169–3178
55. Jakobi, R. (2004) Drug Resist. Update 7, 11–17